

Molecular mechanisms of gastrointestinal protection by quercetin against indomethacin-induced damage: role of NF- κ B and Nrf2

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Abstract

The aim of this study was to determine the gastrointestinal protection by quercetin against indomethacin-induced oxidative stress and inflammation, with specific interest in studying the underlying molecular mechanisms. We hypothesized that the quercetin-protective effect relies on its antioxidant and anti-inflammatory properties. Rats were pretreated with quercetin (50- or 100-mg/kg, ig single dose), 30 min before INDO administration (40-mg/kg ig single dose). Caco-2 cells were treated with INDO (250 and 500 μ M) in the absence or presence of quercetin (10 μ g/ml). Quercetin prevented the decrease in nuclear translocation of Nrf2, a key regulator of the antioxidant response, and the increase in reactive oxygen species levels induced by INDO by inhibiting the enhancement of NADPH oxidase and xanthine oxidase activities as well as the reduction in superoxide dismutase and glutathione peroxidase activities in gastric and ileal tissues. Quercetin also prevented INDO-induced ICAM-1 and P-selectin expressions and the increase of myeloperoxidase activity in gastric and ileal tissues and NF- κ B activation and IL-8 production in Caco-2 cells. Quercetin did not affect the inhibition of TNF α -mediated production of prostaglandin E₂ induced by INDO in Caco-2 cells. The protective effects of quercetin observed in the gastric and ileal mucosa of rats as well as in Caco-2 cells relied on the ability of this flavonol to prevent NF- κ B activation and increase Nrf2 translocation. This study supports the concept that quercetin may be useful in the prevention and/or treatment of nonsteroidal anti-inflammatory drug-associated side effects, without interfering with their therapeutic efficacy. © 2015 Elsevier Inc. All rights reserved.

Keywords: Quercetin; Indomethacin; NF- κ B; Nrf2; Oxidative stress; Inflammation

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their antipyretic, analgesic and anti-inflammatory properties [1]. However, their administration is frequently associated with adverse effects that mainly affect the gastrointestinal (GI) mucosa. Indomethacin (INDO) is one of the most damaging NSAIDs for the GI mucosa; thus, it is frequently used as a paradigm drug to study the adverse effects associated with NSAID use and to evaluate the potential protective

effect of bioactive compounds. We recently reported on Caco-2 cells and animal models of GI damage that the mechanisms associated with the cytotoxic events induced by INDO in the GI tract are linked to mitochondrial dysfunction, oxidative stress and apoptosis [2–7]. Specifically in terms of oxidative stress, we showed that INDO decreased the reduced (GSH)/oxidized glutathione (GSSG) ratio and increased the dichlorofluorescein oxidation, superoxide radical production, xanthine oxidase (XO) activity and lipid peroxidation in Caco-2 cells [2–4]. In addition, we observed that oral administration of INDO to rats induced GI mucosal damage and that these effects were associated with an increase in lipid peroxidation and a decrease in the GSH/GSSG ratio [7]. The molecular mechanisms related to redox homeostasis have not been characterized in these models of GI damage.

On the other hand, the proinflammatory effects of INDO have been linked to activation of nuclear transcription factor kappa B (NF- κ B), the induction of migration/infiltration of the mucosa by polymorphonuclear leukocytes and the increased expression of proinflammatory cytokines and adhesion molecules like ICAM-1 [8,9]. These proinflammatory events are associated with a prooxidant effect, such as increasing NADPH oxidase expression and decreasing catalase activity [10]. Polymorphonuclear migration during an inflammatory response is mediated through interactions between adhesion

Abbreviations: EMSA, electrophoretic mobility shift assay; GCLC, glutamate-cysteine ligase catalytic subunit; GI, gastrointestinal; GSHpx, glutathione peroxidase; ICAM-1, intercellular adhesion molecule-1; I κ B α , inhibitory subunit of kappa B; INDO, indomethacin; MAPK, mitogen activated protein kinase; MPO, myeloperoxidase; NF- κ B, nuclear transcription factor kappa B; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NSAID, nonsteroidal anti-inflammatory drug; NQO1, NAD(P)H dehydrogenase [quinone] 1; O₂^{•-}, superoxide radicals; QUE, quercetin; RFU, relative fluorescence unit; RLU, relative luminescence unit; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF α , tumor necrosis factor alpha; XO, xanthine oxidase.

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molecules present in the membrane of endothelial cells and neutrophils. P-selectin mediates the rolling or slowing of neutrophils, whereas intercellular adhesion molecule-1 (ICAM-1) contributes to the firm adhesion and emigration of polymorphonuclear leukocytes [11]. Polymorphonuclear leukocyte migration, one of the major factors causing a predisposition to NSAID-induced gastric and intestinal lesions, consists of several steps, including interaction with P-selectin from platelets and endothelial cells [12].

Various synthetic antiulcer drugs are currently available, and some, such as prostaglandin analogs and proton pump inhibitors, are employed to manage NSAID-induced gastric ulcers [13]. However, these agents are believed to improve the clinical outcome without directly attacking the etiology of mucosal damage.

Quercetin (QUE) is a ubiquitous polyphenol present in fruits and vegetables. It represents one of the most abundant flavonoids in the western diet, and according to the US Department of Health and Human Services, the average human daily intake of QUE is 25 mg [14], data which are also supported by studies analyzing French and Dutch diets [15,16]. High amounts of QUE may be found in onions (284- to 486-mg/kg fresh edible part) and apples (21–72 mg/kg according the variety) [17]. Due to its low level of intestinal absorption, this flavonoid is accumulated in the GI tract, making this its main site of action [18]. QUE has been shown to exert its antioxidant and antiinflammatory effects by activating nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and inhibiting NF- κ B pathways [19–21]. Nrf2 plays a central role in the redox homeostatic gene regulatory network by inducing the expression of phase II and antioxidant enzymes [22,23]. We have reported that a polyphenol-rich extract reduced the oxidative stress, mitochondrial dysfunction and cell death induced by INDO in *in vitro* and *in vivo* models of GI damage [2,3,7]. QUE has been reported to prevent INDO-induced mitochondrial dysfunction through the antioxidant mechanism in Caco-2 cells [4,6]. Although we have made advances in studying the oxidative and inflammatory damage induced by INDO and the protective effect of polyphenolic compounds, we have not yet characterized the underlying molecular mechanisms of GI protection induced by QUE.

The aim of this study was to determine the GI protection by QUE against INDO-induced oxidative stress and inflammation, with specific interest in studying the underlying molecular mechanisms. We hypothesized that the damage would be attenuated through the induction of antioxidant and antiinflammatory mechanisms. For this purpose, we used an *in vivo* and *in vitro* model of GI damage.

2. Material and methods

2.1. Animals

The study protocol was approved by the Animal Ethics Committee of the University of Chile, Faculty of Medicine; all the procedures were performed in compliance with the Guidelines for Care and Use of Laboratory Animals at the University of Chile. Thirty male Sprague Dawley rats (180–220 g, 7–8 weeks old) purchased from the Pontifical Catholic University, Santiago, Chile, were housed with a 12-h light/dark schedule at room temperature and fed standard rodent chow with *ad libitum* access to water. The animals were fasted for 15 h before the experiments, with water *ad libitum*.

