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Catechin protects against ketoprofen-induced oxidative damage of the gastric mucosa by up-regulating Nrf2 *in vitro* and *in vivo*

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs), including ketoprofen, are widely used in clinical medicine. However, these drugs may damage the gastrointestinal mucosa. Some reports have suggested that intestinal diseases, such as ulcers, are associated with lipid peroxidation and oxidative damage in the mucosa. Phytochemicals, such as polyphenols, are common dietary antioxidants that possess many beneficial characteristics, such as antioxidant and anti-inflammatory capabilities. The objective of this study was to investigate the protective effects of polyphenols on ketoprofen-induced oxidative damage in the gastrointestinal mucosa. We evaluated the effects of catechin, theaflavin, malvidin, cyanidin and apigenin on the activity of antioxidant enzymes in human intestinal-407 (Int-407) cells and rat primary gastric cells treated with ketoprofen. The results indicated that catechin significantly ($P<.05$) decreased the levels of lipid peroxidation (40.5%) and reactive oxygen species (30.0%), and increased the activity of intracellular antioxidant enzymes glutathione peroxidase, glutathione reductase and total sulfhydryl groups. More importantly, the treatment of Sprague–Dawley rats with catechin (35 mg/kg/day) prior to the administration of ketoprofen (50 mg/kg/day) successfully inhibited oxidative damage and reversed the impairment of the antioxidant system in the intestinal mucosa. Western blot analysis revealed that catechin stimulated a time-dependent increase in both the nuclear factor erythroid 2-related factor 2 and total heme oxygenase-1 protein expression in Int-407 cells. These results suggest that catechin may have a protective effect on gastrointestinal ulcers.

Keywords: Catechin; NSAID; Nrf2; HO-1; Ketoprofen; Gastric ulcer; Antioxidant enzymes

1. Introduction

Peptic ulcers are one of the most common gastrointestinal disorders, affecting approximately 8% to 10% of the global population [1]. The gastrointestinal mucosa serves as the first protective barrier against xenobiotics but is easily damaged by toxic agents. Serious ulceration may lead to gastrointestinal bleeding or even perforation [2,3]. The pathophysiology of a peptic ulcer is often described as an imbalance between aggressive factors and protective factors. Major endogenous aggressive factors include gastric acid, pepsin and bile salts, whereas exogenous aggressive factors include the consumption of alcohol, tobacco, *Helicobacter pylori* and nonsteroidal anti-inflammatory drugs (NSAIDs). Relevant protective factors include prostaglandin (PG), mucosal blood flow, mucosal cellular restitution and proliferation [3,4].

NSAIDs, such as ketoprofen, are widely used to alleviate pain, inflammation and fevers in clinical medicine. Epidemiologic studies indicate that NSAIDs users have a higher risk of gastrointestinal ulceration than patients who are not taking NSAIDs [5]. The level of cyclooxygenase (COX)-2 expression provides a useful indicator of the extent of inflammation following ketoprofen treatment. Some reports have suggested that ketoprofen including its enantiomers S(+)- and S(−)-ketoprofen does not have a significant effect on COX-1 expression. However, rats orally administered with S(±)-ketoprofen exhibited a significant increase in the level of COX-2 expression. Therefore, reducing COX-2 enzyme expression may be an effective therapeutic strategy for preventing and treating ketoprofen-induced gastrointestinal mucosal ulcer [6]. Further, van der Vliet and Bast [7] have shown that intestinal diseases, such as ulcers, are associated with lipid peroxidation and oxidative damage in the mucosa. Because reactive oxygen species (ROS) may also cause oxidative damage of biological macromolecules, such as DNA, proteins and lipids [8], it is important to regulate the levels of ROS in the human body.

Another strategy for protecting against oxidative gastrointestinal injury may involve up-regulation of antioxidant enzymes and endogenous antioxidants in the gastrointestinal tract. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates phase II detoxification and antioxidant enzymes such as

Abbreviations: COX, cyclooxygenase; GSH, glutathione; GSSG, glutathione disulfide; GPx, glutathione peroxidase; GRd, glutathione reductase; HO-1, heme oxygenase-1; Int-407 cells, intestinal-407 cells; LDH, lactate dehydrogenase; MDA, malondialdehyde; NSAIDs, nonsteroidal anti-inflammatory drugs; PG, prostaglandin; NQO1, NAD(P)H:(quinone-acceptor) oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SD rats, Sprague–Dawley rats; TBA, thiobarbituric acid; TSH, total sulfhydryl groups.

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NAD(P)H:(quinone-acceptor) oxidoreductase 1 (NQO1), superoxide dismutase, catalase, glutathione peroxidase (GPx), glutathione reductase (GRd) and heme oxygenase-1 (HO-1) [9]. Of these antioxidant enzymes, HO-1 is most notably associated with protection of the gastrointestinal tract. Increased HO-1 expression leads to heme degradation and accumulation of iron, bilirubin and carbon monoxide, followed by reduced sensitivity of the gastrointestinal cells to oxidant damage. Of these metabolites, bilirubin acts as a direct antioxidant, whereas carbon monoxide may exert tissue-protective actions primarily through its vasodilation and antiplatelet effects [10,11]. We recently found that sulforaphane effectively prevents indomethacin-induced injury of human intestinal-407 (Int-407) cells by up-regulating HO-1 expression via Nrf2 pathways [11]. This result suggests that increasing Nrf2 activation and HO-1 expression may reduce oxidative damage caused by ROS in the gastrointestinal tract.

Phytochemicals such as polyphenols are common antioxidants found in food. It has been suggested that phytochemicals possess many beneficial features, such as antioxidant, anti-inflammatory and anticancer properties. However, the role of phytochemicals in protecting against ketoprofen-induced damage of the gastrointestinal mucosa remains unknown. Our preliminary work has shown that pretreatment with anthocyanins (malvidin, cyanidin and apigenin) for 1 h or polyphenols (catechin and theaflavin) for 5 h strongly induces GPx activity in Int-407 cells. Moreover, these polyphenols have been shown to be stable in acidic environments and may be more suitable for the investigating gastrointestinal protection [12,13]. In this study, we investigated the protective effects of catechin, theaflavin, malvidin, cyanidin and apigenin on ketoprofen-induced oxidative damage *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

S(+)-ketoprofen, catechin and theaflavin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Apigenin, cyanidin and malvidin were purchased from Extrasynthese (Lyons, France). 2′-7′-Dichloro-fluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). Eagle's basal medium (BME), bovine serum (BS), trypsin-EDTA, L-glutamine and penicillin–streptomycin–neomycin antibiotic mixture (PSN) were purchased from Gibco (Grand Island, NY, USA). Trizol RNA isolation kit was obtained from Life Technologies (Rockville, MD, USA). Primers for reverse transcription polymerase chain reaction (RT-PCR), deoxyribonucleotide triphosphate (dNTP), reverse transcriptase and Taq polymerase were obtained from Gibco BRL (Cergy Pontoise, France). Anti-β-actin, anti-lamin B1 and anti-Nrf2 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The anti-HO-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-mouse and anti-rabbit antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA). All chemicals and solvents used were of the highest purity available.

