European Journal of Pharmacology 654 (2011) 106–116

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, Gastrointestinal and Urogenital Pharmacology

Zinc protects against indomethacin-induced damage in the rat small intestine

Nageswaran Sivalingam, Suresh Pichandi, Aaron Chapla, Asha Dinakaran, Molly Jacob *

Department of Biochemistry, Christian Medical College, Vellore, 632002, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 9 February 2010
Received in revised form 8 December 2010
Accepted 11 December 2010
Available online 24 December 2010

Keywords:
Anti-inflammatory agents non-steroidal
Bacteria
Indomethacin
Intestine small
Metallothionein
Zinc

ABSTRACT

The clinical utility of nonsteroidal anti-inflammatory drugs (NSAIDs) is often limited by the adverse effects that they produce in the small intestine. Alterations in the composition and functions of the glycocalyx and brush border membranes of the rat small intestine have been shown to occur in response to indomethacin, an NSAID often used in the study of adverse effects of these drugs. The micronutrient, zinc, has been documented to have cytoprotective effects in the gastrointestinal tract. The aim of this study was to evaluate the potential of zinc to reduce indomethacin-induced small intestinal damage. We pre-treated rats with zinc sulphate (50 mg/kg body weight) 2 h before administration of indomethacin (20 mg/kg body weight) and sacrificed the rats 1, 12 or 24 h after indomethacin. The extent of small intestinal mucosal damage and the content of lipids and sugars in the mucosa were determined. Bacterial counts in the intestinal lumen and the mucosa were ascertained. Activities of matrix metalloproteinases (MMPs) and levels of metallothionein in the mucosa were also measured. Pre-treatment with zinc sulphate was found to reduce the extent of indomethacin-induced mucosal damage. It also prevented drug-induced changes in the content of lipids and sugars in the mucosa. Drug-induced increases in activities of the MMPs and bacterial counts in the intestine were also attenuated by zinc. Metallothionein levels were significantly higher in animals pre-treated with zinc. We conclude that zinc was effective in protecting against indomethacin-induced small intestinal damage and suggest that it may do so by induction of metallothionein.

1. Introduction

Long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs), in the treatment of chronic inflammatory diseases, is often associated with enteropathy (Bjarnason et al., 1993), with chronic bleeding and protein loss being features of this condition (Thiefin and Beaugerie, 2005). This often results in poor patient compliance and limits the clinical utility of these drugs.

Mechanisms that have been shown to contribute to NSAID-induced gastrointestinal toxicity include inhibition of cyclooxygenase (Vane, 1971), mitochondrial dysfunction (Nagano et al., 2005; Somasundaram et al., 1997), induction of inducible nitric oxide synthase (iNOS) (Konaka et al., 1999; Tanaka et al., 1999) and oxidative stress (Basivireddy et al., 2002, 2003; Nagano et al., 2005).

Administration of indomethacin has been reported to lead to oxidative stress and alterations in the intestinal brush border membranes (BBM) and glycocalyx of the rat small intestine (Basivireddy et al., 2002, 2003, 2005). Surfactant-like particles (SLP) that form part of the glycocalyx (DeSchryver-Kecskemeti et al., 1989) have a high content of glycoproteins, glycolipids and sugars. The sugar epitopes of these molecules act as attachment sites for normal flora and serve to trap and expel pathogens (Moncada et al., 2003), thus contributing to host-bacterial interactions.

Luminal bacteria are known to play a role in NSAID-induced intestinal injury (Melarange et al., 1992; Robert and Asano, 1977). Germ-free animals treated with indomethacin showed significantly fewer intestinal lesions compared with normal animals (Weissenborn et al., 1985). It is thought that drug-induced alterations in mucosal glycosylation and gut flora may facilitate translocation of luminal bacteria into the mucosa and subsequent development of NSAID-induced enteropathy (Basivireddy et al., 2005).

Zinc, an essential trace metal, has well-documented anti-oxidant properties. Low concentrations of zinc have been associated with slower wound healing (Seymour, 1996). It has been shown to facilitate healing of ulcers in the stomach (Mann et al., 1992) and duodenum (Troskot et al., 1997). It has also been reported to inhibit the growth of enteric pathogens in vitro (Surjawidjaja et al., 2004), attenuate *Helicopylori felis-induced* gastritis in mice (Tran et al., 2005) and ameliorate indomethacin-induced oxidative stress (Basivireddy et al., 2002, 2003).

Metallothionein, a small molecular weight metal-binding protein rich in cysteine (Coyle et al., 2002) has marked anti-oxidant properties and is induced by zinc (Andrews, 2000; Cousins and Lee-Ambrose, 1992; Powell, 2000). It has been shown to scavenge hydroxyl radicals and prevent oxidative stress (Bodiga and Krishnapillai, 2007).

Matrix metalloproteinases (MMP), zinc-dependent endopeptidases actively involved in extracellular matrix degradation (Visse and Nagase, 2003), play an active role in tissue repair and re-modeling

* Corresponding author. Department of Biochemistry, Christian Medical College, Vellore, 632002, Tamil Nadu, India. Tel.: +91 416 2284267; fax: +91 416 2262788.
E-mail address: mjacob@cmcvellore.ac.in (M. Jacob).

0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.12.014

(Egeblad and Werb, 2002). MMP-2 and -9 have been reported to be activated by oxidative stress (Belkhiri et al., 1997; Rajagopalan et al., 1996). Such activation has been held to be involved in the development of damage in the heart and retina (Ali and Schulz, 2009; Kowluru and Kanwar, 2009).

In view of its properties, this study was designed to assess the potential of zinc to protect against indomethacin-induced damage in the rat small intestine and to ascertain the mechanisms by which its effects are mediated.

2. Materials and methods

Bovine serum albumin (BSA), L-cysteine hydrochloride, o-dianisidine, D-fucose, D-galactose, glucosamine, galactosamine, glucose oxidase, indomethacin, lipid standards, poly-L-lysine, sodium meta-periodate, sodium arsenite, thiobarbituric acid and D-mannose were obtained from Sigma Chemical Co., St. Louis, USA. Polyethylene glycol 4000, silica gel and crystal violet were purchased from Sisco Research Laboratory, Mumbai, India. McConkey's media and agar were obtained from Hi-media, Mumbai, India. Specific antibodies were obtained from Stressgen Bioreagents, Michigan, USA (metallothionein-1), Santa Cruz, CA, USA (iNOS), Lab Vision Corporation, CA, USA (MMP-2 and 9), Sigma, St. Louis, USA (beta-actin) and Pierce Biotechnology, CA, USA (anti-mouse IgG conjugated with horse-radish peroxidase). The COX Fluorescent Activity Assay Kit (Cat. no. 700200) was obtained from Cayman Chemical Company, USA. ^{109}Cd was obtained from Board of Radiation and Isotope Technology, Department of Atomic Energy, Government of India. The reverse transcriptase core kit, qPCR Master Mix Plus for SYBR Green kit and the primer sequences were obtained from Eurogentec, Belgium. All chemicals used were of analytical grade.

2.1. Animals

Adult male Wistar rats (200–250 g) bred in the licensed animal house facility of the Christian Medical College, Vellore, India, were used for all the experiments. The animals were housed in standard rat cages (421×290×190 mm), with each cage housing five animals. They were exposed to 12 h dark–light cycles and allowed unlimited access to standard rat chow and water. The experiments done were approved by the institutional animal ethics committee and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

2.2. Protocol for drug administration

Rats were fasted over-night and pre-treated with zinc sulphate (50 mg/kg dissolved in water, equivalent to 11.3 mg of elemental zinc) by oral gavage (Joseph et al., 1999). Two hours later, the animals were given indomethacin (Indo; 20 mg/kg in 5% sodium bicarbonate) by gavage. This dose was chosen as it is documented that indomethacin produces biochemical effects and macroscopic damage in a rat model at this dose (Jacob et al., 2007). Control animals received the vehicles for zinc and indomethacin at the appropriate time periods. Animals were sacrificed by cervical dislocation at different time periods (1, 12 and 24 h) after the dose of indomethacin. The time periods chosen were based on earlier work (Basivireddy et al., 2002, 2005) and are specified in each of the studies carried out. The abdomen of each rat was opened immediately after sacrifice and the entire small intestine was removed.

2.3. Quantitation of ulcers

This was done 24 h after the dose of indomethacin. The isolated intestine was flushed with physiological saline to clear any residual food particles. It was then opened along its anti-mesenteric border and the tissue spread out carefully. The total numbers of mucosal ulcers seen along the entire length of the intestine were counted in all the treatment groups. The numbers of pointed ulcers (less than 5 mm in length) and longitudinal ulcers (more than 5 mm in length) were also determined (Somasundaram et al., 2002). Images of the ulcers seen were obtained using a HP digital camera.

2.4. Isolation of SLP

SLP were isolated as described (Eliakim et al., 1989) with minor modifications, after sacrificing the rats 24 h after the dose of indomethacin. Briefly, the entire small intestine was removed, opened along its anti-mesenteric border and washed with physiological saline. The thin viscous layer on the surface of the mucosa was scraped gently with filter paper (Whatmann No. 3) to remove the gelatinous SLP. This was done along the entire length of the intestine. The filter paper was soaked in 2 ml of phosphate-buffered saline (PBS), pH 7.4, centrifuged at 3000 rpm for 10 min at 4 °C and the supernatant collected. This procedure was repeated and the supernatants collected were pooled to obtain SLP. The SLP preparations were used for the studies described below.

2.5. Preparation of brush border membranes (BBM)

For this part of the study, rats were sacrificed 24 h after the dose of indomethacin and the small intestine isolated. Once opened along its anti-mesenteric border, the whole length of the small intestinal mucosa was scraped with a glass slide. The scrapings obtained were homogenised and brush border membranes (BBM) were prepared from the homogenates as described (Prabhu and Balasubramanian, 2001). The purity of the BBM preparation was determined by enrichment of the marker enzyme, alkaline phosphatase (Dorai and Bachhawat, 1977). The BBM preparations were used for the studies described below.

