

Doxycycline blocks gastric ulcer by regulating matrix metalloproteinase-2 activity and oxidative stress

Laishram Pradeepkumar Singh, Amartya Mishra, Debjit Saha, Snehasikta Swarnakar

Laishram Pradeepkumar Singh, Amartya Mishra, Debjit Saha, Snehasikta Swarnakar, Department of Physiology, Drug Development Diagnostics and Biotechnology Division, Indian Institute of Chemical Biology, Kolkata-700032, India

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Correspondence to: Dr. Snehasikta Swarnakar, PhD, Head, Department of Physiology, Drug Development Diagnostics and Biotechnology Division, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Kolkata-700032, India. snehasiktas@hotmail.com

Telephone: +91-33-24995759 Fax: +91-33-24735197

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compared to control tissues. Similarly, ethanol induced about 22-fold and about 6-fold higher proMMP-9 and proMMP-3 activities, respectively, in rat gastric tissues. Both proMMP-9 and MMP-3 activities were markedly decreased by doxycycline in ulcerogen treated rat gastric tissues. In contrast, the reduced MMP-2 activity in ulcerated tissues was increased by doxycycline during ulcer prevention. On the other hand, doxycycline inhibited significantly proMMP-9, -2 and -3 activities *in vitro*. In addition, doxycycline reduced oxidative load in gastric tissues and scavenged H₂O₂ *in vitro*. Our results suggest a novel regulatory role of doxycycline on MMP-2 activity in addition to inhibitory action on MMP-9 and MMP-3 during prevention of gastric ulcers.

CONCLUSION: This is the first demonstration of dual action of doxycycline, that is, regulation of MMP activity and reduction of oxidative stress in arresting gastric injury.

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Abstract

AIM: To examine the effect of doxycycline on the activity of matrix metalloproteinases (MMPs) and oxidative stress in gastric tissues of rats following gastric injury.

METHODS: Gastric ulcers were generated in rats by administration of 70% ethanol, and activity of doxycycline was tested by administration 30 min prior to ethanol. Similarly, the effect of doxycycline was tested in an indomethacin-induced gastric ulcer model. The activities and expression of MMPs were examined by zymography and Western blot analysis.

RESULTS: Gastric injury in rats as judged by elevated ulcer indices following exposure to ulcerogen, either indomethacin or ethanol, was reversed significantly by doxycycline. Indomethacin-induced ulcerated gastric tissues exhibited about 12-fold higher proMMP-9 activity and about 5-fold higher proMMP-3 activity as

Key words: Doxycycline; Extracellular matrix; Matrix metalloproteinases; Reactive oxygen species; Tissue inhibitor of metalloproteinase

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INTRODUCTION

Matrix metalloproteinases (MMPs) are a class of zinc-de-

pendent endopeptidases that degrade matrix macromolecules and numerous other components such as growth factors, proteinases, and plasma proteins^[1,2]. MMPs are best known for their action on extracellular matrix (ECM) remodeling, wound healing and angiogenesis, and have been implicated in the pathogenesis of various diseases including gastric ulcers^[3-6]. The endogenous inhibitors of MMPs [tissue inhibitors of metalloproteinases (TIMPs) and α -2 macroglobulin] play a major role in balancing protease-antiprotease action^[7,8]. MMPs are synthesized and secreted by gastric epithelial cells, macrophages, and neutrophils^[9,10]. Numerous exogenous factors including nonsteroidal anti-inflammatory drugs (NSAIDs), stress, alcohol and *Helicobacter pylori* infection have been implicated in the etiology of gastric ulceration^[11-14]. We have previously demonstrated that MMP-9 increases significantly in indomethacin-induced as well as ethanol-induced gastric ulcers^[3,4,12]. Overexpression of MMP-9 has been attributed to coronary artery disease, retinopathy, renal disease, chronic obstructive pulmonary disease and atherosclerosis^[15-17]. Involvement of several MMPs including gelatinases and stromelysins in various pathological conditions has been documented^[5]. However, downregulation of MMP-2 activity in retinal and renal vasculature leads to thickening of the basement membrane due to collagen deposition and contributes to diabetic retinopathy and nephropathy, respectively^[18]. Research into the pathophysiology of gastric ulcers suggests a central role of reactive oxygen species (ROS) in the development of the disease. MMP can be activated by ROS *in vitro*, and thus, imbalance of MMP activation and inactivation occurs^[19]. An imbalance of MMP activity can lead to release of growth factors, and therefore, inhibition of MMPs can be a potential therapy for various diseases^[20]. Ulcer healing is a complicated process that requires interactions between ECM proteins including collagens, proteases, cytokines, and growth factors. Repair of gastric wounds encompasses a series of cell-matrix interactions that involve cellular proliferation, migration, and differentiation^[3,4,12,21]. Doxycycline, which belongs to the tetracycline family, is a pluripotent drug that affects many cellular functions including cell proliferation, matrix remodeling and apoptosis, in addition to its use as an antibiotic^[22,23].