2.2. Experimental design

The 30 rats were randomized into five groups. Group 1 (control): a single oral administration of vehicle (through gavage) consisting of 5% NaHCO₃, pH 7.0. Group 2 (QUE 100 mg/kg): a single dose of the flavonoid was administered orally at a dosage of 100-mg/kg body weight (bw), dissolved in 5% NaHCO₃, pH 7.0. Group 3 (INDO 40 mg/kg): a single dose of INDO was administered orally at a dosage of 40-mg/kg bw, dissolved in 5% NaHCO₃, pH 7.0. Group 4 (QUE 50 mg/kg + INDO 40 mg/kg): a single dose of 50-mg/kg bw of QUE was administered orally 30 min before a single dose of 40-mg/kg bw of INDO. Group 5 (QUE 100 mg/kg + INDO 40 mg/kg): a single dose of 100-mg/kg bw of QUE was administered orally 30 min before a single dose of 40-mg/kg bw of INDO. Four hours after INDO administration, the animals were sacrificed by decapitation after ketamine:xylazine anesthesia (100 mg/kg:10 mg/kg, ip), and stomach and ileum were removed immediately (Fig. 1).

2.3. Tissue preparation

The stomach was opened along its greater curvature and the ileum along its antimesenteric border; the tissues were washed in saline solution at 4°C, and segments from both organs were fixed in 4% paraformaldehyde for subsequent immunofluorescence analysis. The intestinal mucosa was scraped with a glass slide and stored at –80°C for further analysis as well as the remaining gastric tissue. For biochemical analysis, the gastric tissue was homogenized with an Ultra-Turrax (IKA T18 basic) and the ileal mucosa with a Teflon Dounce homogenizer (5 strokes). The homogenization process was carried out under a protein inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin and aprotinin; Sigma MO, USA). Myeloperoxidase (MPO), NADPH oxidase, SOD, glutathione peroxidase (GSHpx) activities, superoxide production and Nrf2 expression were quantified in the samples.

2.4. Cell culture conditions

The human intestinal epithelial cell line Caco-2 was cultured in DMEM-F12 with 10% fetal bovine serum added at 37°C (5% CO₂/95% air). Caco-2 cells were trypsinized when they reached 90% confluence, and differentiated cells were used for the experiments.

2.5. Immunofluorescence assays

The presence of ICAM-1 and P-selectin was detected in gastric and ileal tissues by immunofluorescence. Fixed tissue samples were embedded in paraffin, and two serial cross-sections (10 μ m) from each sample were prepared and collected on poly-L-lysine coated glass slides. A total of 60 stomach samples and 60 ileum samples were analyzed. After deparaffinization and rehydration, tissues were subjected to antigen retrieval through incubation with proteinase K and serum blocking solution to prevent nonspecific binding. After this, sections were incubated overnight at 4°C with goat polyclonal anti-ICAM-1 antibody (Santa Cruz Biotechnology, CA, USA) diluted to 1:100 and goat polyclonal anti-P-selectin (Santa Cruz Biotechnology, CA, USA) diluted to 1:100. All sections were washed, stained with 0.25 μ g/ml of DAPI and subsequently incubated with FITC-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature in the dark. After washing, coverslips were mounted with fluorescence mounting medium (Dako), and the immune reactivity was assessed by fluorescence microscopy (Nikon Eclipse E 400). The mean fluorescence intensity was obtained by using ImageJ (NIH Image, <http://www.scioncorp.com>, Scion Corporation, USA), taking into account the area of the tissue under observation according to the magnification. At least six different areas were counted per section, three sections were included per sample.

2.6. MPO activity

Neutrophil infiltration was assessed through the determination of MPO activity. The mucosa was homogenized in 50-mM PBS, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and 10 mM EDTA. An aliquot of homogenate was added to a solution containing 80-mM PBS, pH 5.4, 0.5% HETAB and 1.6-mM 3,3',5,5'-tetramethylbenzidine, and the reaction was started by adding 0.3-mM hydrogen peroxide. Optical density was measured at 655 nm. One unit (U) of MPO activity was defined as the amount of enzyme that produced a change in absorbance of 1.0 unit/min at 37°C [7].

2.7. NF- κ B activation

Caco-2 Cells were plated in 75 cm² flask plates at a density of 2×10^7 cells. After 24 h, cells were incubated for 4 h with TNF α , as a positive control to induce NF- κ B nuclear translocation [24], and/or with INDO and QUE. NF- κ B activation was assessed by the electrophoretic mobility shift assay (EMSA). EMSA remains a powerful experimental tool for detecting the presence of NF- κ B bonded to DNA [25]. Nuclear protein extracts from the samples were prepared as previously described [26]. Nuclear extract (8 μ g) was mixed with double-stranded NF- κ B oligonucleotide 5-GATCTCAGAGGGGACTTCC GAG-3 (GrupoBios SA, Chile) labeled with α -³²P-dCTP using the Klenow DNA Polymerase Fragment I (Invitrogen Corp., Carlsbad, CA, USA) [27]. To confirm the specificity for NF- κ B, a 100-fold excess of unlabeled NF- κ B oligonucleotide was added to the reaction mixture as a competitor. Samples were loaded in nondenaturing conditions. NF- κ B bands were detected by autoradiography and quantified by densitometry using ImageJ.

2.8. IL-8 quantitation

Caco-2 cells were plated in 24-well plates at a density of 1×10^5 cells/well. After 24 h, cells were incubated for 4 h with TNF α as a positive control of induction of IL-8 secretion [28], or with INDO (250 and 500 μ M) alone or in the presence of QUE (10 μ g/ml). Cells were also incubated for 4 h with TNF α and INDO to determine the eventual antiinflammatory effect of the NSAID. This experiment was also conducted in the presence of QUE to evaluate its ability to prevent the IL-8 secretion induced by the positive control (data not shown) as well as by INDO. The media from each well were collected and analyzed for IL-8 secretion using a Human IL-8 Ultrasensitive ELISA Kit (Invitrogen, CA, USA).

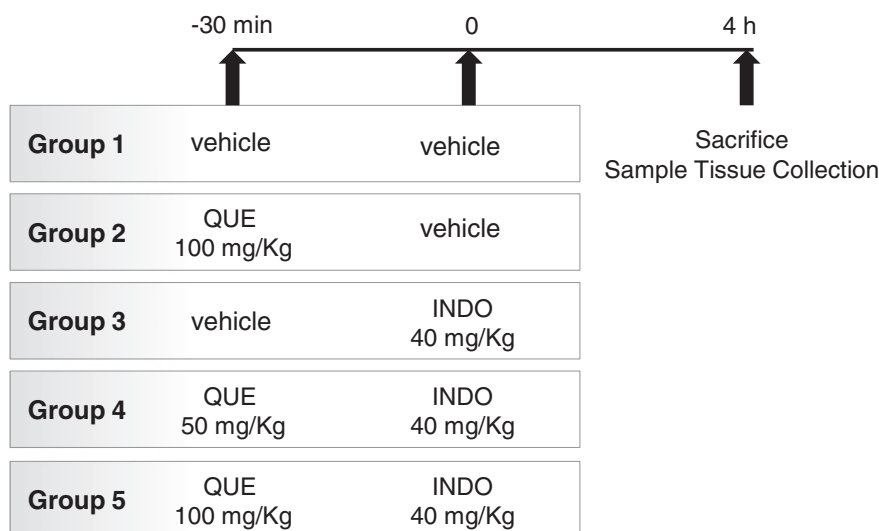


Fig. 1. Scheme of the treatment administration. All the drugs were administered in a single dose orally by gavage. Vehicle consists in 5% NaHCO₃, QUE and INDO. The animals were sacrificed by decapitation under anesthesia.