2.6. Analysis of lipids in SLP and BBM preparations

Lipids were extracted from the SLP and BBM samples by as described (Bligh and Dyer, 1959). Neutral lipids were separated on silica gel G plate, using the solvent system consisting of hexane, diethyl ether and acetic acid (80:20:1 v/v). The separated lipids were identified by exposure to iodine vapour. Spots corresponding to standards were eluted. Cholesteryl esters (CE), free cholesterol (Zlatkis et al., 1953), triacylglycerol (TAG) and diacylglycerol (DAG) (Snyder and Stephens, 1959) levels were estimated.

Phospholipids were separated on silica gel H plates using the solvent system of chloroform, methanol, acetic acid and water (25:14:4:2 v/v). Individual phospholipids were identified by exposure to iodine vapour. Spots corresponding to standards were eluted and quantitated by phosphate estimation after acid hydrolysis (Bartlett, 1959). Phosphatidic acid (PA) was separated on oxalic acid-impregnated silica gel G plates and quantitated by phosphate estimation (Cohen and Derksen, 1969).

2.7. Estimation of sugar content in SLP and BBM

The content of sugars in the SLP and BBM was measured. The level of sialic acid was estimated as described (Saifer and Gerstenfeld, 1962). Levels of hexose and fucose were also determined as described, with minor modifications (Djurdjic and Mandic, 1990). Briefly, to 200 µl of sample (corresponding to 10–20 µg of protein) 1 ml of a cooled mixture, consisting of six parts of concentrated sulphuric acid and one part of water, was added. This was heated for 3 min in a boiling water bath and then immediately cooled in ice. To this, 0.2 ml of CPS reagent (containing 1% of L-cysteine hydrochloride and 0.075% of phenol) was added. The mixture was kept in an ice-bath for 60 min

and then its absorbance was read at 398 nm for fucose and 490 nm for hexoses. Total hexosamines were estimated using Ehrlich's reagent as described (Ludowieg and Benmaman, 1967).

2.8. Isolation and quantitation of bacteria from intestinal contents and mucosa

Twenty four hours after the dose of indomethacin the small intestine was isolated and the small intestine was infused with 15 ml of sterile saline. The contents of the lumen were collected into a sterile tube. This fluid was then serially diluted in saline and cultured on McConkey agar plates. The numbers of *E. coli* grown were quantitated by counting the number of colony forming units (cfu) per milliliter of intestinal washing.

Small intestinal mucosal tissue, (approximately 1 cm^2 in size), was taken from a site 1 cm proximal to the ileo-caecal junction in each animal, washed in sterile saline and homogenized in 1 ml of sterile saline. The homogenate was serially diluted and subjected to quantitative culture analysis as described above. The numbers of bacteria grown were expressed as cfu/cm^2 tissue.

2.9. Assessment of bacterial adherence in vitro

E. coli strains were isolated from the caecal contents of control animals. These were grown on McConkey's media. They were identified using standard microbiological techniques. These organisms were used for in vitro assays to measure bacterial adherence, as described below.

E. coli isolated from the caecal contents of control rats were tested for their ability to adhere to microtitre plates coated with SLP or BBM, isolated from control and treated groups, as described above. Poly L-lysine (PLL) was coated on microtitre plates by adding 10 μg PLL in PBS to each well and leaving it at room temperature for 30 min. The unbound PLL was removed from the wells by gently washing the wells with PBS. The wells were dried at room temperature for 1 h. They were then loaded with either SLP or BBM corresponding to 30 μg of protein and allowed to stand at room temperature for 20 min. Unbound material was removed and its protein content estimated. The percentage of sample bound was calculated from measurements of the protein content in the bound and unbound fractions of the samples. We have found that this resulted in approximately 40–50% of the SLP and 20–30% of BBM added coating each well (data not shown). The wells were washed with PBS. To each of these wells, 0.1 ml of an *E. coli* suspension, containing 3×10^6 bacteria, was added and incubated for 30 min at room temperature. Following this, the media in the wells, containing unbound bacteria, was collected, serially diluted and quantitated by culturing on McConkey agar plates. Colony counts were done to obtain the number of unbound bacteria in the wells. The percentage of bound bacteria was calculated using the following formula:

$$\begin{aligned}&(\text{Total number of bacteria})-(\text{unbound bacteria})\\&\quad=\text{bound bacteria Bound bacteria / total number of bacteria}\\&\quad=\text{percentage of bacteria bound to the sample.}\end{aligned}$$

E. coli bound to the wells was also quantitated using crystal violet staining (Merritt et al., 1998). For this, following removal of unbound bacteria, microtitre wells were washed with PBS. This was followed by addition of 0.1 ml of 10% formalin. This was allowed to stand for 5 min at room temperature. The formalin was then washed out using PBS and then 0.1 ml of 1% crystal violet was added to each well. The dye solution was removed after 3 min and the wells washed twice with PBS. The wells were then dried and 0.3 ml of 95% ethanol added to each well and kept for 5 min. After 5 min, the optical density (OD) of the solution in each well was read at 540 nm. The value of the OD measured indicated the degree of bacterial adherence.

2.10. Determination of matrix metalloproteinase activity

For this part of the study, rats were sacrificed 1, 12 and 24 h after the dose of indomethacin. The small intestine was isolated and opened along its anti-mesenteric border. The entire length of the mucosal surface was scraped with a glass slide. The scrapings obtained were homogenised on ice in 10 mmol/L phosphate-buffered saline that contained serine- and cysteine-protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) 500 μg/ml, leupeptin (0.5 μg/ml), aprotinin (1 μg/ml), and E64 (5 μg/ml). The homogenates were centrifuged at 14,000 *g* for 30 min at 4 °C (Rosario et al., 2004). The supernatant obtained was used for estimation of the activities of matrix metalloproteinases-2 and -9, by gelatin zymography (Keck et al., 2002; Lindsey et al., 2001). Briefly, homogenates containing 5 μg of protein were denatured with SDS under non-reducing conditions and were loaded on to 10% polyacrylamide gels containing 0.1% gelatine. HT1080 cells-conditioned medium was used as a positive control for MMP-2 and -9. Electrophoresis was carried out at 80 V in a cold room. Equal loading of the samples was confirmed by staining the gel with Coomassie blue solution after the separation and looking for equal staining of bands in each lane. The proteins were then re-natured with 2% Triton X-100 solution for 90 min in a cold room, using a rocking platform. The gel was then incubated for 18 h at 37 °C in a developing buffer, pH 7.4, containing 50 mmol/L Tris–HCl, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 0.02% Brij35. The gel was subsequently developed with 0.5% Coomassie blue in 40% methanol and 10% acetic acid. It was then de-stained three times with the same solution without the Coomassie blue. The bands of clearing were visualized and documented using an AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA.).

2.11. Assessment of levels of MMP-2, MMP-9 and iNOS by Western blotting

Intestinal mucosal scrapings were obtained at 1, 12 and 24 h after the dose of indomethacin. They were homogenised as described above and samples corresponding to 50 μg of protein were denatured and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2 μm nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in PBS, containing 0.1% Tween-20, for 2 h. Thereafter, the membranes were incubated, overnight in a cold room, with specific antibodies. The secondary antibodies used were conjugated with horse radish peroxidase. Visualization of the bands was done by chemiluminesce (using Super Signal West Pico chemiluminescent substrate from Pierce Biotechnology, CA) on an AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA.).

2.12. Determination of activities of cyclooxygenase (COX) 1 and 2

Mucosal scrapings (obtained 1, 12 and 24 h after the dose of indomethacin) were homogenised on ice in 100 mM Tris–HCl, pH 7.5, containing leupeptin (0.5 μg/ml) and phenylmethylsulfonyl fluoride (PMSF) (1 mM). The homogenates were subjected to centrifugation at 10,000 *g* for 15 min at 4 °C. The supernatant was used for assays of COX activity, using a commercially available kit (COX Fluorescent Activity Assay Kit, Cayman Chemical Company, USA).

2.13. Determination of metallothionein levels

2.13.1. Cadmium-haemoglobin affinity (Cd-hem) assay

For this part of the study, rats were sacrificed 1, 12 and 24 h after the dose of indomethacin. The metallothionein content of the small

intestinal mucosa was estimated by the cadmium-haemoglobin affinity (Cd-hem) assay (Eaton and Toal, 1982). Briefly, small intestinal mucosal scrapings (obtained from the whole length of the tissue) were homogenized (1:10 w/v) in homogenization buffer (10 mM Tris buffer, pH 8.2). The samples were boiled for 2 min, cooled in ice-cold water and centrifuged at 10,000 *g* for 4 min at 4 °C. Fifty microlitres of the supernatant were added to 200 μl of ^{109}Cd (19.6 μM) and made up to a final volume of 400 μl with homogenization buffer. The samples were incubated at room temperature for 15 min. Excess ^{109}Cd was precipitated by adding 100 μl of 4% haemoglobin prepared from human blood. The samples were centrifuged at 10,000 *g* for 4 min at 4 °C. This was followed by careful removal of 450 μl of the supernatant. The radioactivity of this sample was determined using a PerkinElmer 1470 gamma counter. Total metallothionein concentration was expressed as nanomole of Cd bound per milligram of protein.

2.13.2. Western blot for metallothionein

The level of metallothionein was also determined by western blotting. Samples were prepared for western blotting as described above. Samples, corresponding to 50 μg of protein, were denatured and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2 μm nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in PBS, containing 0.1% Tween-20, for 2 h. Thereafter, the membranes were incubated, overnight in a cold room, with antibodies against metallothionein. The secondary antibodies used were conjugated with horse radish peroxidase. Visualization of the bands was done by use of a chemiluminescent substrate (Super Signal West Pico chemiluminescent substrate from Pierce Biotechnology, CA) on an AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA).