2.2. Cell culture and treatment

The human intestinal epithelial cell line, Int-407 cells (BCRC 60022), was purchased from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Primary gastric mucosa cells were isolated from male Sprague–Dawley (SD) rats (100–125 g) as previously described by Terano et al. [14]. Cells were cultured in BME supplemented with 10% BS and 1% PSN and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was renewed each day. Cells were detached with 0.1% trypsin and 10 mM EDTA in PBS and split weekly. According to a previous preliminary work, human Int-407 and rat primary gastric cells were pretreated with 25 μM cyanidin, malvidin and apigenin for 1 h or 100 μM catechin and theaflavin for 5 h, and then exposed to ketoprofen for 1 h. The cells were collected by centrifugation (2000*g* for 10 min) for the following experiments.

2.3. Transfection of small interfering RNA against Nrf2 (si-Nrf2)

The plasmids containing si-Nrf2 and SuperFECT transfection reagent were purchased from Qiagen (Valencia, CA, USA). The si-Nrf2 was diluted by diethylpyrocarbonate (DEPC) water. Transfection reagent mixed with si-Nrf2 was incubated for 20 min at room temperature and then added to the culture medium at 30 nM for 48 h.

2.4. Experimental animals and procedures

Specific pathogen-free male SD rats (175–200 g) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Animals were housed individually in stainless steel cages. The animals were maintained in an air-conditioned room at 22°C–24°C with 65%–70% relative humidity on a 12-h light–dark cycle and were fed a normal laboratory diet for 1 week. All experimental procedures involving animals were based on the National Institutes of Health guidelines. This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University, Taichung, Taiwan (IACUC Approval No: 100-21). The animals were orally administrated according to a previously reported method with some modifications [3,6]. Briefly, the animals received an oral administration of catechin (14, 35 mg/kg/day) for 21 consecutive days. The control group received vehicle (ddH_2O) only. On the 21st day, ketoprofen (50 mg/kg/day) was orally administrated to all animals, and 24 h later, the rats were sacrificed with CO_2.

2.5. Preparation of intestinal mucosa homogenate

Extraction of the small intestine mucosa was performed according to a previously reported method with a slight modification [15]. Briefly, intestinal tissue was homogenized in ice-cold, 1.15% KCl buffer. The samples were immediately centrifuged (10,000*g* for 10 min) at 4°C to obtain a homogenate of intestinal mucosa. Aliquots of the homogenate were stored at −80°C for the following experiments.

2.6. Intracellular ROS measurement

Intracellular ROS generation was detected using a fluorescent probe, DCF-DA. DCF-DA readily diffuses through the cell membrane, is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH and then, in the presence of ROS, is rapidly oxidized to form highly fluorescent DCF. The intensity of DCF fluorescence corresponds to the level of intracellular ROS formation. Following incubation, the cells were collected and resuspended at 10^6 cells/ml in PBS. An aliquot of the suspension (195 μl) was loaded into a 96-well plate, and 5 μl DCFH-DA was added (final concentration 20 μM). The DCF fluorescence intensity was detected over a series of time intervals using a Fluostar Galaxy (FLUOstar) plate reader (BMG Lab technologies GmbH Inc., Offenburg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.7. Lactate dehydrogenase (LDH) analysis

The intestinal mucosal tissue was first homogenized in KCl buffer, and then the level of LDH leakage assay was performed using a commercial kit (Sigma Chemical Co., MO, USA). The degree of LDH leakage reflects the level of tissue cytotoxicity.

2.8. Measurement of malondialdehyde (MDA)

The MDA concentration in Int-407 cells and rat intestinal mucosa samples was determined using the thiobarbituric acid (TBA) method with modifications [16]. Briefly, following a preincubation, 0.5 ml of cell homogenate was mixed with 1 ml of 15% trichloroacetic acid, 0.375% TBA-reactive substances and 0.25 mM HCl, and then heated in boiling water for 45 min. After centrifugation (2000*g* for 15 min), the absorbance of the butanol phase was read at 535 and 520 nm. The difference between the two values was used to calculate the MDA concentration. An MDA standard was prepared from 1,1,3,3,-tetraethoxypropane. Total protein concentrations were determined by Lowry's method using bovine serum albumin as the standard. The MDA concentration was normalized against the total protein concentration and is expressed in nmol/mg protein.

2.9. Measurement of glutathione (GSH) and the GSH/glutathione disulfide (GSSG) ratio

The intestinal mucosa tissue was first homogenized in KCl buffer, and then the levels of GSH and GSSG were measured using a glutathione assay kit (Cayman Chemical Co., MI, USA). The total amounts of GSH and GSSG were spectrophotometrically calculated by measuring the absorbance at 405 nm on microplate reader (Awareness Technology, Palm City, FL, USA).

2.10. Measurement of GPx

GPx activity was determined spectrophotometrically according to a previously reported method with some modifications [17]. Briefly, intestinal mucosa homogenate solution (100 μl) was mixed with 800 μl of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/ml GSH reductase and 1 mM GSH. After 5 min, 2.5 mM H_2O_2 (100 μl) was added to start the reaction. The change in absorbance measured at 340 nm was recorded over the course of 3 min. Enzyme activity was calculated as E_{340}=6220/*M* per cm (extinction coefficient), and the result is expressed in nmol/min per mg protein.

2.11. Measurement of total sulfhydryl groups (TSH)

The activity of TSH was measured according to a previously reported method with some modifications [18]. Briefly, intestinal mucosa homogenate solution (500 μl) was mixed with 750 μl of Tris/EDTA buffer (pH 8.2), 10 mM 5,5-dithiobis 2-nitrobenzoic acid and 3.16 ml of methanol. The samples were centrifuged (3000*g* for 10 min), and the concentration of TSH in the supernatant was determined at 412 nm by enzyme-linked immunosorbent assay.