Doxycycline as well as tetracycline are non-specific potent MMP inhibitors^[23-27]. Doxycycline has been used at a dose of 100-300 mg/d for the protection of pancreatitis-associated lung injury and abdominal aneurysm^[28,29]. Doxycycline treatment greatly potentiates vasoconstriction, which is attributed to inhibition of MMP-2 and MMP-9 and restoration of elastic fiber integrity during prevention of aortic aneurysm in mice^[29]. However, the mechanism of its actions on gastric ulcers, especially at the MMP level, remains unknown and needs to be resolved. Considering a value of 56 for human/rat conversion coefficient, assuming rat and human weights of 200 and 70 kg, respectively, 5-10 mg/kg doxycycline is equal to the 2-3 mg/kg recommended human dose^[30,31]. It has

been widely available in the market as an antibiotic for over three decades and has become established for long-term use too. However, its use against inhibition of overexpression of MMPs has not been investigated to date in therapeutic trials for gastric ulcers.

This study was conducted to evaluate the ability of the broad-spectrum MMP inhibitor doxycycline to prevent gastric ulcers in rats. In this paper, we report that doxycycline inhibited MMP-3 and MMP-9 activity and expression during prevention of indomethacin- and ethanol-induced gastric ulcers. Remarkably, doxycycline up-regulated MMP-2 activity and expression *in vivo*, whereas it downregulated the same activity *in vitro*. Finally, we suggest that doxycycline might exert its regulatory role in addition to its inhibitory role on MMPs *in vivo*, which may be associated with ROS scavenging activity. These results provide an insight into the gastroprotective mechanisms of doxycycline that underlie the dual actions, antioxidant and MMP modulatory role. Hence, establishment of use of doxycycline as an effective therapy will be a boon to gastric ulcer patients.

MATERIALS AND METHODS

Materials

Gelatin from porcine skin, casein, Triton X-100 (TX), 1, 10 phenanthroline, catalase and protease inhibitor cocktail were obtained from Sigma Chemicals (St. Louis, MO, USA). MMP-9 and MMP-2 were purchased from Chemicon (UK). Polyclonal anti-MMP-2, anti-MMP-3, anti-MMP-9, anti-TIMP-1, anti-TIMP-2, and anti- β -tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Doxycycline was purchased from Dr. Reddy's Laboratory (India), and all other chemicals were purchased from Sisco Research Laboratory (Mumbai, India).

Animals used

Sprague-Dawley rats (180-220 g) bred in-house with free access to food and water were used for all experiments, which were carried out following the guidelines of the animal ethics committee of the institute. Experiments were designed to minimize animal suffering and to use the minimum number associated with valid statistical evaluation. Animals were anesthetized by ketamine (12 mg/kg), followed by cervical dislocation for killing. Animals in control and experimental groups were fasted overnight with free access to water before each experiment.

Gastric ulceration and protection studies

Indomethacin (48 mg/kg) was dissolved in distilled water with a minimum amount of NaOH and was orally fed to rats to induce a maximum level of acute gastric ulcer. The control group received the vehicle only. After 4 h, the animals were sacrificed, gastric lesions in the fundic stomach were scored and expressed as ulcer index as follows: 0 =

no pathology; 1 = a small pinhead ulcer spot; and 2-5 = a band-like lesion of 2-5 mm in length. The sum of the total scores divided by the number of animals was expressed as the mean ulcer index^[3]. Again, rats were orally fed with 70% ethanol at 4 mL/kg to induce maximum level of acute gastric ulcer or 2 mL/kg to induce moderate ulcer, while the control group received sterile water only. Animals were sacrificed after 3 h, and stomachs were scored for ulcer index as described by Pradeepkumar Singh *et al.*^[12]. The sum of the total scores divided by the number of animals indicates the mean ulcer index. Doxycycline at a dose of 50 mg/kg was administered orally 30 min prior to indomethacin or ethanol treatment to test the gastro-protective effects.

Measurement of glutathione level

A homogenate of stomach tissue without the connective tissue layer was made in 0.2 mol/L Tris, pH 8.2, which contained 20 mmol/L EDTA, and centrifuged at 12000 r/min for 15 min at 4°C. An aliquot of 1.0 mL homogenate was precipitated with 10% TCA and centrifuged at 14000 r/min for 3 min at 4°C. The supernatant (1 mL) was added to 2 mL of 0.8 mol/L Tris-HCl, pH 9.0, which contained 20 mmol/L EDTA and mixed with 0.1 mL 10 mmol/L 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The intense yellow color of nitromercaptobenzoate was read at 412 nm. For calibration, a standard curve was prepared treating varied concentrations of reduced glutathione with DTNB under identical conditions^[3].