2.14. Real time PCR assays

These were carried out for metallothionein and iNOS. Snap-frozen mucosal scrapings (obtained from the whole length of the intestine) isolated after 1, 12 and 24 h after the dose of indomethacin were used to isolate total RNA using TRI-reagent (Sigma, USA). RNA was reverse-transcribed to cDNA (Reverse Transcriptase Core kit, Eurogentec, Belgium). Expression levels of mRNA for metallothionein, iNOS and beta-actin (used as a housekeeper gene) were analyzed by real-time quantitative polymerase chain reaction (PCR), using a qPCR Master Mix Plus for SYBR green I dNTP kit (Eurogentec, Belgium), according to the manufacturer's protocol. The primers used for metallothionein-1 and beta-actin (Jiang et al., 2005) and iNOS (Okazaki et al., 2009) were as follows:

Metallothionein-1
Forward — 5′-GCG ATC TCT CGT TGA TCT CC-3′
Reverse — 5′-CAG CTG CAC TTG TCC GAA G-3′
iNOS
Forward — 5′-CAT TGG AGG TGA AGC GTT TCG-3′
Reverse — 5′-CAG CTG GGC TGT ACA AAC CTT-3′
Beta-actin
Forward — 5′-ATC TGG CAC CAC ACC TTC-3′
Reverse — 5′-AGC CAG GTC CAG ACG CA-3′.

Expression levels of the 2 genes were calculated using the modified comparative CT method (Pfaffl, 2001) and normalized to that of beta-actin.

2.15. Statistical analysis

Data obtained in the various groups were analyzed by analysis of variance (ANOVA), using the Statistical Package for the Social Scientist (SPSS), version 11. A P value of less than 0.05 was taken to indicate statistical significance.

3. Results

3.1. Assessment of small intestinal damage

Rats treated with indomethacin developed ulcers 24 h later. The ulcers were seen scattered throughout the length of the intestine. The number of ulcers increased from the proximal to the distal end of the intestine (Fig. 1). The numbers of pointed and longitudinal ulcers that were seen are shown in Table 1. Pre-treatment of rats with zinc resulted in a significant decrease in the number and size of the ulcers, when compared with indomethacin-treated rats (Table 1). Control rats that received either only the vehicles or zinc alone did not develop any ulcers (Fig. 1).

3.2. Effects on the lipid content of SLP and BBM

Rats treated with indomethacin showed significant decreases in the levels of cholesteryl esters, cholesterol and triacylglycerol in SLP, with significant increases in levels of monoacylglycerol 24 h later (Table 2). In the case of BBM, there were significant decreases in levels of cholesteryl esters, triacylglycerol and diacylglycerol and significantly increased levels of monoacylglycerol levels in the indomethacin-treated rats when compared with control rats. Pre-treatment with zinc resulted in significant reversal of the drug-induced changes seen in the lipids in SLP and BBM. Levels of these neutral lipids did not change significantly in response to zinc alone.

Significant changes were also seen in the content of phospholipids in SLP and BBM in response to indomethacin. Levels of phosphatidylcholine and phosphatidylethanolamine were significantly decreased in SLP and BBM of indomethacin-treated animals when compared with control animals (Table 3). Levels of lysophosphatidylcholine and lysophosphatidylethanolamine were significantly higher in drug-treated SLP (Table 3). In the case of BBM, only lysophosphatidylethanolamine levels were significantly elevated (Table 3). Other phospholipids, such as phosphatidylinositol, sphingomyelin and phosphatidic acid, were not significantly affected (data not shown). Pre-treatment of the animals with zinc resulted in reversal of the drug-induced effects seen in the levels of phospholipids in SLP and BBM (Table 3). Administration of zinc alone did not affect these parameters significantly.

3.3. Effects on the sugar content of SLP and BBM

Twenty four hours after the dose of indomethacin, significant increases were seen in the content of fucose, galactose, hexosamine and sialic acid in SLP from rats treated with the drug, when compared with data from control rats (Table 4). There were significant decreases in the content of these sugars in drug-treated BBM (Table 4). Pre-treatment with zinc significantly reversed the effects of indomethacin on the levels of sugars in the SLP and BBM (Table 4). Administration of zinc alone did not affect the levels of the sugars in the SLP or the BBM.

3.4. Effects on bacterial counts in the small intestine

Twenty four hours after the dose of indomethacin, the numbers of bacteria in the intestinal contents and mucosa of the small intestine were significantly higher in indomethacin-treated rats when compared with control animals (Fig. 2A–D). Similar increases were seen in the number of bacteria in the caecal contents and caecal tissue of the drug-treated animals. Pre-treatment with zinc significantly reversed these effects. Administration of zinc alone did not affect the bacterial content of the small intestine or caecum significantly.

3.5. Effects on bacterial adherence to SLP and BBM in vitro

Significantly higher numbers of bacteria adhered to SLP isolated from indomethacin-treated rats, 24 h after the dose of the drug. These were observed by both methods (culture of unbound bacteria and quantification of bound bacteria by crystal violet staining) used to quantify the degree of adherence (Fig. 3A and B). In the case of BBM, significantly fewer bacteria tended to bind to the BBM from drug-dosed animals (Fig. 3C and D). The drug-induced effects in both SLP and BBM were reversed by pre-treatment with zinc (Fig. 3A–D). Administration of zinc alone did not significantly affect bacterial adherence to SLP or BBM, when compared with control data.

3.6. Effects on activity of MMP 2 and 9

Activities of MMP-2 and MMP-9 were determined at 1, 12 and 24 h after the dose of the drug. It was found that both MMP-2 and 9 activities were significantly increased 1 h after the dose of indomethacin (Fig. 4A), but not at the other time periods studied (data not shown). Pre-treatment with zinc was found to reverse these drug-induced effects (Fig. 4A). However, there were no significant changes in the protein content of MMP-2 and MMP-9, 1 h after the dose of indomethacin, as assessed by western blots (Fig. 4B).

3.7. Effects on iNOS

Animals treated with indomethacin showed significantly higher levels of iNOS in the intestine 12 h after the dose of the drug, but not at other time periods measured (data not show). This was seen at the level of mRNA and protein. Pretreatment with zinc did not affect these changes (Fig. 5).

3.8. Effects on activities of COX 1 and 2

Activities of COX-1 (1.36 ± 21 nmol/min/mg protein) and COX-2 (1.36 ± 21 nmol/min/mg protein) were significantly decreased in indomethacin-treated rats, 1 h after the dose of the drug, when compared with activities in control tissue (2.49 ± 0.41 nmol/min/mg protein for COX-1 and 2.83 ± 0.19 nmol/min/mg protein for COX-2). Pretreatment with zinc did not affect these values in these animals (1.94 ± 0.48 nmol/min/mg protein for COX-1 and 1.50 ± 0.37 nmol/min/mg protein for COX-2).

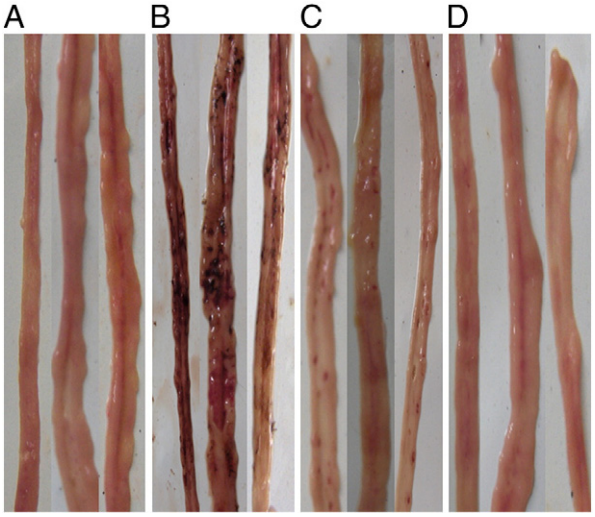

Fig. 1. Macroscopic damage produced in the small intestine by indomethacin, with and without pretreatment with zinc, 24 h after the dose of the drug. Each figure shows views of the intestine at proximal, middle and distal sections. (A) control, (B) indomethacin only, (C) zinc + indomethacin and (D) zinc alone.

Table 1
Numbers of pointed and longitudinal ulcers in the small intestine at 24 h, produced by indomethacin, with and without pre-treatment with zinc. Results are expressed as means ± S.D.; $n = 6$.

Groups	Number of pointed ulcers (<5 mm in length>)	Number of longitudinal ulcers (>5 mm in length)
Control	0	0
Indomethacin	89.25 ± (2.79)[a]	3.25 ± (0.95)[a]
Zinc + indo	49.75 ± (3.48)[b]	1.00 ± (0.81)[b]
Zinc alone	0	0

[a] Denotes $P<0.05$ when compared with control.
[b] Denotes $P<0.05$ when compared with the indomethacin-treated group.

3.9. Effects on levels of metallothionein

There was no significant effect of indomethacin on the level of metallothionein at any of the time periods studied. In the animals that received zinc, it was found that there was significant induction of metallothionein 1 h after the dose of indomethacin (as assayed by the cadmium-hemoglobin affinity assay) (Fig. 6A). Western blot analysis showed evidence of significant induction at 1 and 12 h after the drug (Fig. 6B). At the level of RNA, increased expression of metallothionein was seen only at 1 h (Fig. 7).

4. Discussion

Studies have reported that zinc has protective effects in the gastrointestinal tract (Mahmood et al., 2007; Tran et al., 2005). However, there are no reports of studies that have assessed the ability of zinc to protect against NSAID-induced enteropathy. Our results show that pre-treatment with zinc resulted in a significant decrease in indomethacin-induced intestinal damage. This is in keeping with results of earlier studies, which have shown that administration of zinc was effective in attenuating indomethacin-induced oxidative stress in the rat small intestine (Basivireddy et al., 2002, 2003). The dose used in the current study was chosen as it has been documented to have protective effects against the effects of indomethacin in other organs (Joseph et al., 1999; Varghese et al., 2009).

Administration of indomethacin and other NSAIDs is well known to be associated with increases in the permeability of the intestinal mucosa, allowing entry of luminal contents into the mucosa. This occurs due to these drugs breaching the barrier function of the intestinal mucosa (Sigthorsson et al., 2000; Thiefin and Beaugerie, 2005). Mechanisms postulated to be involved in such effects include interaction of the drug with membrane phospholipids (Lichtenberger et al., 1995), drug-induced disruption of the integrity of SLP and BBM (Basivireddy et al., 2003, 2005) and mitochondrial dysfunction (Basivireddy et al., 2002; Somasundaram et al., 1997). The resultant increased intestinal permeability that ensues allows bacterial invasion and entry of other noxious agents from the intestinal lumen. This, in turn, leads to mucosal inflammation and eventually results in macroscopic damage (Lanas and Scarpignato, 2006).