2.12. Measurement of GRd

The activity of GRd was determined from the rate of oxidation of NADPH during the reduction of GSSG [17]. A 1-cm spectrophotometric cuvette containing 0.9 ml of 0.10 M phosphate buffer (pH 7.0) with 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG and 0.1 mM NADPH was preincubated for 5 min at 37°C. The reaction was started by adding 0.1 ml of homogenate, and the decrease in absorbance measured at 340 nm was monitored for 5 min.

2.13. Measurement of 8-hydroxy-deoxyguanosine (8-OHdG) in DNA

The intestinal mucosa tissue was homogenized by lysis buffer containing Pronase E (Merck Co., Darmstadt, Germany) and butylated hydroxytoluene. DNA was immediately digested by deoxyribonuclease I (DNase I), nuclease 5′-nucleotide hydrolase, 3′-phosphohydrolase and alkaline phosphatase as described by Frenkel and Klein [19]. The sample was analyzed by high-performance liquid chromatography equipped with UV and electrochemical detectors for the presence of deoxyguanosine (dG) and 8-OHdG, respectively. The amount of 8-OHdG in DNA was expressed as the number of 8-OHdG molecules per 10^5 dG.

2.14. Western blotting analysis

Protein fractions were isolated from Int-407 cells following treatment with catechin (100 μM) for 1–4 h. Total proteins were extracted using a Total Protein Extraction Kit (Millipore, Bedford, MA, USA), and the nuclear and cytosolic proteins were extracted with a Nuclear/Cytosol Fractionation Kit (BioVision Research Products, Mountain View, CA, USA) following the manufacturer's instructions. Total protein extraction and quantification were performed using a BCA protein assay kit as directed (BCA protein assay, Pierce, USA). Total protein and compartmental protein extracts (20–25 μg of protein) were separated on 10% sodium dodecyl sulfate–polyacrylamide mini gels for detection of Nrf2 and HO-1, and then immobilized on polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) using a transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine and 20% methanol. The membrane was blocked with Starting Block Blocking Buffer (Pierce, Rockford, IL, USA) for 30 min at room temperature and then incubated overnight at 4°C with indicated primary antibodies (1:1000 dilutions). After hybridization with primary antibodies, the membrane was washed three times with Tris-buffered saline containing Tween-20 (TBST), incubated with horseradish-peroxidase-labeled secondary antibody for 60 min at room temperature and washed with TBST three times. The immunoblotting signals were detected by chemiluminescence according to the manufacturer's instructions (The ECL Western Blotting System, Amersham Pharmacia Biotech). The protein bands were quantified using UVP VisionWorks LS Image Acquisition and Analysis software (UVP, Upland, CA, USA).

2.15. RNA extraction and real-time RT-PCR

The levels of gene expression in Int-407 cells were determined by real-time RT-PCR. Cells (1×10^6 cells in 10-ml medium) were seeded in 100-mm tissue culture dishes and treated with 100 μM catechin for the indicated times. Total RNA was prepared using the TRIzol method (Life Technologies, Rockville, MD, USA) as described in the manufacturer's instructions. The following sense and antisense primer sequences were used for RT-PCR analysis: COX-1: 5′-AACCGTGTGTGTGACTTGCTGAA-3′ (forward) and 5′-AGAAAGAGGCCCTCAGAGCTAG-3′ (reverse); COX-2: 5′-TGATGACTGCCCAACTCC-CATG-3′ (forward) and 5′-AATGTTGAAGGTGTCCGGCAGC-3′ (reverse); GAPDH: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ (forward) and 5′-CTAGAAG-CATTTGCGGGGACGATGGAGGG-3′ (reverse). RT-PCR for detection of gene expression levels was conducted using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction mixture (total volume of 25 μl) contained 1× SYBR green PCR master mix, 300 nM forward primer, 300 nM reverse primer, cDNA, DEPC-H_2O and commercial reagents (Applied Biosystems, Foster City, CA, USA). The thermal profile followed the recommended protocol of the manufacturer: 95°C for 10 min for enzyme activation, followed by denaturing at 95°C for 15 s, and annealing and elongation at 60°C for 1 min, for a total of 40 cycles.

2.16. Histopathologic studies

The stomach and intestinal tissues of SD rats were fixed in 10% formaldehyde immediately following sacrifice, processed for histological examination according to the conventional method, and stained with hematoxylin and eosin (H&E). The morphology of any lesions observed was classified and recorded.

2.17. Statistical analysis

Statistical analysis was performed according to the SAS User's Guide. Analysis of variance (ANOVA) was performed using the ANOVA procedure. Significant differences ($P<.05$) between means were determined using Duncan's multiple range test.

3. Results

3.1. The protective effects of polyphenols on ketoprofen-induced changes in lipid peroxidation and ROS levels in Int-407 and rat primary gastric cells

As shown in Fig. 1A, exposure of Int-407 cells to 200 μM ketoprofen for 1 h significantly ($P<.05$) increased the level of total lipid peroxidation (MDA). Moreover, rat primary gastric cells treated with 200 μM ketoprofen for 1 h displayed significantly ($P<.05$) increased level of intracellular ROS (Fig. 2B). In human Int-407 cells, catechin produced the most dramatic reduction of MDA, a decrease of