Measurement of lipid peroxidation

The mitochondrial membrane fraction from gastric tissue homogenate was used for measurement of lipid peroxide content as thiobarbituric acid reactive species (TBARS). Briefly, 1 mL of the membrane fraction was allowed to react with 2 mL of TCA/TBA/HCl (15% TCA, 0.375% TBA, 0.25N HCl) reagent, heated in a boiling water bath for 15 min, cooled and centrifuged at 1000 r/min for 10 min at 4°C. The absorbance of the supernatant was measured at 535 nm and nmol TBARS produced was determined from a standard curve using tetraethoxypropane as standard^[3,12].

Measurement of protein carbonyl content

Protein oxidation was measured as carbonyl content in the low speed supernatant of the gastric tissue homogenate^[3,12]. The stomach from control, indomethacin-treated and doxycycline (50 mg/kg) pretreated indomethacin-treated rats was homogenized in 50 mmol/L sodium phosphate buffer, pH 7.4, in a Potter-Elvehjem glass homogenizer for 2 min, to obtain 20% homogenate. After centrifugation at 600 g for 10 min, the proteins from 1.0 mL of the supernatant were precipitated with 10% TCA and allowed to react with 0.5 mL 10 mmol/L 2,4-dinitrophenylhydrazine for 1 h. After precipitation with 20% TCA, the protein was washed three times with a mixture of ethanol-ethyl acetate (1:1), dissolved in 1.0 mL of a solution that contained 6 mol/L guanidine HCl in 20 mmol/L potassium phosphate, adjusted to pH 2.3

with TCA, centrifuged at 1000 r/min for 5 min at 4°C, and the supernatant was read for carbonyl content at 362 nm ($\epsilon = 22000/\text{M}$ per centimeter).

Tissue extraction

The whole stomach (including fundic, body and pyloric parts) was washed with saline and used for extraction. Gastric tissues were suspended in 10 mmol/L phosphate buffered saline (PBS) containing protease inhibitors, minced, and incubated for 10 min at 4°C. After centrifugation at 12000 g for 15 min, the supernatant was collected as PBS extracts. The pellet was then extracted in lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% TX and protease inhibitors) and centrifuged at 12000 g for 15 min to obtain TX extracts. PBS and TX extracts were preserved at -70°C^[3,12].

Gelatin and casein zymography

For assay of MMP-2 and MMP-9 activity, PBS and/or TX extracts were electrophoresed in 8% SDS-polyacrylamide gel containing 1 mg/mL gelatin under non-reducing conditions. For assay of MMP-3 activity, tissue extracts were electrophoresed in casein gel instead of gelatin. The gels were washed in 2.5% TX and incubated in calcium assay buffer (40 mmol/L Tris-HCl, pH 7.4, 0.2 mol/L NaCl, 10 mmol/L CaCl₂) for 18 h at 37°C, and stained with 0.1% Coomassie Blue followed by destaining^[3,12,13]. The zones of gelatinolytic or caseinolytic activity came as negative staining.

MMP inhibition assay

Equal amount of PBS extract from ulcerated tissue were incubated with two different concentrations of doxycycline (100 and 200 $\mu\text{mol/L}$) in calcium assay buffer (40 mmol/L Tris-HCl, pH 7.4, 0.2 mol/L NaCl, 10 mmol/L CaCl₂) for 1.5 h at 37°C. Gelatin and casein zymography were performed from the samples as described previously^[3,12,13]. Parallel assays were performed with the addition of phenanthroline (100 and 200 $\mu\text{mol/L}$ in 10% DMSO) to show that inhibition could be attributed to MMP-2, MMP-3 and MMP-9 activity. PBS extract incubated with requisite volume of 10% DMSO was used as a control^[32].

Effect of H₂O₂ on MMP-2 activity in vitro

To determine the effect of H₂O₂, equal amounts of PBS extract from control stomach (60 μg) were incubated with 500 $\mu\text{mol/L}$ concentration of H₂O₂ at 37°C for 1 h. To examine the protective effect of doxycycline on H₂O₂ mediated changes in MMP-2 activity, equal amounts of PBS extract were pretreated with doxycycline (200 $\mu\text{mol/L}$) prior to incubation with 500 $\mu\text{mol/L}$ H₂O₂. Pretreatment with catalase followed by incubation with H₂O₂ was used as a positive control. Gelatin zymography was performed with the samples as described previously^[19].

Western blotting

PBS extracts (100 $\mu\text{g/lane}$) were resolved by 8% reducing SDS-PAGE and processed for Western blotting^[13].