In our study, the contents of various lipids and sugars in the BBM and SLP were altered in response to indomethacin. These alterations are thought to contribute to the loss of integrity of the intestinal mucosa with subsequent loss of its barrier function. Zinc pre-treatment was able to reverse many of these drug-induced changes. Zinc limitation has been reported to affect the composition of lipids in tissue (Gomez et al., 2006) and to inhibit the activity of phospholipases A_2, C and D (Hatakeyama et al., 2002). (Southon et al., 1984) have reported significant morphological changes in the small intestine of zinc-deficient rats, with increased uptake of sugars occurring across the mucosal membrane. In addition, Mahmood et al. (2007) have shown that the administration of zinc carnosine was successful in stabilizing the gut mucosa, possibly by preventing indomethacin-induced increases in intestinal permeability, an event

Table 2
Content of phospholipids in SLP and BBM isolated from intestinal tissue at 24 h, from rats treated with indomethacin, with and without pre-treatment with zinc.. Each value represents mean ± S.D. of duplicate estimations from six different animals.

(Phospholipids) nanomoles mg^{-1} protein	SLP				BBM			
	Cont	Indo	Zinc + Indo	Zinc alone	Cont	Indo	Zinc + Indo	Zinc alone
1. Phosphatidylcholine	63.6 ± 61	45.5[a] ± 9.1	60.8[b] ± 3.8	64.3 ± 4.4	65.3 ± 8.1	46.8[a] ± 7.2	60.1[b] ± 7.0	63.3 ± 5.9
2. Lysophosphatidylcholine	19.5 ± 5.2	30[a] ± 6.2	20.3[b] ± 4.2	18.3 ± 2.5	17.8 ± 10.1	29.8 ± 9.4	20 ± 9.3	16.8 ± 4.0
3. Phosphatidylethanolamine	30.8 ± 2.6	20.3[a] ± 2.8	28.6[b] ± 2.0	30.5 ± 3.0	35.3 ± 4.6	24.6[a] ± 3.4	32.1[b] ± 4.0	32.3 ± 3.3
4. Lysophosphatidylethanolamine	11.3 ± 2.4	21.1[a] ± 1.7	13.5[b] ± 2.6	11.5 ± 1.8	16.6 ± 2.6	28.8[a] ± 2.9	18.5[b] ± 2.8	16.6 ± 2.5

[a] $P<0.05$ when compared with control.
[b] $P<0.05$ when compared with the indomethacin-treated group.

known to precede the development of indomethacin-induced enteropathy. Thus, levels of zinc in tissues have been shown to affect their composition and function. Our results, which show a protective effect of zinc in preserving the integrity of the intestinal mucosa by preventing indomethacin-induced effects in the composition of the SLP and BBM and subsequent tissue damage, are in keeping with these reports.

It has been reported that indomethacin failed to produce small intestinal damage in germ-free animals, suggesting that enterobacteria play a role in producing the intestinal lesions (Robert and Asano, 1977). The numbers of enterobacteria in the rat small intestine have been shown to increase in response to administration of indomethacin (Konaka et al., 1999). An increase in the number of gram-negative bacteria has been shown to be causative in the development of ulcers during the use of NSAIDs (Hagiwara et al., 2004). Bacterial invasion of the intestinal mucosa is known to occur in rats treated with indomethacin (Takeuchi et al., 2002). In addition, studies have shown that antibiotics against gram-negative bacteria and inhibition of bacterial invasion by a cysteinyl-leukotriene receptor agonist were effective against indomethacin-induced small intestinal damage in rats (Nishio et al., 2007; Watanabe et al., 2008). These reports underscore the role of enteric bacteria in the pathogenesis of intestinal damage produced by indomethacin.

The precise mechanisms by which luminal bacteria contribute to the development of indomethacin-induced intestinal damage are not clear. Our results show that administration of indomethacin resulted in increased bacterial numbers in the small intestine and caecum, findings that are in keeping with earlier work (Basivireddy et al., 2005). We postulate that these findings are a result of drug-induced changes in normal peristalsis in the gastrointestinal tract (Shahbazian et al., 2001). Disturbances in normal intestinal flora during antibiotic treatment have been shown to result in bacterial overgrowth with pathogenic bacteria as main colonizers (Sullivan et al., 2001). Under such pathological conditions, attachment of bacteria to receptors on the surface of epithelial cells rich in sugars (such as sialic acid, hexose, fucose and hexosamine) occurs. This facilitates translocation of the bacteria into the mucosa (Helander et al., 1997). We postulate that indomethacin-induced changes in the glycocalyx of the intestinal mucosa resulted in altered host-microbial interactions in the gut, resulting in changes in adherence of bacteria to the intestinal mucosa, as observed in our study. Such changes have been postulated to increase binding of bacteria to enterocytes in vivo (Basivireddy et al., 2005; Helander et al., 1997) and predispose to bacteria invading the mucosa and cause subsequent damage.

Administration of zinc decreased indomethacin-induced increases in the number of bacteria in the small intestine and caecum, both in the intestinal washings and that adherent to the mucosa. (Surjawidjaja et al., 2004) have reported that zinc supplementation inhibited the growth of enteric bacteria in vitro while (Roselli et al., 2003) have shown that zinc oxide prevents *E. coli* adhesion and internalisation of bacteria in Caco-2 cells. Such mechanisms may account for the protective effect of zinc seen in our study. We found that indomethacin-induced alterations in the content of sugars in the SLP and BBM were reversed by the administration of zinc. We postulate that this reduced the ability of bacteria to adhere to the mucosa. The zinc-induced decreases in the number of *E. coli* were associated with reduced mucosal damage. Protection afforded by zinc may also be accounted for by the fact that zinc has been shown to improve intestinal permeability in experimental colitis in rats (Sturniolo et al., 2002), thereby reducing the probability of enteropathy developing subsequently.

Indomethacin is known to activate MMP-2 and -9 expression and secretion in gastric tissue (Ganguly et al., 2005). We found that indomethacin treatment resulted in increased activities of MMP-2 and -9, in keeping with the above findings. Such activation is possibly due to drug-induced oxidative stress, a phenomenon that has been previously reported (Valentin et al., 2005; Viappiani et al., 2009). Studies have shown that anti-oxidants, such as curcumin, prevent indomethacin-induced up-regulation of MMP-2 and -9 expression and activity in the rat gastric tissue (Swarnakar et al., 2005). In our study, we found that zinc significantly decreased the activation of indomethacin-induced MMP-2 and MMP-9, an hour after the dose of the drug. The changes in activity of the MMPs seen were not associated with corresponding changes in the levels of the proteins concerned, suggesting that the changes were due to direct effects on enzyme action. The ability of zinc to reverse the activation of these enzymes is likely to be due to its effect as an antioxidant, thereby decreasing the oxidative stress produced. Our findings are supported by those reported by Szuster-Ciesielska et al. (2009), who have shown that

Table 3
Content of neutral lipids in SLP and BBM isolated from intestinal tissue at 24 h, from rats treated with indomethacin, with and without pre-treatment with zinc. Each value represents mean ± S.D. of duplicate estimations from six different animals.

(Neutral lipids) nanomoles mg^{-1} protein	SLP				BBM			
	Cont	Indo	Zinc + Indo	Zinc alone	Cont	Indo	Zinc + Indo	Zinc alone
1. Cholesterol esters	29.3 ± 8.3	18[a] ± 7.0	29.8[b] ± 5.9	25.1 ± 2.6	38.8 ± 9.7	19.6[a] ± 6.7	32.6[b] ± 7.7	27.1 ± 1.7
2. Cholesterol	85.3 ± 14.3	65.1[a] ± 8.2	83[b] ± 11.3	79.6 ± 3.0	100.5 ± 24.5	98.3 ± 9.3	98.3 ± 17.8	93.5 ± 5.2
3. Triacylglycerol	40.03 ± 6.3	25.5[a] ± 3.1	36.3[b] ± 5.9	36.6 ± 1.0	51.6 ± 4.9	33.5[a] ± 5.3	50.1[b] ± 5.1	47.6 ± 3.1
4. Diacylglycerol	17.5 ± 4.3	12.1 ± 3.8	16.8 ± 3.3	16.8 ± 2.6	21.6 ± 3.8	15.5[a] ± 2.5	20 ± 3.0	20.1 ± 1.7
5. Monoacylglycerol	9.1 ± 3.4	23[a] ± 2.0	12[b] ± 3.1	9.8 ± 1.4	14.1 ± 2.8	27[a] ± 2.0	15.3[b] ± 3.0	13.5 ± 1.5

[a] $P<0.05$ when compared with control.
[b] $P<0.05$ when compared with the indomethacin-treated group.

Table 4
Content of various sugars in SLP and BBM at 24 h, isolated from intestinal tissue from rats treated with indomethacin, with and without pre-treatment with zinc. Each value represents mean ± S.D. of duplicate estimations from six different animals.

(Sugar levels) nanomoles mg^{-1} protein	SLP				BBM			
	Cont	Indo	Zinc + Indo	Zinc alone	Cont	Indo	Zinc + Indo	Zinc alone
1. Fucose	118.5 ± 36.3	199.1[a] ± 38.4	141[b] ± 36.7	115 ± 14.3	226 ± 92.9	126[a] ± 11.2	187[b] ± 21.5	201 ± 13.7
2. Galactose	341.8 ± 29.3	452.0[a] ± 77.4	300[b] ± 74.1	306.8 ± 56.3	90.1 ± 8.3	56.5[a] ± 6.3	75.8[b] ± 12.7	92.6 ± 6.9
3. Hexosamine	371.6 ± 55.5	461[a] ± 48.0	388.6[b] ± 31.8	328 ± 21.64	257.5 ± 25.7	174.6[a] ± 21.5	238.5[b] ± 25.3	216.3 ± 19.11
4. Sialic acid	32.1 ± 11.7	71.6[a] ± 17.1	39.5[b] ± 12.2	32.8 ± 3.8	73.8 ± 8.5	56.8[a] ± 7.3	72.8[b] ± 9.4	77.5 ± 10.5

[a] $P<0.05$ when compared with control.
[b] $P<0.05$ when compared with the indomethacin-treated group.

zinc inhibited the activity of MMP in hepatic stellate cells, by reducing generation of reactive oxygen species and inhibition of nuclear factor kappa-B (NFkB) and mitogen-activated protein kinase (MAPK) signalling pathways.