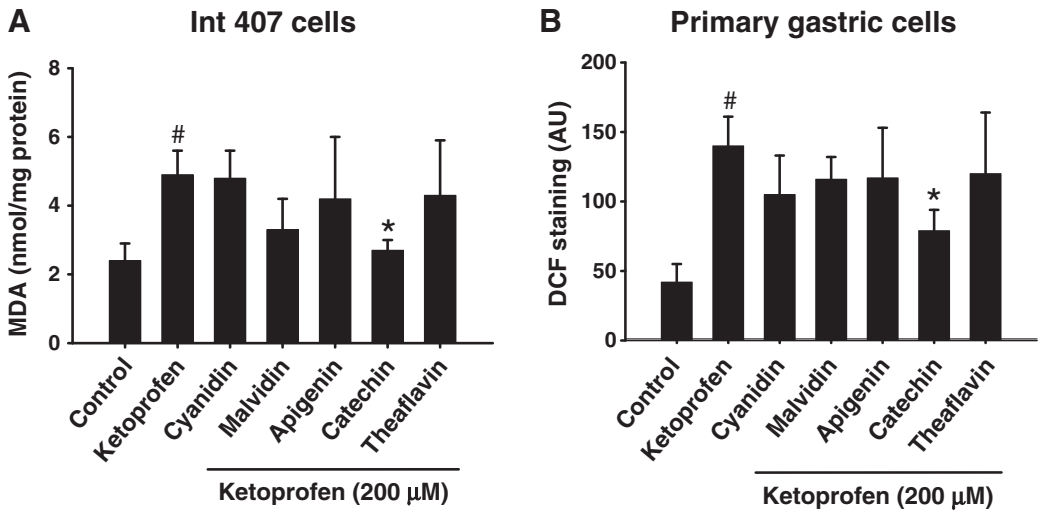

Fig. 1. The protective effects of polyphenols on ketoprofen-induced changes in the levels of lipid peroxidation and ROS in Int-407 and rat primary gastric cells. Cells were pretreated with 25 μM cyanidin, malvidin and apigenin for 1 h or 100 μM of catechin and theaflavin for 5 h and then exposed to ketoprofen for 1 h. Results are shown as the mean±SD for $n=3$. #Significant difference ($P<.05$) from the control group. *Significant difference ($P<.05$) from the ketoprofen-only group.

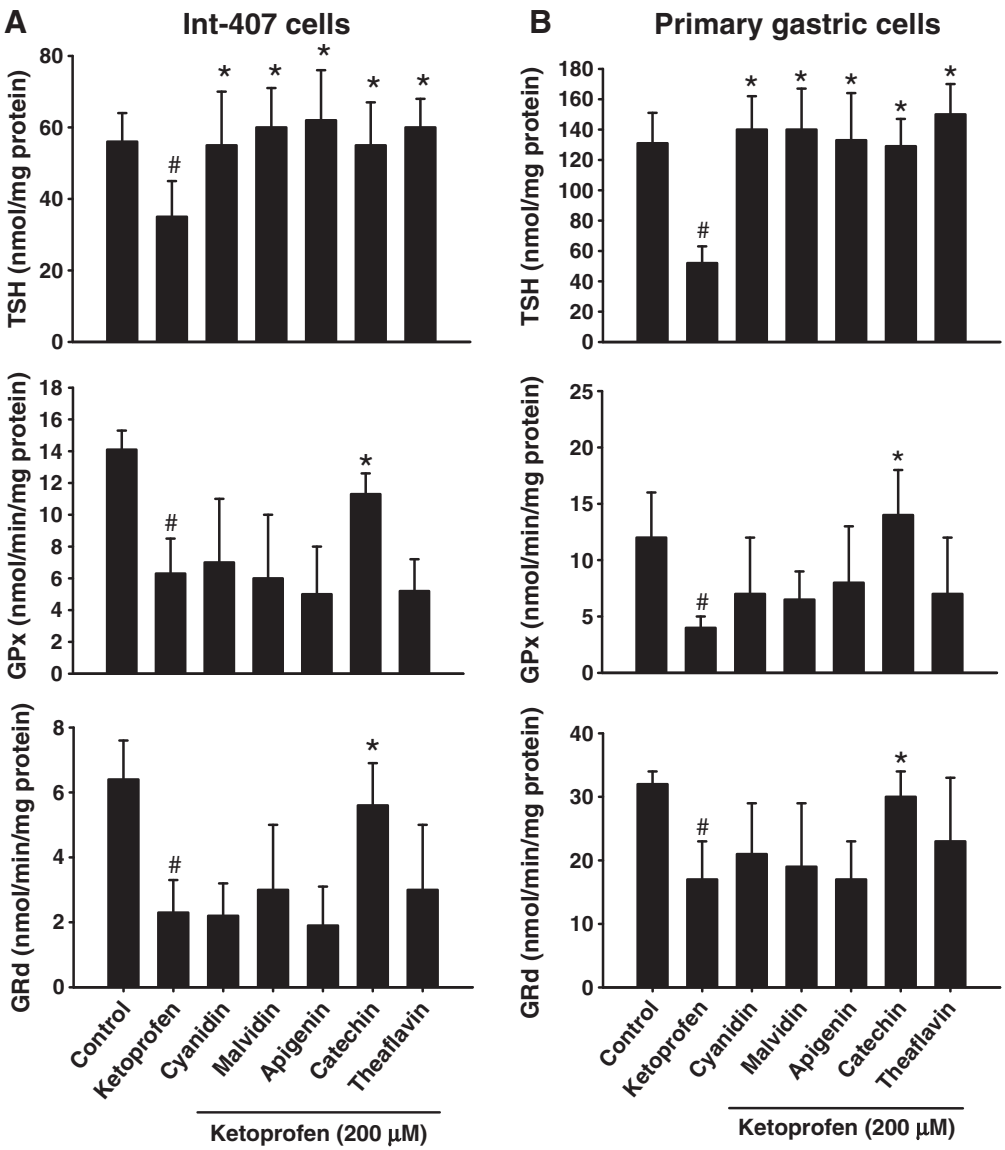

Fig. 2. The protective effects of polyphenols on ketoprofen-induced changes in the levels of (A) TSH, (B) GPx and (C) GRd activity in Int-407 and rat primary gastric cells. Cells were pretreated with 25 μM cyanidin, malvidin and apigenin for 1 h or 100 μM catechin and theaflavin for 5 h, and then exposed to ketoprofen for 1 h. Results are shown as the mean±SD for $n=3$. #Significant difference ($P<.05$) from the control group. *Significant difference ($P<.05$) from the ketoprofen-only group.

40.5% relative to the ketoprofen-only group (Fig. 1A, $P<.05$). In rat primary gastrointestinal cells, the catechin treatment reduced ROS production by 30.0% relative to the ketoprofen-only group (Fig. 1B, $P<.05$).

3.2. The effect of polyphenols on ketoprofen-induced changes in the levels of TSH, GRx and GRd activity in Int-407 and rat primary gastric cells

Previous research has suggested that up-regulation of antioxidant enzymes may be the most critical cytoprotective mechanism against oxidative stress [9]. As shown in Fig. 2A, Int-407 cells treated with ketoprofen displayed reduced activity of GPx and GRd by 55.3% and 64.1%, respectively, relative to the control group ($P<.05$). Similarly, rat primary gastric cells treated with ketoprofen, GPx and GRd activity decreased by 66.7% and 46.9%, respectively, relative to the control group (Fig. 2B, $P<.05$). The levels of TSH play an important role in maintaining intracellular redox status as well as antioxidation or detoxification of ROS [20]. Our results also indicated that the five polyphenols contributed to an increase in TSH expression in Int-407 cells and rat primary gastrointestinal cells treated with ketoprofen (Fig. 2A and Fig. 2B, $P<.05$). Among these polyphenols, catechin showed the most significant ($P<.05$) restoration of TSH, GPx and GRd activity in Int-407 cells and rat primary gastrointestinal cells treated with ketoprofen (Fig. 2A and Fig. 2B). These results suggest that catechin may provide a potential therapeutic approach for oxidative-stress-induced gastrointestinal injury.