2.9. Superoxide anion radical production assay

Superoxide radical (O₂^{•-}) production was assessed by measuring the oxidation of 10- μ M DHE (470Ex/590Em) after incubation for 30 min at 37°C with the tissue homogenate (20–50 μ g of protein). To establish the role of NADPH oxidase and XO on O₂^{•-} production, incubations in presence of apocynin, an inhibitor of NADPH oxidase, and/or allopurinol, an XO inhibitor, were also evaluated.

2.10. NADPH oxidase activity

NADPH oxidase activity was determined based on the rate of NADPH consumption monitored at 340 nm at 37°C. The NADPH oxidase enzymatic activity was determined in 50 μ g of the total fraction. The assay was performed on a solution containing 50-mM phosphate buffer, pH 7.0, 1-mM EDTA, 150-mM sucrose and tissue homogenate (20–50 μ g of protein). The enzymatic reaction was initiated by adding 0.1-mM NADPH. NADPH activity was calculated as μ moles of NADPH oxidized/mg protein/min. Only a slight oxidation of NADPH could be detected in the presence of 0.1-mM apocynin (0.04- μ moles NADPH oxidized/mg protein/min).

2.11. Antioxidant enzymes activities

Samples of gastric or ileal mucosa were homogenized in 0.25-M sucrose to determine SOD activity or in 0.01-M KCl-Tris buffer pH 7.4 to determine GSHpx activity. The SOD assay is based on the SOD-mediated increase in the rate of autooxidation of catechols in an aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm [29]. One SOD unit is defined as the activity that doubles the autooxidation background, and results are expressed as U/mg protein. Soluble GSHpx activity was measured spectrophotometrically in the cytosolic fraction (100,000g supernatant) by the reduction of GSSG coupled to NADPH oxidation by GSH reductase [30]. One GSHpx unit is defined as the activity that oxidizes 1 μ mol of NADPH/min and expressed as U/mg protein.

2.12. Nrf2 quantitation

Nrf2 quantitation was performed by western blotting as we previously described [31]. Gastric tissue homogenate was suspended in a buffer solution (pH 7.9) containing 10-mM HEPES, 1.5-mM MgCl₂, 10-mM KCl, 0.2-mM PMSF, 0.5-mM DTT and 0.5% Igepal and centrifuged at 17,000 g for 10 s. The cytosolic supernatant was collected for Nrf2 quantitation. The pellet was suspended in a buffer solution (pH 7.9) containing 20-mM HEPES, 5% glycerol, 420-mM NaCl, 1.5-mM MgCl₂, 0.2-mM EDTA, 0.5-mM DTT and 0.2-mM PMSF and centrifuged at 17,000g for 10 min. The supernatant was collected for Nrf2 quantitation in the nuclear extract. The protein content was determined in the supernatants by using the Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). Protein extracts (35 μ g) were resolved on a 10% SDS-PAGE, western blotted and probed with rabbit polyclonal antibody for Nrf2 (1:500; Abcam, Cambridge, MA, USA), lamin A/C (1:1000; 70 kDa; Cell Signaling Technology, MA, USA) and monoclonal antibody for β -actin (1:1000; 45 kDa; Cell Signaling Technology, MA, USA) and for α -tubulin (HRP conjugated) (1:1000; 52 kDa; Cell Signaling Technology, MA, USA). In all determinations, anti- β -actin was used as control for cytosolic fractions, whereas antilamin A/C was used as control for nuclear fractions. In addition, the extracts were western blotted

with anti- α -tubulin or antilamin A/C to confirm contamination of the cytosolic and nuclear fractions, respectively. The antigen-antibody complexes were detected using horseradish peroxidase goat antirabbit IgG (1:2000; Cell Signaling Technology, MA, USA) and Super Signal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA). Bands were quantified by densitometry using ImageJ.

2.13. Prostaglandin E₂ production

Caco-2 cells were plated in 12-well plates at a density of 2×10^5 cells/well. After 24 h, cells were incubated for 24 h with TNF α or with INDO (250 and 500 μ M) alone or in the presence of QUE (10 μ g/ml). Since TNF α induces the expression of COX-2 in Caco-2 cells, it was used as a positive control to induce prostaglandin E₂ (PGE₂) production [32]. The medium was collected from each well and analyzed for PGE₂ using an enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical Co., Inc. Ann Arbor, MI, USA). The PGE₂ production was expressed as pg/ml.

2.14. Statistical analysis

A sample size calculation was performed on the basis on the reduction of macroscopic damage to the gastric mucosa induced by INDO after apple peel polyphenol extract administration (APPE), with powerful antioxidant and antiinflammatory effects [7]. The assumption used for this purpose included an expected 50% reduction in macroscopic damage with APPE compared with INDO group (40 mg/kg). The sample size calculation was based on the differences in the mean value between two groups of equal sample size, 5% alpha error and 80% power. The resulting sample size was 6.3 rats in each treatment group (Stata V. 10.0 for Windows).

Values are expressed as mean \pm standard deviation. The statistical differences between groups were analyzed by one way ANOVA and Dunnett's post hoc test in GraphPad Prism V. 6 statistical software (La Jolla, CA, USA). In the figures, statistically significant differences in the post-hoc test relative to the control are indicated as #, and those compared to the INDO treatment are shown as * or §. In order to determine if the two doses of QUE have different effects, the significant differences between INDO + QUE 100-mg/kg group and INDO + QUE 50-mg/kg group were expressed as &. More details are found in each figure legend. Unless otherwise indicated, the experiments were performed three times and in triplicate.

3. Results

3.1. QUE attenuates INDO-induced P-selectin and ICAM-1 expressions in gastric and ileal mucosa

The oral administration of INDO induced a 2.8-fold increase in P-selectin expression in the ileal mucosa compared to the control (Fig. 2A and B), which was greater than the ICAM-1 expression (data not shown). By contrast, in the gastric mucosa, INDO exhibited only a weak effect on both P-selectin and ICAM-1 expression (data not shown). The increase in P-selectin and ICAM-1 expression was prevented by QUE in a

dose-dependent manner in both the stomach and the ileum. The highest dose of QUE, 100 mg/kg, completely prevented the increase in protein expression (Fig. 2A and B). Control animals (Fig. 2A and B) as well as those treated with only QUE 100 mg/kg (data not shown) did not show any positive immunostaining for P-selectin or ICAM-1.

3.2. QUE prevents the increase of INDO-induced MPO activity in gastric and ileal mucosa

INDO caused a 100% and 233% increase of MPO activity in the gastric (Fig. 3A) and ileal mucosa of the rats, respectively (Fig. 3B). This increase was completely prevented in both tissues by QUE at a dose of 50 mg/kg. QUE 100 mg/kg (without INDO) had no effect on MPO activity in the GI mucosa.