Indomethacin is a non-selective inhibitor of COX (Roberts and Morrow, 2001). Inhibition of this enzyme by the drug is an important event in the pathogenesis of indomethacin-induced enteropathy (Whittle, 2003). Similarly, induction of iNOS by indomethacin has been reported to contribute to the adverse effects of the drug (Konaka et al., 1999; Tanaka et al., 1999). We found that pretreatment with zinc did not affect indomethacin-induced effects on COX and iNOS. Thus, the protective effects of zinc do not appear to be mediated by changes in COX activity or iNOS levels.

The acute antioxidant properties of zinc are thought to be due to its ability to stabilize sulfhydryl groups and to antagonize redox-active transition metals like iron and copper (Powell, 2000). Its long-term

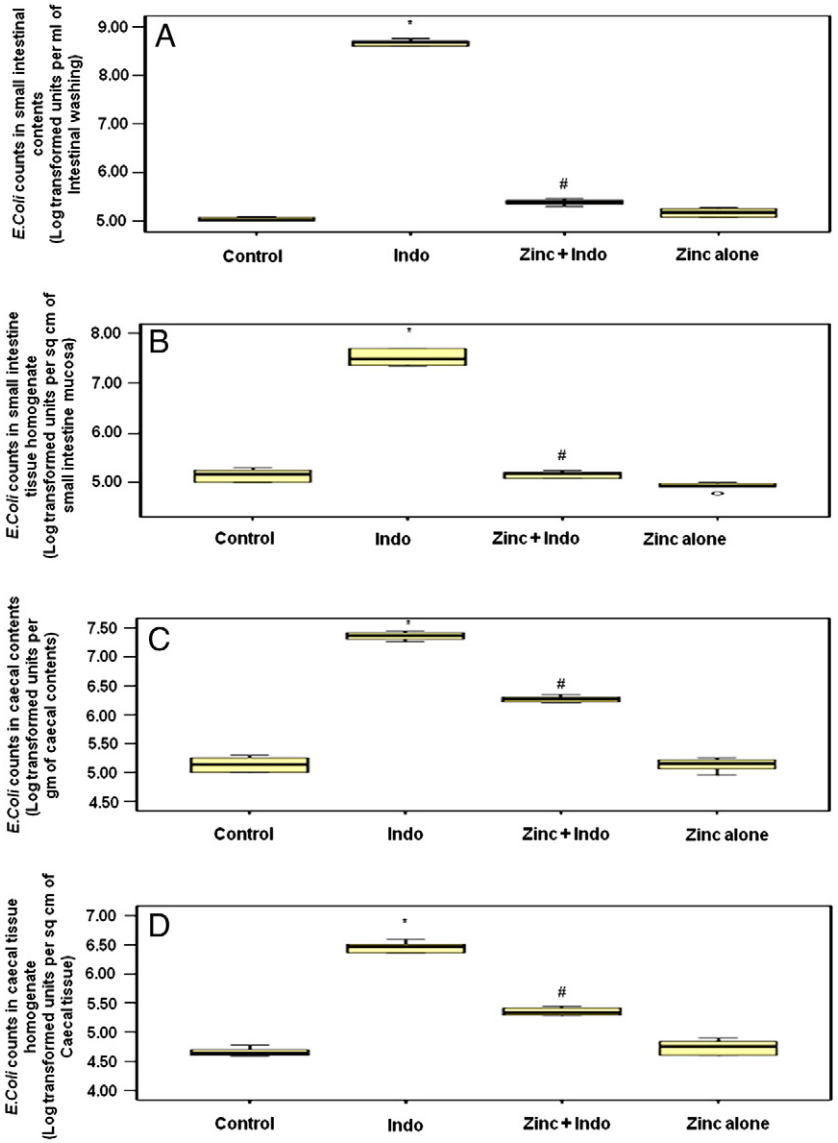

Fig. 2. Number of colony forming units (cfu) of *E. coli* (log-transformed data) cultured from small intestinal washings (A), small intestinal mucosal homogenate (B), caecal contents (C) and caecal tissue homogenates (D), from indomethacin-treated rats, with and without zinc pretreatment, 24 h after the dose of indomethacin. Each value represents mean ± S.D. of duplicate estimations from six different animals. $^{*}P<0.05$ when compared with control, $^{\#}P<0.05$ when compared with the indomethacin-treated group.

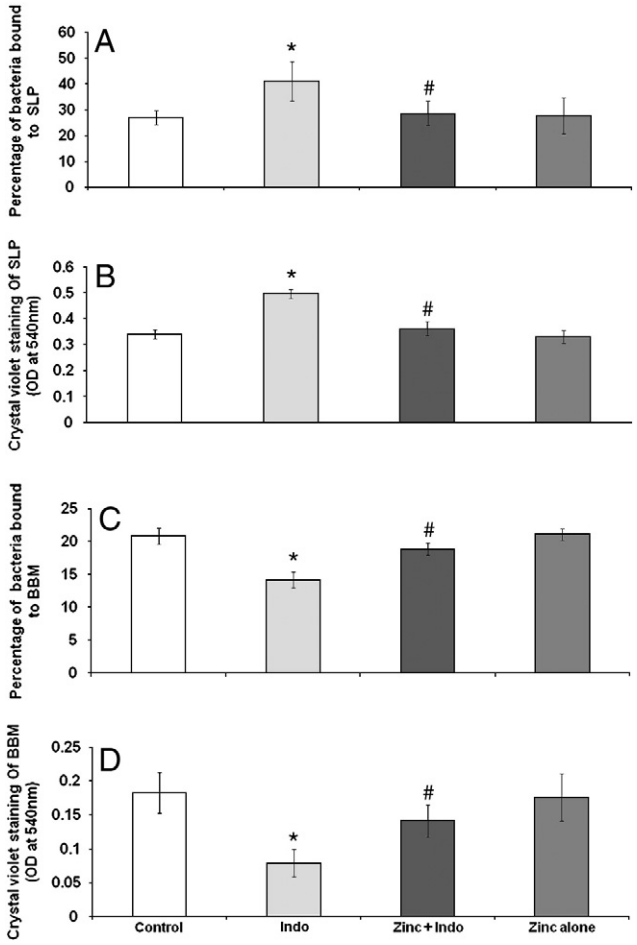

Fig. 3. Quantitation of *E. coli* bound in vitro to SLP and BBM isolated from indomethacin-treated rats, with and without zinc pretreatment, 24 h after the dose of indomethacin. Percentage of bacteria bound to SLP (A) and crystal violet staining of SLP (B). Percentage of bacteria bound to BBM (C) and crystal violet staining of BBM (D). Each value represents mean ± S.D. of triplicate estimations from six separate animals. $^{*}P<0.05$ when compared with control, $^{\#}P<0.05$ when compared with the indomethacin-treated group.

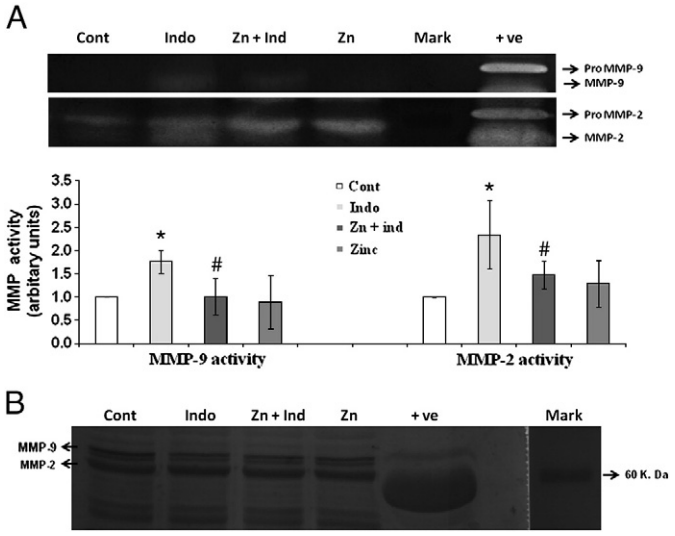

Fig. 4. Activities of matrix metalloproteinases-2 and -9 (A), as quantitated by zymography, and protein content of MMP-2 and MMP-9 (B), as assessed by western blots, in tissue from indomethacin-treated rats, with and without zinc pretreatment, 1 h after the dose of indomethacin. Each value, expressed in arbitrary units, represents mean ± S.D. of data from six separate animals. $^{*}P<0.05$ when compared with control, $^{\#}P<0.05$ when compared with the indomethacin-treated group.

antioxidant effects are postulated to be mediated by metallothionein (Maret, 2000; Maret and Vallee, 1998), which is induced in response to metal ions, like copper and zinc. In our studies, we found that the expression of metallothionein was significantly increased in response to zinc, at the 1 and 12 h periods studied. Oxidative stress has been shown to decrease metallothionein levels (Arda-Pirincci et al., 2006), while zinc can induce the expression of metallothionein by interacting with a metal-sensitive transcription factor (Andrews, 2000; Cousins and Lee-Ambrose, 1992). It has been shown that zinc-induced metallothionein in beta-cells of the pancreas was found to protect these cells by scavenging hydroxyl radicals (Ohly et al., 1998). In view of these reports, we postulate that in our study, zinc, possibly by inducing metallothionein, was able to alleviate the oxidative stress produced by indomethacin. This is also in keeping with the results of Yang et al. (2009) who have shown that over-expression of metallothionein protected against adriamycin-induced oxidative stress and with those of Lazo et al. (1995) who have demonstrated that cells deficient in metallothionein show enhanced sensitivity to oxidative stress.