3.3. Catechin affects the regulation of HO-1 by Nrf2

Because Nrf2 is a critical transcription factor involved in the regulation of phase II detoxification enzymes, we examined whether catechin affected Nrf2 translocation and activation of HO-1 expression in Int-407 cells. Treatment of the cells with 100 μM catechin for 1–4 h resulted in a time-dependent increase in the nuclear/cytosol Nrf2 ratio and total HO-1 protein expression, and also decreased the amount of cytosol Nrf2 protein expression (Fig. 3A–C). To determine whether the increased HO-1 activity induced by Nrf2 could confer

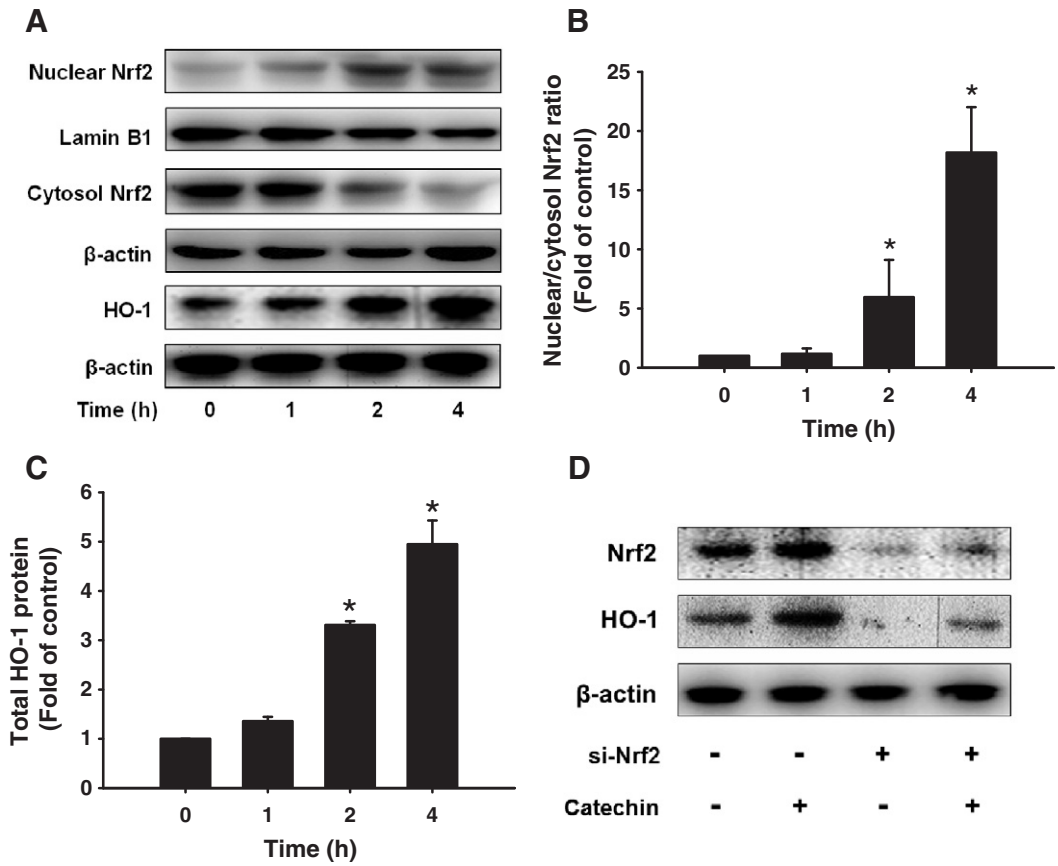

Fig. 3. Catechin affects the regulation of HO-1 by Nrf2 in Int-407 cells. Cells were treated with catechin (100 μM) for the indicated amount of time. (A) Cytosol, nuclear extracts and whole cell lysates were subjected to Western blot analysis using antibodies specific to HO-1, Nrf2, β-actin and lamin B1. (B) The Nrf2 nuclear/cytosol ratio and (C) total HO-1 protein expression were quantified by densitometry using UVP VisionWorks LS Image Acquisition and Analysis software. Results are shown as the mean±SD for $n=3$. *Significant difference ($P<.05$) from the other time points. (D) Cells were pretreated with si-Nrf2 (30 nM) for 48 h and then exposed to catechin (100 μM) for 4 h, and the levels of Nrf2 and HO-1 protein expression were analysis by Western blot.

with catechin, cells were pretreated with the silence Nrf2 (si-Nrf2). As shown in Fig. 3D, catechin-mediated increase in HO-1 protein expression was moderately blocked by si-Nrf2. These results indicate that Nrf2 might be involved in the regulation of HO-1 expression by catechin.

3.4. The effect of catechin on ketoprofen-induced changes in the levels of LDH leakage, lipid peroxidation (MDA), DNA damage (8-OHdG), GRd and GPx activity, and GSH and GSH/GSSG ratio in intestinal mucosa

Administration of ketoprofen (50 mg/kg/day) caused a significant increase in the levels of LDH leakage, MDA and DNA damage. In addition, the administration of ketoprofen resulted in a significant reduction in the activity of GRd, GPx, GSH and GSH/GSSH in the intestinal mucosa of SD rats (Fig. 4). Animals treated with catechin (14 and 35 mg/kg/day) exhibited a significant ($P<.05$) dose-dependent inhibition of oxidative damage. These results suggest that catechin was able to reverse the ketoprofen-induced impairment of the antioxidant system in the intestinal mucosa of these SD rats.

3.5. The effect of catechin on the gene expression of COX-1 and COX-2 in the intestinal mucosa

The level of COX-1 and COX-2 expression provides a useful indicator of the extent of inflammation following ketoprofen treatment. Neither ketoprofen nor catechin caused a significant difference in the level of COX-1 expression (Fig. 5A). However, animals dosed with ketoprofen exhibited a significant increase in the level of COX-2 expression ($P<.05$) that was partly abrogated by pretreatment with catechin (35 mg/kg/day) (Fig. 5B, $P<.05$).