3.3. QUE prevents the INDO-induced translocation of NF- κ B to the nucleus in Caco-2 cells

NF- κ B DNA binding as an indirect measure of the factor activation is shown in Fig. 4. The negative control conducted with 32 P-labeled oligonucleotide without sample (pb) showed no detectable signal (Fig. 4A). Suppression of the EMSA NF- κ B in control bands by 100-molar excess of the respective unlabeled DNA probes (Cp) confirmed the specificity of the reaction (Fig. 4A). The higher intensity in EMSA bands in the positive control reflects a higher activation of this transcription factor induced by LPS-stimulation (C+) [33]. Treatment with 10-nM TNF α , 250- μ M and 500- μ M INDO showed a level of DNA binding 2.6, 2.5 and 2.8-fold higher than that of the control samples, respectively ($P < 0.05$) (Fig. 4A and B). Incubations with 500- μ M INDO plus TNF α increased 3.5-fold the activity of NF- κ B, while 10- μ g/ml QUE totally attenuated the activation of NF- κ B induced by 250- μ M and 500- μ M INDO (Fig. 4B).

3.4. QUE prevents the production of IL-8 induced by INDO in Caco-2 cells

When the cells were incubated with 10-nM TNF α or with 250- and 500- μ M INDO, the basal levels of IL-8 release increased by 210%, 160% and 318%, respectively (Fig. 5). INDO at a concentration of 500 μ M achieved the maximum release of IL-8 (12 pg/ml) in Caco-2 cells under these experimental conditions. TNF α in association with 500- μ M INDO did not increase the secretion of IL-8 any further. TNF α in association with 250- μ M INDO increased the secretion of IL-8 similarly to that induced by 500- μ M INDO. However, QUE totally prevented TNF α - and INDO-induced IL-8 secretion. QUE (10 μ g/mL) alone had no effect on IL-8 production (Fig. 5).

3.5. QUE prevents the increase of superoxide anion radical production induced by INDO in the gastric and ileal mucosa

Compared to the control group, oral administration of INDO caused a 2.6- and 3.8-fold increase in $O_2^{\bullet -}$ production in the gastric (Fig. 6A) and ileal mucosa (Fig. 6B), respectively. This increase was totally prevented by QUE at a dose of 50 mg/kg. QUE 100 mg/kg (without INDO) had no effect on $O_2^{\bullet -}$ production in the GI mucosa.

Apocynin and allopurinol separately or together caused a 26%, 47% and 77% decrease, respectively, in the oxidation of DHE induced by the gastric homogenate from INDO-treated rats (Fig. 6A, inset) and a 18%, 64% and 91% decrease in this parameter, respectively, induced by the ileal homogenate (Fig. 6B, inset).

3.6. QUE prevents the increase of INDO-induced NADPH oxidase activity in gastric and ileal mucosa

The INDO-treated group exhibited a 1.6- and 2.13-fold increase in the NADPH oxidase activity in the gastric (Fig. 7A) and ileal mucosa

compared to the control group, respectively (Fig. 7B). Tissues of rats treated with both INDO and 50-mg/kg QUE showed no increase in the prooxidant enzyme activity. The administration of QUE 100 mg/kg (without INDO) had no effect on NADPH oxidase activity in the GI mucosa.

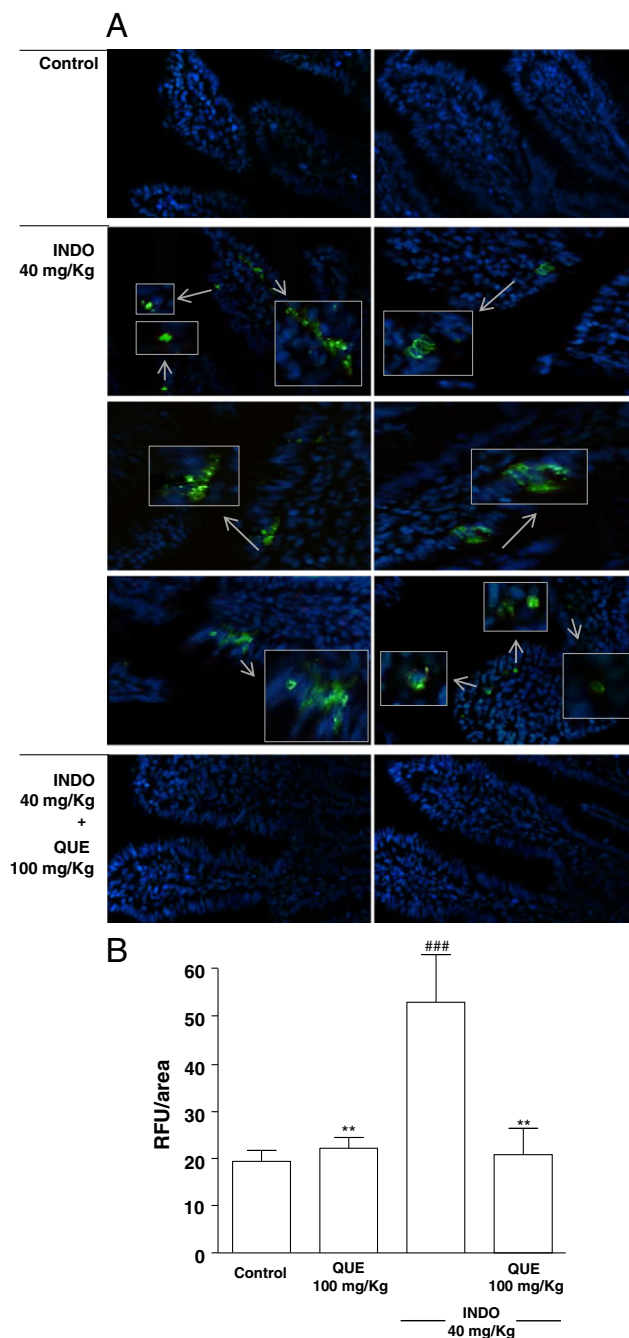


Fig. 2. Expression of P-selectin induced by INDO on rat ileum mucosa. Immunofluorescence images (A) of ileum mucosa from control rats, rats treated with INDO or INDO + QUE 100 mg/kg are shown. Paraffin-fixed ileum tissue section, using antirat P-selectin antibody; the bound antibody was visualized with a fluorescein-conjugated secondary antibody (green fluorescence), and nuclei were stained with DAPI (blue). Magnification $\times 40$. Quantification of p-selectin expression (B); values are expressed as relative fluorescence unit/area. ANOVA $P = 0.0008$, significantly different to control rats ### $P < 0.001$ and to INDO-treated rats ** $P < 0.01$. $N = 6$.

3.7. QUE prevents the increase of INDO-induced superoxide dismutase and GSHpx activities in gastric and ileal mucosa

Oral administration of INDO induced a 45% and 32% decrease of the SOD activity in the gastric (Fig. 8A) and ileal mucosa, respectively (Fig. 8B). This increase was totally prevented by QUE at a dose of 100 mg/kg in both tissues.

Administration of INDO induced a 40% and 31% decrease in the GSHpx activity in the gastric (Fig. 9A) and ileal mucosa, respectively (Fig. 9B). This decrease was completely prevented by QUE at a dose of 100 mg/kg in both tissues.

QUE 100 mg/kg (without INDO) had no effect on the antioxidant enzymes activities in the GI mucosa (Figs. 8AB and 9AB).