It is possible that zinc and indomethacin, both of which were given orally, may form a complex (Zhou et al., 2000) in the gut, leading to a reduction in the amount of drug absorbed and subsequent damage in the intestine. We do not think this is likely to have occurred under the conditions under which the current studies were carried out. Zinc was given to the experimental rats 2 h before the dose of indomethacin. Keyzer et al. (1983) have shown that maximum zinc absorption takes place within two hours of administration under fasting conditions and that absorption is inhibited when zinc was taken along with food. In our model, rats were fasted overnight, dosed with zinc, kept fasting and then given indomethacin 2 h later, followed by access to food. In view of these conditions, we think that zinc would not have been available to complex with indomethacin in the intestine and form a complex that prevented the absorption of indomethacin into the mucosa, thus reducing mucosal damage by this NSAID. However, to rule out the possibility that oral zinc may interfere with the absorption of indomethacin and its subsequent effects on the intestinal mucosa, rats were given zinc intra-peritoneally, instead of orally, 2 h before the indomethacin. Mucosal damage was assessed

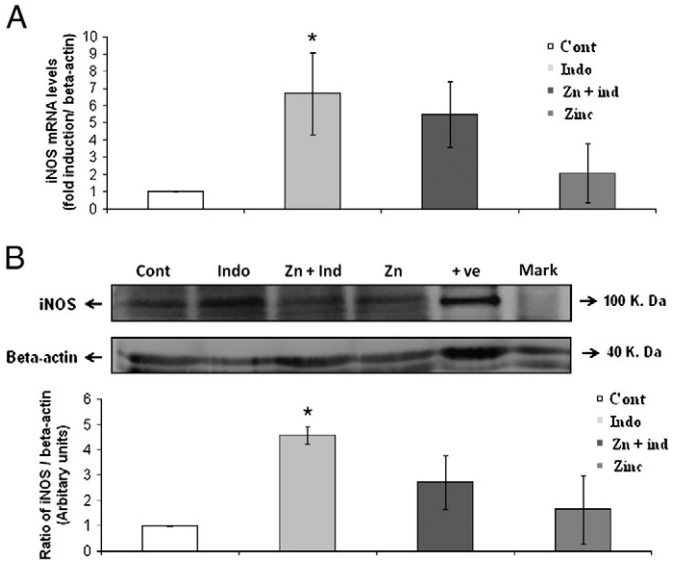

Fig. 5. Levels of iNOS in intestine from indomethacin-treated rats, with and without zinc pretreatment, 12 h after the dose of indomethacin, as measured by real-time PCR assays (A) and western blots (B). Each value represents mean ± S.D. of data from six separate animals. $^{*}P<0.05$ when compared with control, $^{\#}P<0.05$ when compared with the indomethacin-treated group.

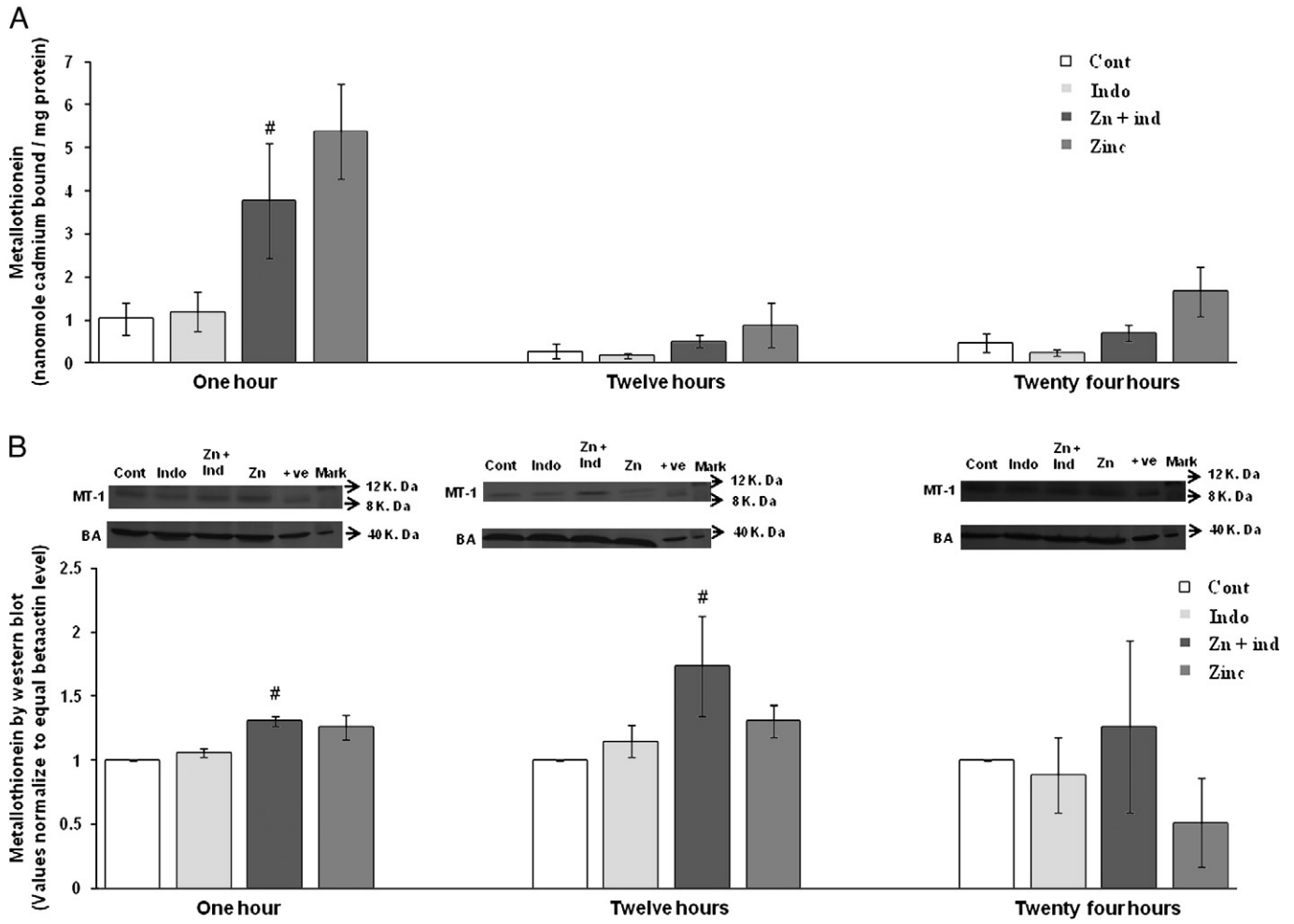

Fig. 6. Levels of metallothionein in the intestine from indomethacin-treated rats, with and without zinc pretreatment, at 1, 12 and 24 h after the dose of indomethacin, as assessed by cadmium heme assay (A) and Western blots (B). Each value represents mean ± S.D. of data from six separate animals. $^{*}P<0.05$ when compared with control, $^{\#}P<0.05$ when compared with the indomethacin-treated group.

after 24 h, as described. It was found that rats that were pre-treated with zinc before the dose of indomethacin had markedly less damage when compared with those that had received only the drug (71.3 ± 26 ulcers in the indomethacin-treated group when compared with 27.3 ± 9.29 ulcers in the group pre-treated with zinc before administration of indomethacin). Thus, zinc protected against drug-induced damage even when administered parenterally. Hence, it is unlikely that the protective effect of oral zinc was due to it forming a complex with indomethacin, thereby preventing its absorption into the mucosa.

Other mechanisms such as mast cell involvement and induction of heat-shock protein have also been studied in connection with indomethacin-induced damage small intestinal damage (Okayama

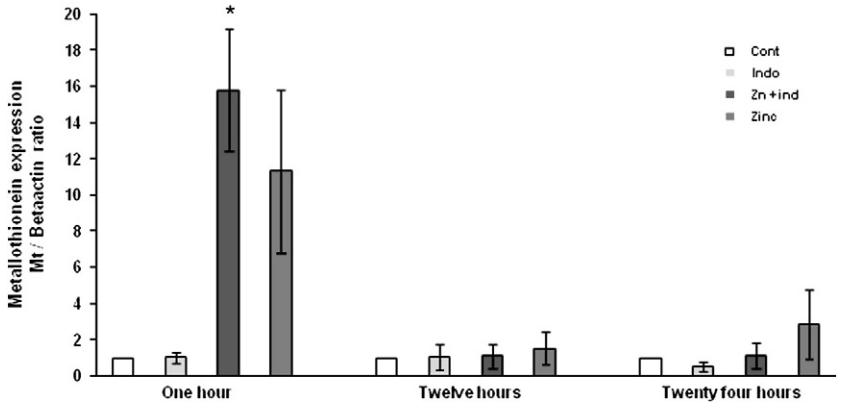

Fig. 7. Levels of metallothionein mRNA in intestine from indomethacin-treated rats, with and without zinc pretreatment, at 1, 12 and 24 h after the dose of indomethacin, as measured by real-time PCR assays. Each value represents mean ± S.D. of triplicate estimations done on six separate animals. $^{*}P<0.05$ when compared with control, $^{\#}P<0.05$ when compared with the indomethacin-treated group.

et al., 2009; Jin et al., 1997). However, it is not known whether zinc affects any of these events. Studies on these aspects may be warranted in the future.

5. Conclusion

We conclude that indomethacin-induced damage in the intestinal mucosa can be reduced by prior administration of zinc. Zinc was found to preserve the integrity of the intestinal glycocalyx against effects of the drug and inhibit the growth of enterobacteria and their adherence to the mucosa. We postulate that these events prevented the entry of pathogens into the mucosa and subsequent enteropathy. Pretreatment with zinc resulted in induction of metallothionein. It is possible that this protein may mediate some of the protective effects of zinc seen in this study.

Statement of conflicts of interest

None.