3.6. Pathological features of the inner surface of the stomach and intestine of rats dosed with ketoprofen

The protective effects of catechin against damage to the stomach and intestinal mucosa were investigated by H&E staining. Animals that were pretreated with catechin (35 mg/kg/day) for 21 consecutive days prior to the administration of ketoprofen exhibited reduced incidence of gastric and intestinal mucosal lesions (Fig. 6).

4. Discussion

NSAIDs such as ketoprofen are widely used in clinical medicine to alleviate the pain and swelling of rheumatoid arthritis and other inflammatory disorders. However, despite their benefit, the use of these drugs may cause gastrointestinal bleeding and perforation [2,4]. Digestive diseases, such as ulcers, are associated with hydrochloric acid, pepsin, refluxed bile and ROS. Recent reports have associated ROS with the pathogenesis of acute gastric mucosal injuries induced by ischemia–reperfusion, stress, HCl, *Helicobacter pylori*, ethanol and NSAIDs in rats [4,7,18,21]. Therefore, some researchers have focused on the use of natural antioxidants to protect the gastrointestinal mucosa of patients using NSAIDs. Vegetables and fruits are important dietary sources of natural antioxidants and have been shown to act as

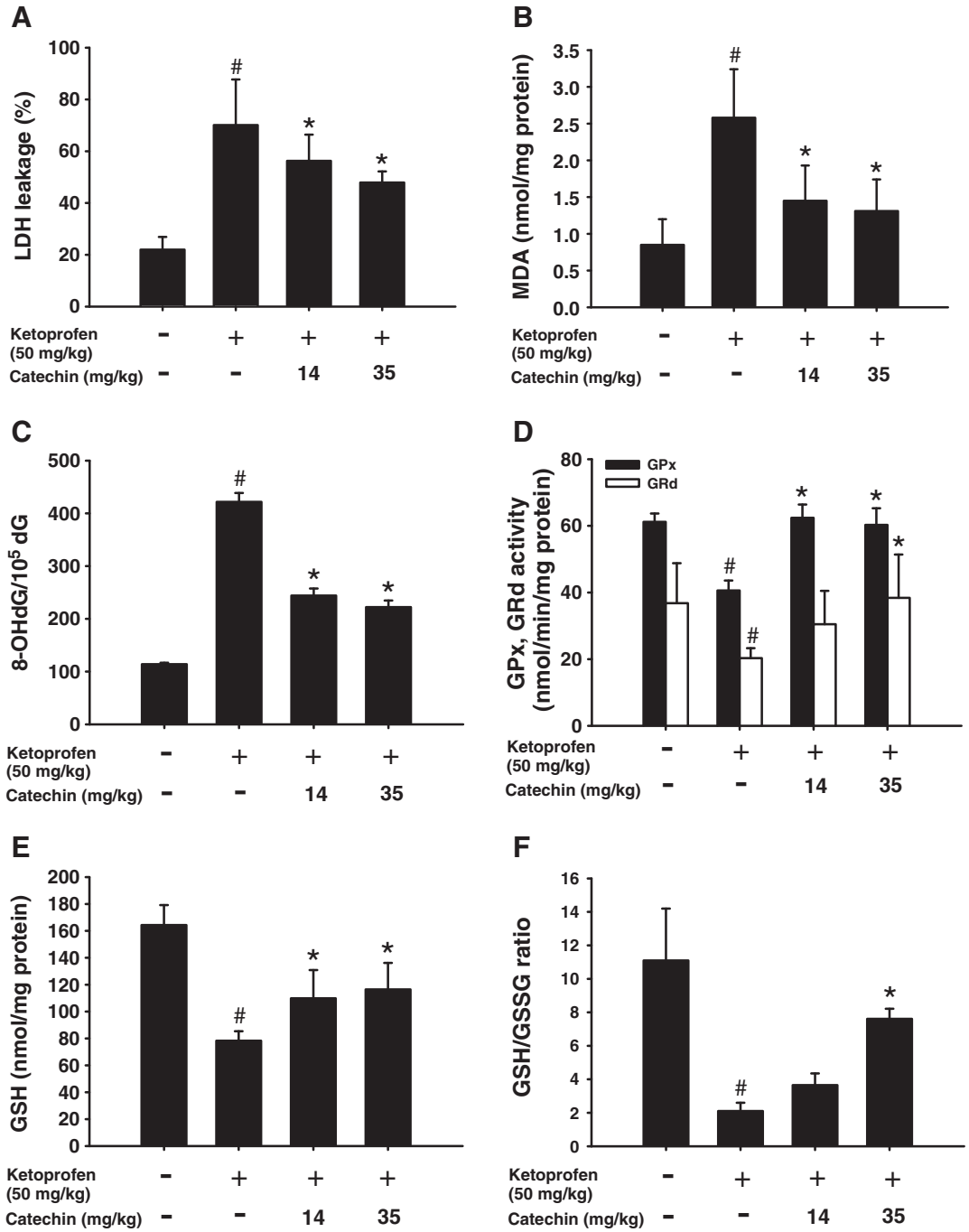

Fig. 4. The effect of catechin on ketoprofen-induced changes in the levels of (A) LDH leakage, (B) lipid peroxidation (MDA), (C) DNA damage (8-OHdG), (D) GR and GPx activity, (E) GSH and (F) GSH/GSSG ratio in the intestinal mucosa of SD rats. Data are shown as the mean±SD for $n=6$. #Values were significantly different ($P<.05$) from the control group. *Values were significantly different ($P<.05$) from the ketoprofen-only group.

effective ROS scavengers, metal ion chelators and trapping agents for reactive carbonyl species, and they may play an important role in gastrointestinal protection [22]. Therefore, naturally occurring antioxidants may be useful for the prevention of and/or therapeutic intervention against gastrointestinal disorders resulting from oxidative damage. Brigelius-Flohe [23] has shown that gastrointestinal isoenzyme (GI-GPx) is most related to classical enzyme, which might serve as barrier against hydroperoxide form metabolism of xenobiotics. Our preliminary work has shown that pretreatment with anthocyanins (malvidin, cyanidin and apigenin) for 1 h or polyphenols (catechin and theaflavin) for 5 h strongly induces GPx activity in Int-407 cells, suggesting that they play an important role in preventing gastrointestinal oxidative injury. The aims of this study were to evaluate the protective effects of polyphenols or anthocyanins on ketoprofen-induced oxidative damage *in vitro* and *in vivo*.