3.8. QUE increases the drop in Nrf2 activation induced by INDO in gastric mucosa

As Nrf2 plays a central role in the redox homeostatic gene regulatory network, the prooxidant effect of INDO and the antioxidant protection exerted by QUE were evaluated in terms of Nrf2 activation. Activation of Nrf2 depends on its translocation from the cytosol to the nucleus; thus, Nrf2 was quantitated in cytosolic and nuclear extracts from the gastric mucosa of rats. The higher nuclear/cytosolic Nrf2 ratio, the greater Nrf2 activation; therefore, higher expression of antioxidant enzymes can be induced. The purity of the nuclear isolation of nuclei was confirmed by their low content in α -tubulin (Fig. 10A). As shown in Fig. 7B, the administration of INDO caused a 3.3-fold decrease in the nuclear/cytosolic Nrf2 ratio; however, when QUE was co-administered, the ratio increased threefold compared with the control. QUE alone caused a 3.3-fold increase in the nuclear/cytosolic Nrf2 ratio (Fig. 10A).

3.9. QUE and INDO prevent the synthesis of PGE₂ induced by TNF α in Caco-2 cells

When cells were incubated with 10-nM TNF α for 24 h, the production of PGE₂ increased up to 4.4-fold from its basal level. However, this increase was prevented when TNF α was co-incubated in the presence of 250- or 500- μ M INDO or 10- μ g/mL QUE (Fig. 11).

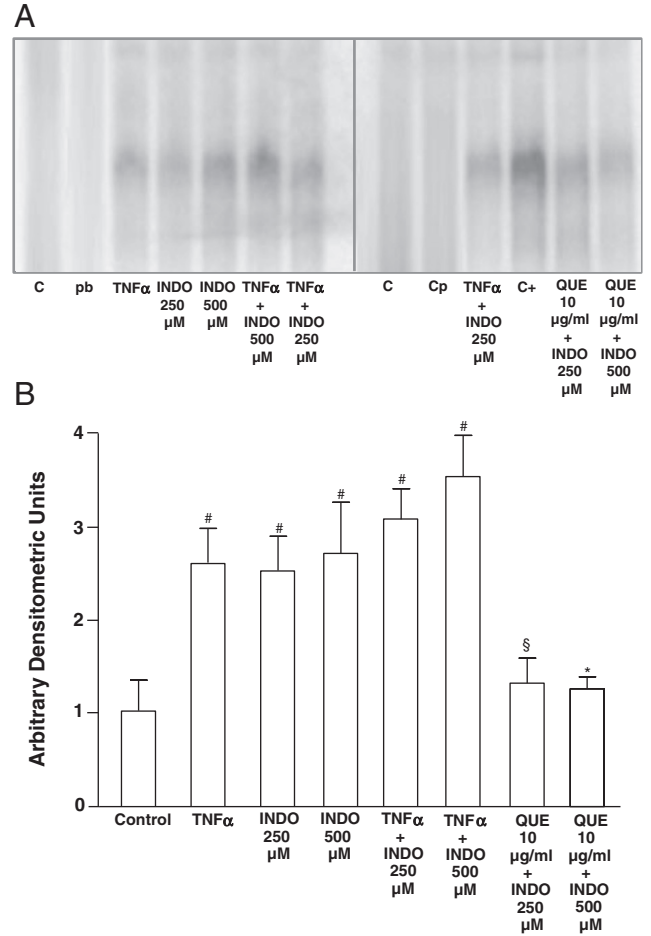


Fig. 4. Effect of INDO and QUE on NF- κ B activation in Caco-2 cells. Cells were treated for 4 h with the specified experimental condition (see materials and methods). The nuclear fractions were used for EMSA assay. (A) Autoradiograph representing lanes loaded with 8 μ g of nuclear protein: ³²P-labeled oligonucleotide without sample (pb), sample with cold competition oligonucleotides with unlabeled DNA probe (Cp), leucocytes stimulated with LPS (positive control C+), nontreated Caco-2 cells (control, cont). (B) Bar graphs corresponding to densitometric quantitation of relative NF κ B DNA binding. ANOVA $P=0.0021$; significantly different to control cells [#] $P<0.05$; to 250- μ M INDO-treated cells [§] $P<0.05$ and to 500- μ M INDO-treated cells ⁺ $P<0.05$. $N=3$.

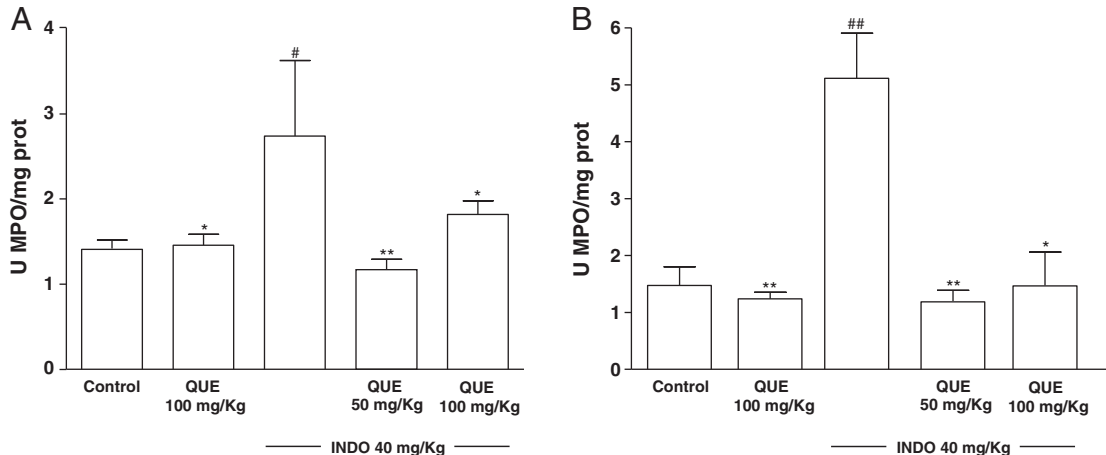


Fig. 3. Effect of INDO and QUE on the MPO activity in gastric (A) and ileum (B) mucosa. MPO activity was measured in mucosal homogenates of rats treated with: INDO, 50-mg/kg QUE + INDO, 100-mg/kg QUE + INDO or 100-mg/kg QUE. Results are expressed as units of MPO per milligram of protein. ANOVA $P=0.0019$ (A) and $P=0.0008$ (B); significantly different to control rats [#] $P<0.05$, ^{##} $P<0.01$ and to INDO-treated rats ^{*} $P<0.05$, ^{**} $P<0.01$. $N=6$.

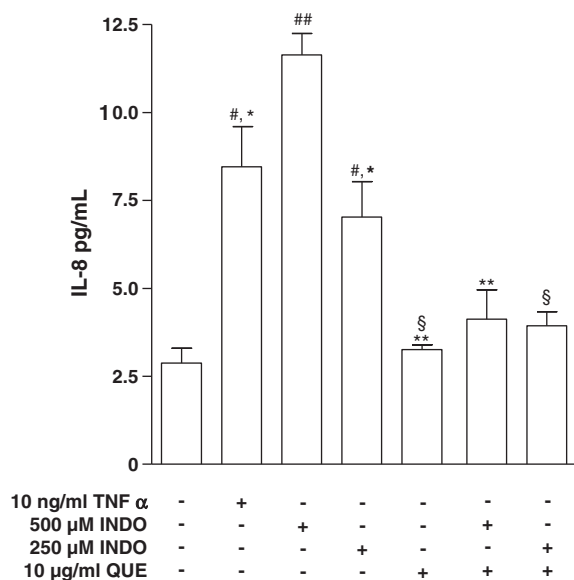


Fig. 5. Effect of INDO and QUE on IL-8 leakage in Caco-2 cells. Cells were treated for 4 h in the specified experimental condition (see materials and methods). ANOVA $P=0.0011$; significantly different to control cells # $P<0.05$, ## $P<0.01$; to 250- μ M INDO-treated cells § $P<0.05$ and to 500- μ M INDO-treated cells * $P<0.05$, ** $P<0.01$. $N=3$.