Role of the funding source

The authors declare that the funding agency provided only financial support for the study. It had no role in study design or in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

Acknowledgements

The authors would like to acknowledge Research Committee of the Christian Medical College, Vellore, India and the Council for Scientific and Industrial Research (CSIR), New Delhi, India for financial assistance for the study. Nageswaran Sivalingam is a Senior Research Fellow funded by the CSIR.

References

Ali, M.A., Schulz, R., 2009. Activation of MMP-2 as a key event in oxidative stress injury to the heart. Front. Biosci. 14, 699–716.
Andrews, G.K., 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol. 59, 95–104.
Arda-Pirincci, P., Bolkent, S., Yanardag, R., 2006. The role of zinc sulfate and metallothionein in protection against ethanol-induced gastric damage in rats. Dig. Dis. Sci. 51, 2353–2360.
Bartlett, G.R., 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234, 466–468.
Basivireddy, J., Vasudevan, A., Jacob, M., Balasubramanian, K.A., 2002. Indomethacin-induced mitochondrial dysfunction and oxidative stress in villus enterocytes. Biochem. Pharmacol. 64, 339–349.
Basivireddy, J., Jacob, M., Ramamoorthy, P., Pulimood, A.B., Balasubramanian, K.A., 2003. Indomethacin-induced free radical-mediated changes in the intestinal brush border membranes. Biochem. Pharmacol. 65, 683–695.
Basivireddy, J., Jacob, M., Ramamoorthy, P., Balasubramanian, K.A., 2005. Alterations in the intestinal glycocalyx and bacterial flora in response to oral indomethacin. Int. J. Biochem. Cell Biol. 37, 2321–2332.
Belkhiri, A., Richards, C., Whaley, M., McQueen, S.A., Orr, F.W., 1997. Increased expression of activated matrix metalloproteinase-2 by human endothelial cells after sublethal H_2O_2 exposure. Lab. Invest. 77, 533–539.
Bjarnason, I., Hayllar, J., MacPherson, A.J., Russell, A.S., 1993. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology 104, 1832–1847.
Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.
Bodiga, S., Krishnapillai, M.N., 2007. Concurrent repletion of iron and zinc reduces intestinal oxidative damage in iron- and zinc-deficient rats. World J. Gastroenterol. 13, 5707–5717.
Cohen, P., Derksen, A., 1969. Comparison of phospholipid and fatty acid composition of human erythrocytes and platelets. Br. J. Haematol. 17, 359–371.
Cousins, R.J., Lee-Ambrose, L.M., 1992. Nuclear zinc uptake and interactions and metallothionein gene expression are influenced by dietary zinc in rats. J. Nutr. 122, 56–64.
Coyle, P., Philcox, J.C., Carey, L.C., Rofe, A.M., 2002. Metallothionein: the multipurpose protein. Cell. Mol. Life Sci. 59, 627–647.
DeSchryver-Kecskemeti, K., Eliakim, R., Carroll, S., Stenson, W.F., Moxley, M.A., Alpers, D.H., 1989. Intestinal surfactant-like material. A novel secretory product of the rat enterocyte. J. Clin. Invest. 84, 1355–1361.
Djurdjic, V., Mandic, L., 1990. A spectrophotometric method for simultaneous determination of protein-bound hexoses and fucose with a mixture of L-cysteine and phenol. Anal. Biochem. 188, 222–227.
Dorai, D.T., Bachhawat, B.K., 1977. Purification and properties of brain alkaline phosphatase. J. Neurochem. 29, 503–512.
Eaton, D.L., Toal, B.F., 1982. Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. Toxicol. Appl. Pharmacol. 66, 134–142.
Egeblad, M., Werb, Z., 2002. New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer 2, 161–174.
Eliakim, R., DeSchryver-Kecskemeti, K., Nogee, L., Stenson, W.F., Alpers, D.H., 1989. Isolation and characterization of a small intestinal surfactant-like particle containing alkaline phosphatase and other digestive enzymes. J. Biol. Chem. 264, 20614–20619.
Ganguly, K., Maity, P., Reiter, R.J., Swarnakar, S., 2005. Effect of melatonin on secreted and induced matrix metalloproteinase-9 and -2 activity during prevention of indomethacin-induced gastric ulcer. J. Pineal Res. 39, 307–315.
Gomez, N.N., Biaggio, V.S., Rozzen, E.J., Alvarez, S.M., Gimenez, M.S., 2006. Zn-limited diet modifies the expression of the rate-regulatory enzymes involved in phosphatidylcholine and cholesterol synthesis. Br. J. Nutr. 96, 1038–1046.
Hagiwara, M., Kataoka, K., Arimochi, H., Kuwahara, T., Ohnishi, Y., 2004. Role of unbalanced growth of gram-negative bacteria in ileal ulcer formation in rats treated with a nonsteroidal anti-inflammatory drug. J. Med. Invest. 51, 43–51.
Hatakeyama, D., Kozawa, O., Otsuka, T., Shibata, T., Uematsu, T., 2002. Zinc suppresses IL-6 synthesis by prostaglandin F2alpha in osteoblasts: inhibition of phospholipase C and phospholipase D. J. Cell. Biochem. 85, 621–628.
Helander, A., Hansson, G.C., Svennerholm, A.M., 1997. Binding of enterotoxigenic *Escherichia coli* to isolated enterocytes and intestinal mucus. Microb. Pathog. 23, 335–346.
Jacob, M., Foster, R., Sigthorsson, G., Simpson, R., Bjarnason, I., 2007. Role of bile in pathogenesis of indomethacin-induced enteropathy. Arch. Toxicol. 81, 291–298.
Jiang, P., Chang, L., Pan, C.S., Qi, Y.F., Tang, C.S., 2005. Protective role of metallothionein in stress-induced gastric ulcer in rats. World J. Gastroenterol. 11, 2739–2743.
Jin, M., Otaka, M., Otani, S., Okuyama, A., Itoh, S., Iwabuchi, A., Sasahara, H., Fujimori, S., Itoh, H., Tashima, Y., Masamune, O., 1997. Effect of pre-induction of heat shock proteins on indomethacin-induced small-intestinal lesion in rats. J. Gastroenterol. 32, 34–39.
Joseph, R.M., Varela, V., Kanji, V.K., Subramony, C., Mihas, A.A., 1999. Protective effects of zinc in indomethacin-induced gastric mucosal injury: evidence for a dual mechanism involving lipid peroxidation and nitric oxide. Aliment. Pharmacol. Ther. 13, 203–208.
Keck, T., Balcom, J.H.T., Fernandez-del Castillo, C., Antoniu, B.A., Warshaw, A.L., 2002. Matrix metalloproteinase-9 promotes neutrophil migration and alveolar capillary leakage in pancreatitis-associated lung injury in the rat. Gastroenterology 122, 188–201.
Keyzer, J.J., Oosting, E., Wolthers, B.G., Muskiet, F.A., 1983. Zinc absorption after oral administration of zinc sulfate. Pharm. Weekbl. Sci. 5, 252–253.
Konaka, A., Kato, S., Tanaka, A., Kunikata, T., Korolkiewicz, R., Takeuchi, K., 1999. Roles of enterobacteria, nitric oxide and neutrophil in pathogenesis of indomethacin-induced small intestinal lesions in rats. Pharmacol. Res. 40, 517–524.
Kowluru, R.A., Kanwar, M., 2009. Oxidative stress and the development of diabetic retinopathy: contributory role of matrix metalloproteinase-2. Free Radic. Biol. Med. 46, 1677–1685.
Lanas, A., Scarpignato, C., 2006. Microbial flora in NSAID-induced intestinal damage: a role for antibiotics? Digestion 73 (Suppl. 1), 136–150.
Lazo, J.S., Kondo, Y., Dellapiazza, D., Michalska, A.E., Choo, K.H., Pitt, B.R., 1995. Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. J. Biol. Chem. 270, 5506–5510.
Lichtenberger, L.M., Wang, Z.M., Romero, J.J., Ulloa, C., Perez, J.C., Giraud, M.N., Barreto, J.C., 1995. Non-steroidal anti-inflammatory drugs (NSAIDs) associate with zwitterionic phospholipids: insight into the mechanism and reversal of NSAID-induced gastrointestinal injury. Nat. Med. 1, 154–158.
Lindsey, M., Wedin, K., Brown, M.D., Keller, C., Evans, A.J., Smolen, J., Burns, A.R., Rossen, R.D., Michael, L., Entman, M., 2001. Matrix-dependent mechanism of neutrophil-mediated release and activation of matrix metalloproteinase 9 in myocardial ischemia/reperfusion. Circulation 103, 2181–2187.
Ludowieg, J., Benmaman, J.D., 1967. Colorimetric differentiation of hexosamines. Anal. Biochem. 19, 80–88.
Mahmood, A., FitzGerald, A.J., Marchbank, T., Ntatsaki, E., Murray, D., Ghosh, S., Playford, R.J., 2007. Zinc carnosine, a health food supplement that stabilises small bowel integrity and stimulates gut repair processes. Gut 56, 168–175.
Mann, N.S., Mann, S.K., Brawn, P.N., Weaver, B., 1992. Effect of zinc sulfate and acetylcysteine on experimental gastric ulcer: in vitro study. Digestion 53, 108–113.
Maret, W., 2000. The function of zinc metallothionein: a link between cellular zinc and redox state. J. Nutr. 130, 1455S–1458S.
Maret, W., Vallee, B.L., 1998. Thiolate ligands in metallothionein confer redox activity on zinc clusters. Proc. Natl Acad. Sci. USA 95, 3478–3482.
Melarange, R., Moore, G., Blower, P.R., Coates, M.E., Ward, F.W., Ronaasen, V., 1992. A comparison of indomethacin with ibuprofen on gastrointestinal mucosal integrity in conventional and germ-free rats. Aliment. Pharmacol. Ther. 6, 67–77.
Merritt, K., Gaind, A., Anderson, J.M., 1998. Detection of bacterial adherence on biomedical polymers. J. Biomed. Mater. Res. 39, 415–422.
Moncada, D.M., Kammanadiminti, S.J., Chadee, K., 2003. Mucin and Toll-like receptors in host defense against intestinal parasites. Trends Parasitol. 19, 305–311.
Nagano, Y., Matsui, H., Muramatsu, M., Shimokawa, O., Shibahara, T., Yanaka, A., Nakahara, A., Matsuzaki, Y., Tanaka, N., Nakamura, Y., 2005. Rebamipide