Ketoprofen has previously been shown to cause cellular damage via oxidative stress *in vivo* [24,25]. Our results show that treatment with ketoprofen not only increased the levels of lipid peroxidation (MDA) and ROS (Fig. 1) but also decreased the levels of intracellular antioxidants such as GPx, GRd and TSH in human Int-407 or rat primary gastric cells (Fig. 2). The selenium-dependent enzyme GPx is thought to act as barrier against hydroperoxide attack. GRd is

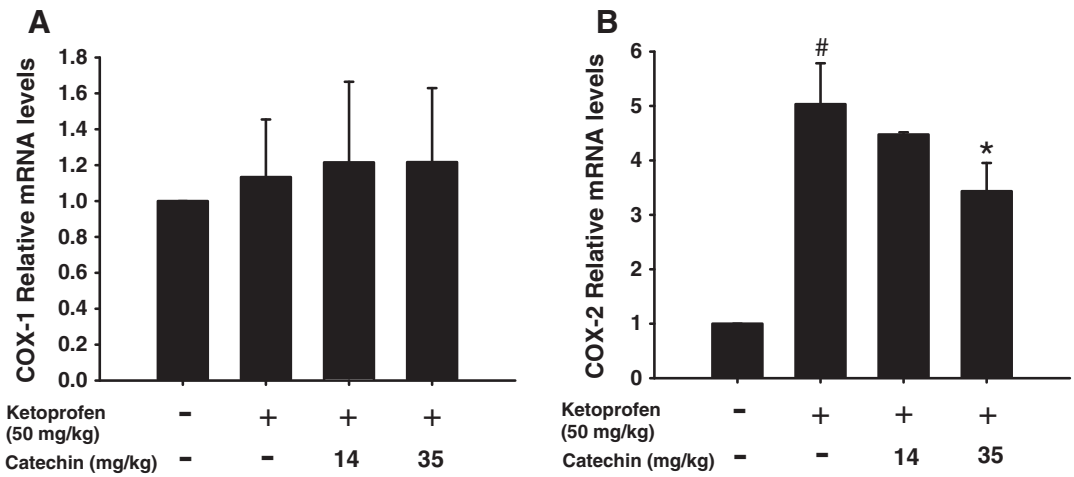

Fig. 5. The effect of catechin on ketoprofen-induced changes in the level of COX-1 and COX-2 expression in rat intestinal mucosal tissue. Data are shown as the mean±SD for $n=6$. #Values are significantly different ($P<.05$) from the control group. *Values are significantly different ($P<.05$) from the ketoprofen-only group.

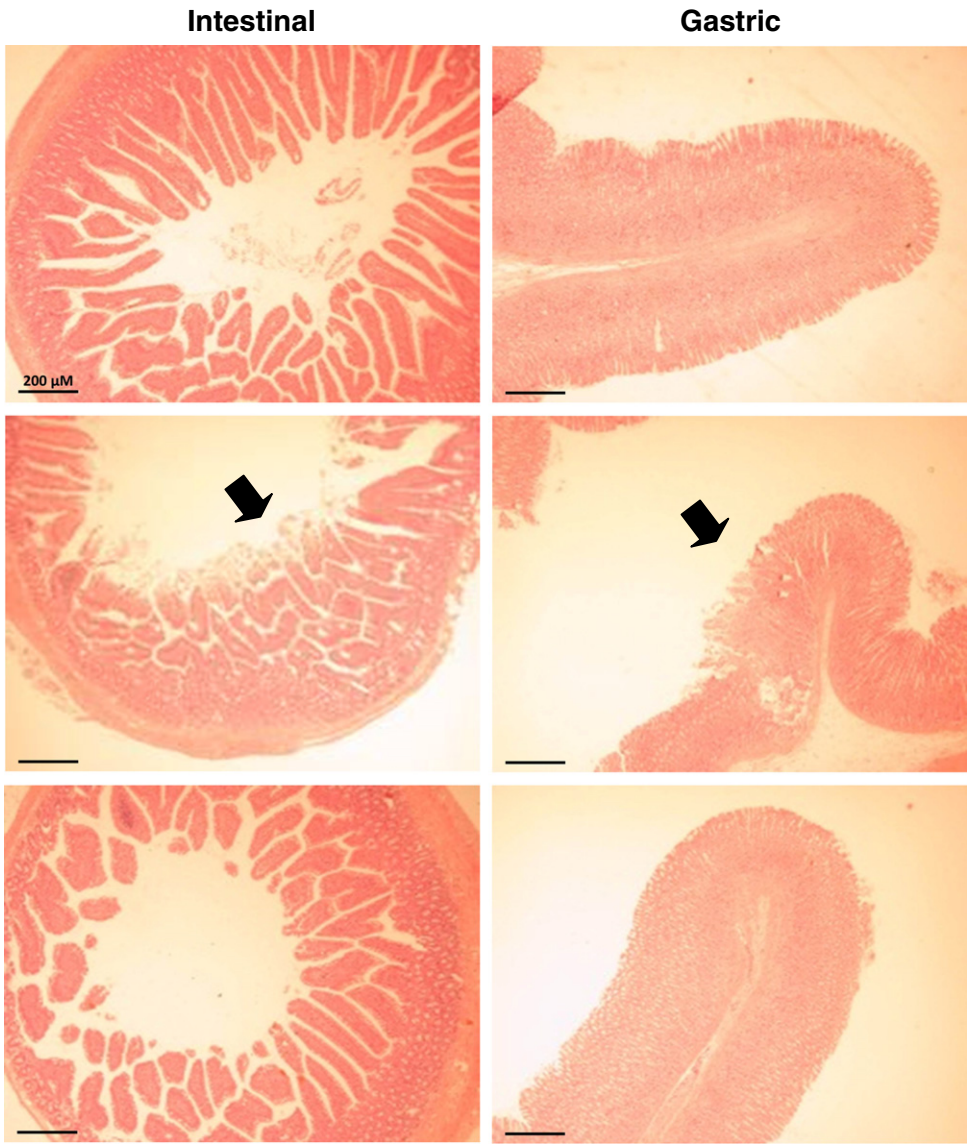

Fig. 6. The morphology of ketoprofen-induced gastrointestinal ulcers in SD rats with and without pretreatment with catechin for 21 days. Ketoprofen-induced damage to the gastric and intestinal mucosa is indicated by the arrows. Photomicrographs of H&E staining were imaged at 100× magnification.