Discussion

Considering the high consumption of NSAIDs worldwide (about 30 million people take them daily) and the high prevalence of adverse effects, particularly in the GI tract, there is considerable interest in clarifying the mechanisms underlying the side effects of these drugs and in evaluating preventive strategies. We show here that inflammation and oxidative stress arise as important mechanisms underlying INDO-induced GI mucosal injury and that the QUE protective effect is associated with its antioxidant and antiinflammatory properties. The damage of NSAIDs are not just limited to the gastric mucosa, they are also detected in the intestinal tract; in fact, the effect of NSAIDs on the terminal ileum range from an early increase in mucosal permeability to changes in bile acid malabsorption, ulceration,

bleeding and less commonly strictures and perforation. Interestingly, this condition has many similarities to those observed in patients with inflammatory bowel disease [34]. We recently found that INDO increases the proinflammatory damage and neutrophil infiltration in the GI mucosa [7], which is consistent with the higher MPO activity in stomach and ileum (Fig. 3) observed in our study. With respect to the inflammatory process, adhesion molecules like ICAM-1 and P-selectin are necessary for the adhesion of leukocytes to the vascular endothelium and their subsequent migration into the inflammatory perivascular tissues [35]. INDO may exert these proinflammatory effects by inducing the expression of ICAM-1 and especially P-selectin more intensely in the ileal than in the gastric tissue (Fig. 2). The expression of adhesion molecules can be induced by inflammatory signals, specifically through NF- κ B and IL-8 pathways [36–38]. We also found consistently that INDO activates the NF- κ B pathway, resulting in IL-8 production in Caco-2 cells (Figs. 4 and 5).

The expression of adhesion molecules can be also modulated by redox processes. It is noteworthy that reactive oxygen species (ROS) can act as signal transduction molecules to activate IL-8, ICAM-1 and NF- κ B pathways [37,39]. NADPH oxidase induces ICAM-1 expression via the PI3K/Akt pathway [40], and SOD prevents the TNF α -induced ICAM-1 expression [36] in human endothelial cells. Thus, adhesion molecule expression and neutrophil infiltration seems to be triggered by ROS such as $O_2^{\cdot-}$. We found that INDO increased the $O_2^{\cdot-}$ -oxidant status in gastric and ileal tissues (Fig. 6) by increasing NADPH oxidase and XO activities (Figs. 7 and 6 inset) and decreasing SOD activity (Fig. 8).

QUE has been shown to reduce the expression of adhesion molecules induced by inflammatory stimulus in *in vitro* and *in vivo* models. QUE down-regulates the expression of ICAM-1 induced by TNF α and IL-1 β in human endothelial ECV304 cells [41] and in alveolar epithelial A549 cells [42]. QUE inhibits P-selectin expression induced by NF- κ B in a model of airway allergic inflammation in mice lungs [43]. In our work, we found that QUE inhibited the expression of ICAM-1 and P-selectin and prevented the increase of MPO activity induced by INDO in the gastric and ileal mucosa (Figs. 2 and 3). Since the increase of $O_2^{\cdot-}$ status triggers the expression of cytokines and adhesion molecules, the antioxidant properties of QUE may be one of the main mechanisms underlying the protective effect of this flavonol against the inflammation induced by INDO in the GI mucosa. We found that QUE prevented the increase of $O_2^{\cdot-}$ status (Fig. 6), attenuating the

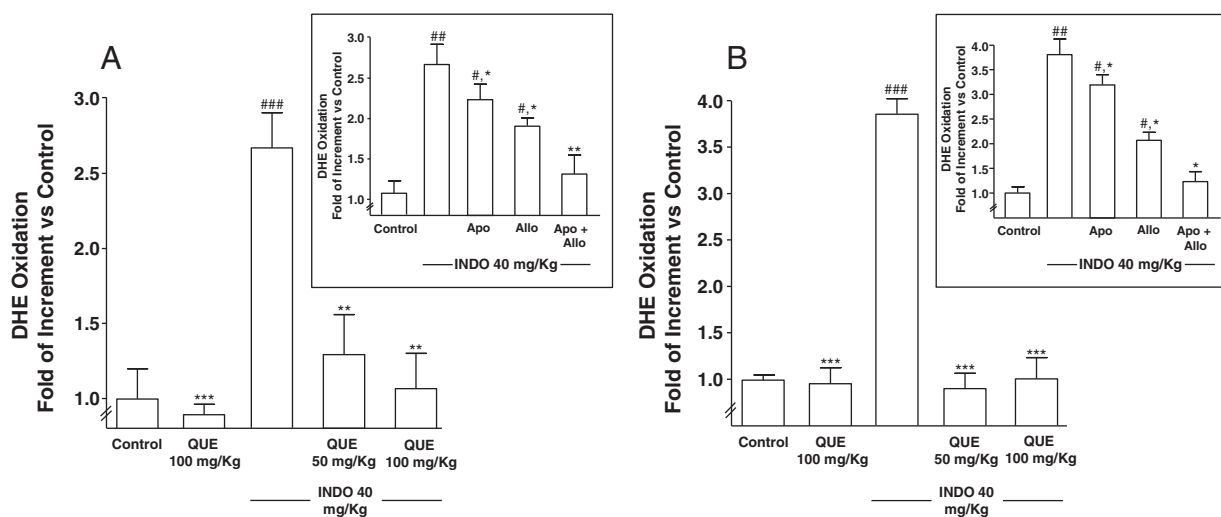


Fig. 6. Effect of INDO and QUE on $O_2^{\cdot-}$ production in gastric (A) and ileum mucosa (B). Superoxide production, evaluated through the oxidation of DHE, was measured in mucosal homogenates of rats treated with INDO or QUE, or both together. Superoxide production was also evaluated in gastric and ileum mucosa from INDO-treated rats in the presence or in the absence of apocynin (Apo) and/or allopurinol (Allo) (inserts). ANOVA $P=0.0009$ (inserts, ANOVA $P=0.0015$) (A); $P=0.0003$ (inserts, ANOVA $P=0.0012$) (B); significantly different to control rats ## $P<0.01$, ### $P<0.001$ and to INDO-treated rats * $P<0.05$, ** $P<0.01$, *** $P<0.001$. $N=6$.