significantly inhibits indomethacin-induced mitochondrial damage, lipid peroxidation, and apoptosis in gastric epithelial RGM-1 cells. Dig. Dis. Sci. 50 (Suppl. 1), S76–S83.
Nishio, H., Hayashi, Y., Terashima, S., Takeuchi, K., 2007. Protective effect of pranlukast, a cysteinyl-leukotriene receptor 1 antagonist, on indomethacin-induced small intestinal damage in rats. Inflammopharmacol. 15, 266–272.
Ohly, P., Wang, Z., Abel, J., Gleichmann, H., 1998. Zincsulphate induced metallothionein in pancreatic islets and protected against the diabetogenic toxin streptozotocin. Talanta 46, 355–359.
Okayama, T., Yoshida, N., Uchiyama, K., Takagi, T., Ichikawa, H., Yoshikawa, T., 2009. Mast cells are involved in the pathogenesis of indomethacin-induced rat enteritis. J. Gastroenterol. 44 (Suppl. 19), 35–39.
Okazaki, S., Nishitani, Y., Nagoya, S., Kaya, M., Yamashita, T., Matsumoto, H., 2009. Femoral head osteonecrosis can be caused by disruption of the systemic immune response via the toll-like receptor 4 signalling pathway. Rheumatol. Oxf. 48, 227–232.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
Powell, S.R., 2000. The antioxidant properties of zinc. J. Nutr. 130, 1447S–1454S.
Prabhu, R., Balasubramanian, K.A., 2001. A novel method of preparation of small intestinal brush border membrane vesicles by polyethylene glycol precipitation. Anal. Biochem. 289, 157–161.
Rajagopalan, S., Meng, X.P., Ramasamy, S., Harrison, D.G., Galis, Z.S., 1996. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. J. Clin. Invest. 98, 2572–2579.
Robert, A., Asano, T., 1977. Resistance of germfree rats to indomethacin-induced intestinal lesions. Prostaglandins 14, 333–341.
Roberts II, L.J., Morrow, J., 2001. Analgesic, antipyretic and anti-inflammatory agents and drugs employed in the treatment of gout. In: Hardman, J.G. (Ed.), In Goodman and Gilman's The Pharmacological Basis of Therapeutics. McGraw Hill, Newyork, pp. 687–732.
Rosario, H.S., Waldo, S.W., Becker, S.A., Schmid-Schonbein, G.W., 2004. Pancreatic trypsin increases matrix metalloproteinase-9 accumulation and activation during acute intestinal ischemia-reperfusion in the rat. Am. J. Pathol. 164, 1707–1716.
Roselli, M., Finamore, A., Garaguso, I., Britti, M.S., Mengheri, E., 2003. Zinc oxide protects cultured enterocytes from the damage induced by *Escherichia coli*. J. Nutr. 133, 4077–4082.
Saifer, A., Gerstenfeld, S., 1962. Photometric determination of sialic acids in serum and cerebrospinal fluid with the thiobarbituric acid method. Clin. Chim. Acta 7, 467–475.
Seymour, C., 1996. Trace metal disorders. In: Warreel, D.J., L.J, Warrel, D.A. (Eds.), Oxford Textbook of Medicine. Oxford University Press, England, pp. 1423–1424.
Shahbazian, A., Schuligoi, R., Heinemann, A., Peskar, B.A., Holzer, P., 2001. Disturbance of peristalsis in the guinea-pig isolated small intestine by indomethacin, but not cyclo-oxygenase isoform-selective inhibitors. Br. J. Pharmacol. 132, 1299–1309.
Sigthorsson, G., Crane, R., Simon, T., Hoover, M., Quan, H., Bolognese, J., Bjarnason, I., 2000. COX-2 inhibition with rofecoxib does not increase intestinal permeability in healthy subjects: a double blind crossover study comparing rofecoxib with placebo and indomethacin. Gut. 47, 527–532.
Snyder, F., Stephens, N., 1959. A simplified spectrophotometric determination of ester groups in lipids. Biochim. Biophys. Acta 34, 244–245.
Somasundaram, S., Rafi, S., Hayllar, J., Sigthorsson, G., Jacob, M., Price, A.B., Macpherson, A., Mahmod, T., Scott, D., Wrigglesworth, J.M., Bjarnason, I., 1997. Mitochondrial damage: a possible mechanism of the "topical" phase of NSAID induced injury to the rat intestine. Gut 41, 344–353.
Somasundaram, S., Simpson, R., Rafi, S., Shergill, J.K., Bjarnason, I., Wrigglesworth, J., 2002. 2,4-diamino-6-hydroxy pyrimidine inhibits NSAIDs induced nitrosyl-complex EPR signals and ulcer in rat jejunum. BMC Gastroenterol. 2, 8.
Southon, S., Gee, J.M., Johnson, I.T., 1984. Hexose transport and mucosal morphology in the small intestine of the zinc-deficient rat. Br. J. Nutr. 52, 371–380.
Sturniolo, G.C., Fries, W., Mazzon, E., Di Leo, V., Barollo, M., D'Inca, R., 2002. Effect of zinc supplementation on intestinal permeability in experimental colitis. J. Lab. Clin. Med. 139, 311–315.
Sullivan, A., Edlund, C., Nord, C.E., 2001. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect. Dis. 1, 101–114.
Surjawidjaja, J.E., Hidayat, A., Lesmana, M., 2004. Growth inhibition of enteric pathogens by zinc sulfate: an in vitro study. Med. Princ. Pract. 13, 286–289.
Swarnakar, S., Ganguly, K., Kundu, P., Banerjee, A., Maity, P., Sharma, A.V., 2005. Curcumin regulates expression and activity of matrix metalloproteinases 9 and 2 during prevention and healing of indomethacin-induced gastric ulcer. J. Biol. Chem. 280, 9409–9415.
Szuster-Ciesielska, A., Plewka, K., Daniluk, J., Kandefer-Szerszen, M., 2009. Zinc supplementation attenuates ethanol- and acetaldehyde-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS) production and by influencing intracellular signaling. Biochem. Pharmacol. 78, 301–314.
Takeuchi, K., Miyazawa, T., Tanaka, A., Kato, S., Kunikata, T., 2002. Pathogenic importance of intestinal hypermotility in NSAID-induced small intestinal damage in rats. Digestion 66, 30–41.
Tanaka, A., Kunikata, T., Mizoguchi, H., Kato, S., Takeuchi, K., 1999. Dual action of nitric oxide in pathogenesis of indomethacin-induced small intestinal ulceration in rats. J. Physiol. Pharmacol. 50, 405–417.
Thiefin, G., Beaugerie, L., 2005. Toxic effects of nonsteroidal antiinflammatory drugs on the small bowel, colon, and rectum. Joint Bone Spine 72, 286–294.
Tran, C.D., Campbell, M.A., Kolev, Y., Chamberlain, S., Huynh, H.Q., Butler, R.N., 2005. Short-term zinc supplementation attenuates Helicobacter felis-induced gastritis in the mouse. J. Infect. 50, 417–424.
Troskot, B., Simicevic, V.N., Dodig, M., Rotkvic, I., Ivankovic, D., Duvnjak, M., 1997. The protective effect of zinc sulphate pretreatment against duodenal ulcers in the rat. Biometals 10, 325–329.
Valentin, F., Bueb, J.L., Kieffer, P., Tschirhart, E., Atkinson, J., 2005. Oxidative stress activates MMP-2 in cultured human coronary smooth muscle cells. Fundam. Clin. Pharmacol. 19, 661–667.
Vane, J.R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat. New Biol. 231, 232–235.
Varghese, J., Faith, M., Jacob, M., 2009. Zinc prevents indomethacin-induced renal damage in rats by ameliorating oxidative stress and mitochondrial dysfunction. Eur. J. Pharmacol. 614, 114–121.
Viappiani, S., Nicolescu, A.C., Holt, A., Sawicki, G., Crawford, B.D., Leon, H., van Mulligen, T., Schulz, R., 2009. Activation and modulation of 72 kDa matrix metalloproteinase-2 by peroxynitrite and glutathione. Biochem. Pharmacol. 77, 826–834.
Visse, R., Nagase, H., 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ. Res. 92, 827–839.
Watanabe, T., Higuchi, K., Kobata, A., Nishio, H., Tanigawa, T., Shiba, M., Tominaga, K., Fujiwara, Y., Oshitani, N., Asahara, T., Nomoto, K., Takeuchi, K., Arakawa, T., 2008. Non-steroidal anti-inflammatory drug-induced small intestinal damage is Toll-like receptor 4 dependent. Gut 57, 181–187.
Weissenborn, U., Maedge, S., Buettner, D., Sewing, K.F., 1985. Indometacin-induced gastrointestinal lesions in relation to tissue concentration, food intake and bacterial invasion in the rat. Pharmacology 30, 32–39.
Whittle, B.J., 2003. Gastrointestinal effects of nonsteroidal anti-inflammatory drugs. Fundam. Clin. Pharmacol. 17, 301–313.
Yang, L., Zheng, S., Epstein, P.N., 2009. Metallothionein over-expression in podocytes reduces adriamycin nephrotoxicity. Free Radic. Res. 43, 174–182.
Zhou, Q., Hambley, T.W., Kennedy, B.J., Lay, P.A., Turner, P., Warwick, B., Biffin, J.R., Regtop, H.L., 2000. Syntheses and characterization of anti-inflammatory dinuclear and mononuclear zinc indomethacin complexes. Crystal structures of [Zn2 (indomethacin)4(L)2] (L = N, N-dimethylacetamide, pyridine, 1-methyl-2-pyrrolidinone) and [Zn(indomethacin)2(L1)2] (L1 = ethanol, methanol). Inorg. Chem. 39, 3742–3748.
Zlatkis, A., Zak, B., Boyle, A.J., 1953. A new method for the direct determination of serum cholesterol. J. Lab. Clin. Med. 41, 486–492.