responsible for maintaining equal amounts of reduced GSH and oxidized GSH (GSSG). Therefore, the ratio of GSH/GSSG is a well-known index for the level of oxidative stress. The activation of GRd plays an important role in elevating the concentration of GSH, which maintains the redox status of the organism [26]. Bansal et al. [20] reported that the levels of TSH play an important role in maintaining intracellular redox status as well as antioxidation or detoxification from ROS. We therefore sought to elucidate the potential protective effects of catechin, theaflavin, malvidin, cyanidin and apigenin against ketoprofen-induced oxidative damage. Of these compounds, catechin was the most effective in significantly decreasing the levels of lipid peroxidation and ROS and increasing the levels of intracellular GPx, GRd and TSH in ketoprofen-damaged human Int-407 and rat primary gastric cells. Supplementation with catechin appears to increase the antioxidant capacity of the cells not only through its own natural antioxidant properties but also through stimulating the expression of antioxidant and detoxifying enzymes. Our results may indicate a protective role of catechin that is worthy of further investigation.

We further investigated the relationship between antioxidant systems and the protective effects of catechin against ketoprofen-induced gastrointestinal injury *in vivo* by orally administering catechin to SD rats followed by gavage with ketoprofen. Proton pump inhibitors including omeprazole, pantoprazole and lansoprazole have been shown to effectively prevent NSAIDs-induced damage of stomach tissue [27,28]. Fornai et al. [24] reported that administration of lansoprazole in a 60-μg/kg/day dose also reversed the effects of indomethacin, diclofenac, piroxicam or ketoprofen on the expression of myeloperoxidase, MDA and GSH in the mucosa. Similarly, we found that administration of catechin at 35 mg/kg/day reduced or reversed ketoprofen-induced damage to the intestinal mucosa of SD rats. Catechin inhibited LDH leakage, lipid peroxidation and DNA damage and reversed the ketoprofen-induced reduction in the activities of GPx, GRd, GSH and the GSH/GSSG ratio (Fig. 4). These results suggest that catechin protects against NSAIDs-induced oxidative injury to gastrointestinal tissue.

Several studies have suggested that the gastrointestinal toxicity of NSAIDs in rats arises from a combination of COX-1 inhibition and stimulation of COX-2 enzyme production in gastric mucosal lesions. COX-1 converts arachidonic acid into PGs. PGs are potent vasodilators and control almost all aspects of gastric mucosal defense and healing. Inhibitors of COX-1 and PG synthesis have been shown to cause serious gastrointestinal damage. Enhanced COX-2 gene expression is associated with the inflammatory response [6,25,29]. Some studies have suggested that ketoprofen is a photodegradation product including enantiomers S(+)- and S(−)-ketoprofen that does not have a significant effect on COX-1 expression. However, the rats orally administered with S(±)-ketoprofen exhibited a significant increase in the level of COX-2 expression [6,30–32]. Therefore, reducing COX-2 enzyme expression may be an effective therapeutic strategy for preventing and treating photodegradation products of ketoprofen-induced gastrointestinal mucosal ulcer. In this study, our data also show that COX-1 gene expression was not significantly affected by either ketoprofen or catechin, but the increase in COX-2 gene expression in rats dosed with ketoprofen was partially abrogated by treatment with catechin (Fig. 5). Shan et al. [33] reported that the administration of epigallocatechin-3-gallate at 40 and 80 mg/kg/day dose-dependently reduced the formation of cholesterol gallstones in a C57BL/6 mouse model by down-regulating the activity of proinflammatory nuclear factor (NF-κB) and up-regulating the activity of peroxisome proliferator activated receptor gamma (PPARγ). A previous study demonstrated that administration of catechin (50, 100 and 200 mg/kg/day) for 7 and 15 consecutive days significantly inhibited the induction of gastrointestinal mucosal lesions caused by acetic acid in SD rats [34]. Moreover, some reports have suggested that oral administrant of catechin prevent ethanol, water immersion (stress), ischemia–reperfusion and *Helicobacter pylori*-infected stomach ulcer *in vivo* [3,35,36]. Furthermore, as demonstrated in the histopathological staining of the stomach and intestinal tissues found in Fig. 6, we found that administration of catechin (35 mg/kg/day) strongly inhibited ketoprofen-induced gastrointestinal mucosal injury. Taken together, these studies suggest that catechin may provide a potential therapeutic approach to protect patients with inflammation-associated disorders from developing gastrointestinal ulcers. Taken together, these studies suggest that catechin may provide a potential therapeutic approach to protect patients with inflammation-associated disorders from developing gastrointestinal ulcers.

Previous research has suggested that up-regulation of antioxidant enzymes may be the most critical cytoprotective mechanism against oxidative stress [9]. The transcription factor Nrf2 plays an important role in this process by forming heterodimers with small muscle aponeurotic fibrosarcoma proteins and binding to antioxidant responsive elements in the genome. Proteins that are up-regulated by this pathway include NQO1, glutathione *S*-transferases, GPx, GRd and HO-1 [9,10]. Of these enzymes, HO-1 may be the most crucial in reversing the damage caused by oxidative stress because it is the major enzyme involved in the degradation of heme [10]. We recently found that sulforaphane effectively prevents indomethacin-induced injury of Int-407 cells and the induction of HO-1 by sulforaphane via the ERK and Nrf2 pathways plays a key role in this cytoprotective effect [11]. Yu et al. [37] reported that green tea polyphenol extract strongly stimulated the expression of antioxidant enzymes via the mitogen-activated protein kinase pathway in HepG2 cells. We found that catechin is capable of strongly inducing Nrf2 activity and HO-1 protein expression in a time-dependent manner (Fig. 3A–C). To determine whether the increased HO-1 activity induced by Nrf2 could confer with catechin, cells were pretreated with the si-Nrf2. As shown in Fig. 3D, catechin-mediated increase in HO-1 protein expression was moderately blocked by si-Nrf2. Together, these studies suggest that Nrf2 plays a key role in the induction of HO-1 expression by exogenous antioxidant compounds.

Our observations suggest that catechin effectively prevents ketoprofen-induced intestinal epithelial cell injury and induces expression of HO-1 via modulation of Nrf2 protein translocation. More importantly, treatment of SD rats with catechin prior to the administration of ketoprofen successfully inhibited oxidative damage and reversed the impairment of the antioxidant system in the intestinal mucosa. Our results support the possible use of catechin as a dietary preventive agent against intestinal injuries caused by oxidative stress.

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