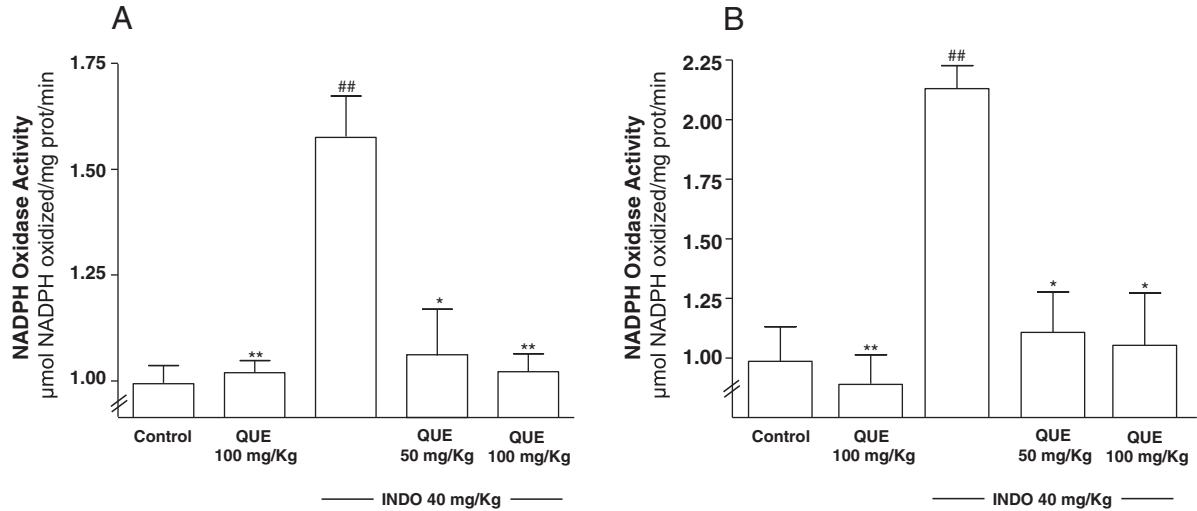


Fig. 7. Effect of INDO and QUE on NADPH oxidase activity in gastric (A) and ileum mucosa (B). NADPH oxidase activity, evaluated through the oxidation of NADPH, was measured in mucosal homogenates of rats treated with INDO; 50-mg/kg QUE + INDO; 100-mg/kg QUE + INDO and 100-mg/kg QUE. Results are expressed as fold of increment with respect to control group. ANOVA $P=0.0005$ (A) and $P=0.0009$ (B); significantly different to control rats ## $P<0.01$ and to INDO-treated rats * $P<0.05$, ** $P<0.01$. $N=6$.

increase of NADPH oxidase and XO activities (Figs. 7 and 6, inset) and the decrease of SOD activity (Fig. 8). QUE has consistently been shown to protect against oxidative damage caused by ethanol in gastric mucosa [44,45], and a QUE-rich apple peel extract has been reported to protect against INDO-induced GI mucosal lesions by preventing lipid peroxidation and neutrophil infiltration [7]. In addition, the prevention observed by QUE against INDO-induced NF- κ B activation and IL-8 production (Figs. 4 and 5) may be another underlying mechanism in the inhibition of adhesion molecule expression and neutrophil infiltration in the GI mucosa. Considering that NF- κ B and IL-8 may be activated by oxidative stress [37,39], the antioxidant properties of QUE are key in the prevention of the inflammatory damage induced by INDO.

Nrf2 plays a central role in the redox homeostatic gene regulatory network; the Nrf2 signaling pathway is activated to enhance the expression of multiple antioxidant enzymes such as SOD and GSHpx, among others [22,23]. Under basal conditions, Nrf2 is sequestered in the cytosol by a Keap1 homodimer. Posttranslational modifications of

Nrf2, including phosphorylation (via MAPK/ERK, p38 pathway), alter its interaction with Keap1, allowing the translocation of Nrf2 to the nucleus [46–48]. Our results show that the gastric tissue from INDO-treated rats exhibits a lower nuclear translocation of Nrf2 (Fig. 10). Consequently, this event may result in lower induction of antioxidant defenses; in fact, we observed a decrease in the activity of SOD and GSHpx in gastric tissue (Figs. 8 and 9). INDO has been reported to decrease the expression of heme oxygenase-1 (an antioxidant enzyme that is expressed downstream Nrf2 activation) in the gastric mucosa, which is consistent with a lower Nrf2 activation [9]. This effect was also associated with an increased ICAM-1 expression [9]. QUE has been shown to exert a Keap1-independent regulation of Nrf2 through posttranslational modifications which induce its translocation [49,50]. We found that QUE, in the absence or presence of INDO, increased the translocation of Nrf2 (Fig. 10). Supplementation with QUE consistently protected against the decrease in the antioxidant enzymes activities induced by INDO (Figs. 8 and 9), probably due to their expressions being increased and/or their activities being preserved as

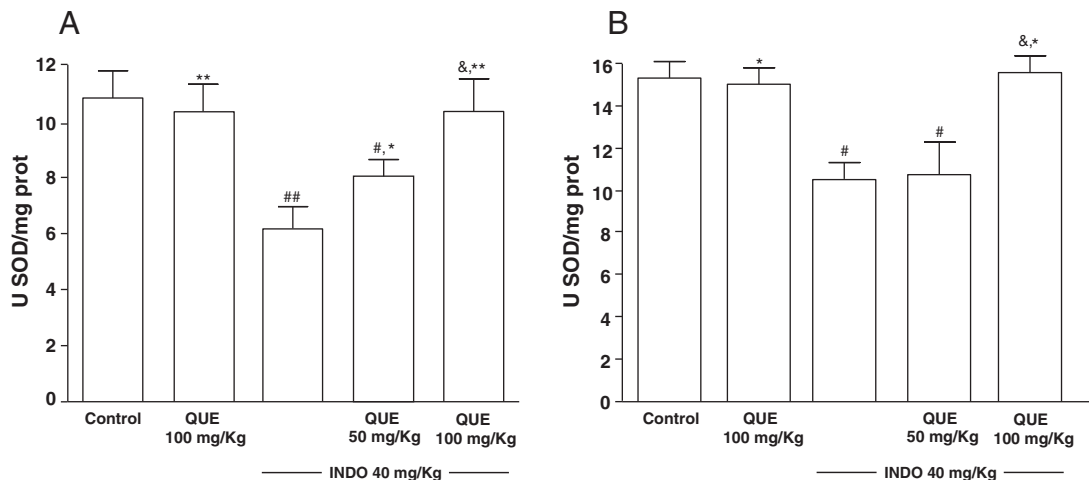


Fig. 8. Effect of INDO and QUE on the SOD activity in gastric (A) and ileum mucosa (B). SOD activity was measured in mucosal homogenates of rats exposed to the different treatments. ANOVA $P=0.0034$ (A) and $P=0.0028$ (B); significantly different to control rats # $P<0.05$, ## $P<0.01$; to INDO-treated rats * $P<0.05$, ** $P<0.01$ and to INDO + 50-mg/kg QUE-treated rats & $P<0.05$. $N=6$.

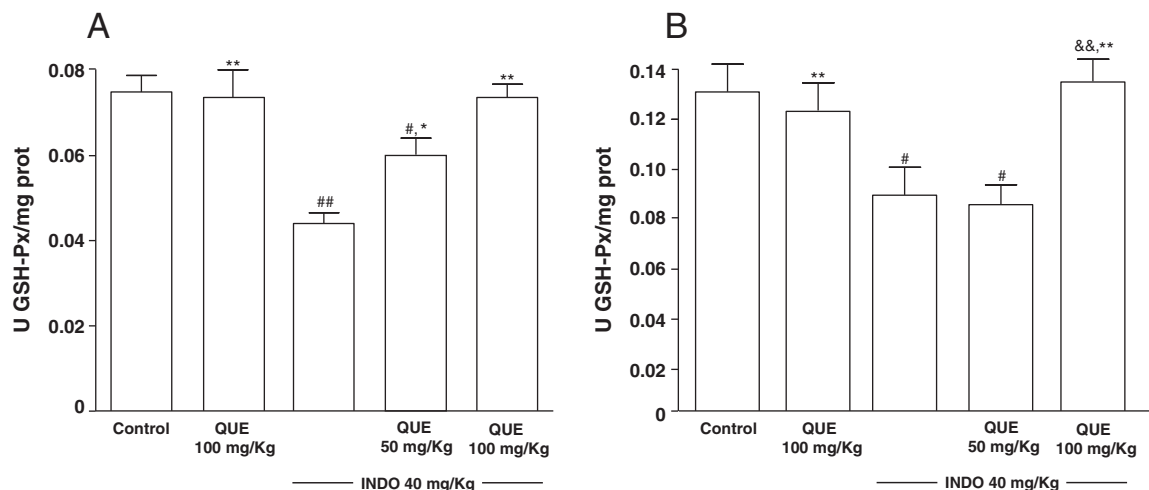


Fig. 9. Effect of INDO and QUE on the GSHpx activity in gastric (A) and ileum mucosa (B). GSHpx activity was measured in mucosal homogenates of rats treated with INDO or QUE, or both together. ANOVA $P=0.0029$ (A) and $P=0.0017$ (B); significantly different to control rats # $P<0.05$, ## $P<0.01$; to INDO-treated rats * $P<0.05$, ** $P<0.01$ and to INDO + 50-mg/kg QUE-treated rats && $P<0.01$. $N=6$.

a result of a reduced INDO-induced oxidative status. The GI protective effect of QUE may rely on its Nrf2 activation-mediated antioxidant activity, as reported with other antioxidant gastro-protective molecules such as pantoprazole [9] and sulforaphane [51]. Interestingly, Nrf2 has also been involved in cytoprotection against inflammation, which has been related to its ability to antagonize the activation of transcription factor NF- κ B [52,53].

As QUE did not interfere with the inhibitory effect of INDO on TNF α -induced PGE $_2$ production in Caco-2 cells (Fig. 11), the protective effect of QUE seems not to be linked to the PGE $_2$ -dependent mucosal protection.

Although the consumption of QUE can be increased by eating foods rich in this flavonol, such as onions [17], specifically those belonging to the pink, yellow and red varieties (ranging 719–927 mg/kg) [54], the dose used in our study is difficult to achieve through diet. However, it would be possible to achieve this dose through the consumption of nutraceuticals based on QUE [55]. A dose of 50 mg/kg of QUE in a rat is equivalent to a dose of 486 mg for a 60-kg human [56].

In conclusion, QUE protects against INDO-induced oxidative stress and inflammation in the gastric and ileal mucosa and in Caco-2 cells.

These protective effects may rely in the ability of QUE to prevent NF- κ B activation while increasing Nrf2 translocation. Moreover, QUE does not interfere with the inhibition of prostaglandin synthesis induced by INDO (Fig. 11); therefore, it is possible to use this flavonoid as a co-adjuvant in NSAID treatments without altering the antiinflammatory systemic therapeutic effects. Other intracellular signaling pathways that result in potential pharmacological targets from these effects should be characterized in future studies.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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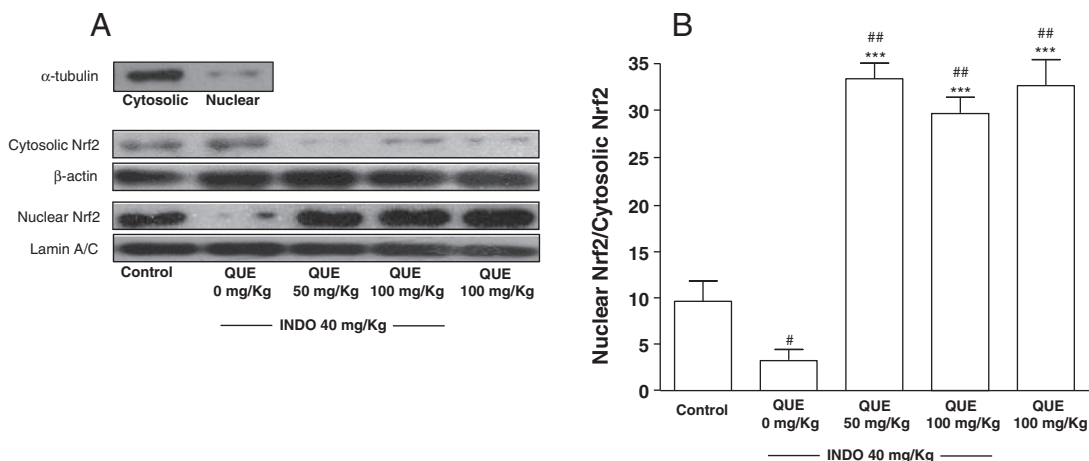


Fig. 10. Effect of INDO and QUE on Nrf2 activation in gastric mucosa. (A) Representative blots of nuclear and cytosolic Nrf2; β -actin, Lamin A/C and α -tubulin expression in gastric tissue homogenate of untreated control rats, or rats treated with INDO, 50-mg/kg QUE + INDO, 100-mg/kg QUE + INDO or only with 100-mg/kg QUE. (B) Nuclear Nrf2/Cytosolic Nrf2 content ratios: these ratios are based on the densitometric quantitation of cytosolic Nrf2 (normalized by β -actin) and nuclear Nrf2 (normalized by Lamin). ANOVA $P=0.0002$; significantly different to control rats # $P<0.05$, ### $P<0.001$ and to INDO-treated rats *** $P<0.001$. $N=6$.

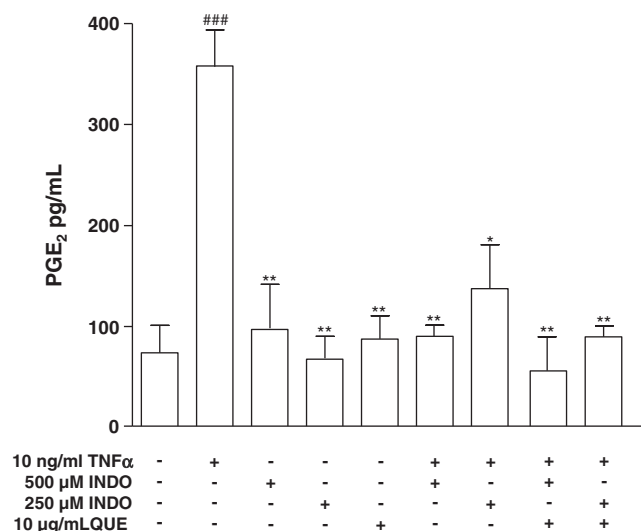


Fig. 11. Effect of INDO and QUE on PGE₂ synthesis in Caco-2 cells. Cells were treated for 24 h with the specified experimental condition (see materials and methods). ANOVA $P=0.0001$; significantly different to control rats ### $P<0.001$ and to INDO-treated rats ** $P<0.01$, *** $P<0.001$. $N=3$.

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