Proteins were transferred to membranes, blocked in 3% BSA solution in 20 mmol/L Tris-HCl, pH 7.4, containing 150 mmol/L NaCl and 0.02% Tween 20 (TBST), and incubated at 4°C in 1:200 dilutions of the respective primary antibodies in TBST containing 0.2% BSA. The membranes were then washed with TBST, incubated with alkaline-phosphatase-conjugated secondary antibody, and bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution.

Statistical analysis

Ulcer index data were fitted using Sigma plot software. Data were presented as the mean \pm SE. Statistical analysis was performed using Student-Newman-Keuls test (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of doxycycline on gastric ulceration induced by indomethacin or ethanol in rats

Indomethacin- and ethanol-induced gastric ulcer models in rat were used to test the protective effect of doxycycline against gastric damage. Doxycycline, a tetracycline analogue, has a number of non-antibiotic properties including inhibition of connective tissue breakdown. The action of doxycycline treatment on gastric damage was evaluated in rats by measuring ulcer index. One single dose of doxycycline (50 mg/kg) was selected for our study. Gastric ulceration was not detected in doxycycline-pretreated, indomethacin-treated rat gastric tissues. Figure 1A illustrates the effect of doxycycline on significant reduction in ulcer indices that occurred due to indomethacin-induced ulceration. In addition, doxycycline caused a significant decrease in ulcer index score from 50 to 15 when administered to high-dose ethanol-treated rats. Similarly, ulcer index score was reduced by less than 5 from 20 in low-dose ethanol-treated rats (Figure 1B). Thus, doxycycline arrested gastric damage by $> 70\%$ in rats with severe or moderate ulceration.

Effect of doxycycline on secreted and synthesized proMMP-9 and -2 activities

The gastroprotective action of doxycycline and association with MMP-2 and MMP-9 activities were examined in rat gastric tissues. Figure 2A and C shows that doxycycline inhibited proMMP-9 activity in PBS- and TX-extracted gastric tissues during protection against indomethacin-induced ulcer. PBS and TX extracts represented secreted and synthesized proMMP-9, respectively. ProMMP-9 activity is an inflammatory indicator of gastric damage and it increased with increasing gastric indices. Secreted and synthesized proMMP-9 activity was increased by about 12-fold and about 16-fold in indomethacin-ulcerated gastric tissues as compared to the controls, and doxycycline reduced the activity by 80% and 70%, respectively (Figure 2B and D). In contrast, doxycycline upregulated proMMP-2 activity at the level of secretion and synthesis, while protecting against indomethacin-induced gastric ulcer (Figure 2A and C). Indomethacin treatment de-

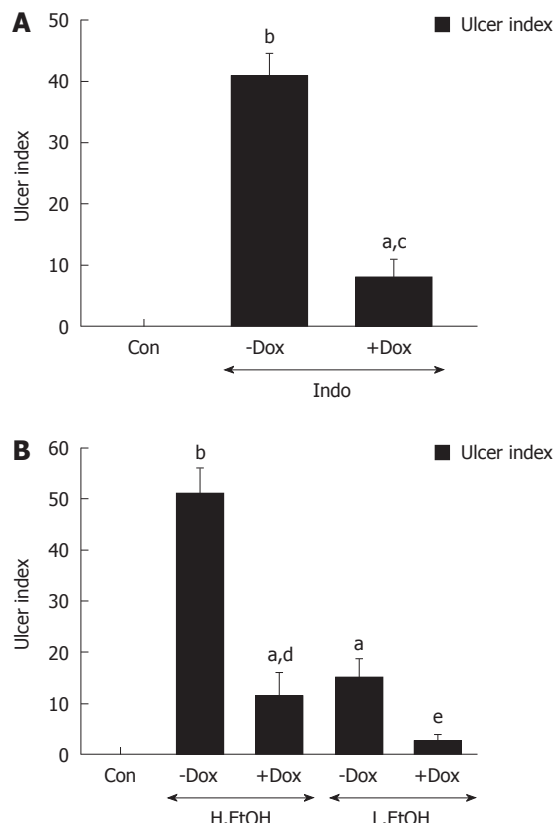


Figure 1 Protective effect of doxycycline on gastric injury. Gastric ulcers were induced in rats by oral administration of 48 mg/kg indomethacin or 70% ethanol (low and high) doses. Doxycycline (50 mg/kg) was administered orally prior to ulcerogen treatment to assess the protective effect. Control rats received sterile water only. After 3 h, rats were sacrificed and ulcer indices were scored. Mean ulcer index for indomethacin-induced (A) and ethanol-induced (B) ulceration in each group of rats was plotted. Dox: Doxycycline; Con: Control; Indo: Indomethacin; L.EtOH: Low dose ethanol; H.EtOH: High dose ethanol. Data expressed as mean \pm SE. ^a $P < 0.05$, ^b $P < 0.001$ vs control; ^c $P < 0.001$ vs indomethacin; ^d $P < 0.001$ vs high dose ethanol; ^e $P < 0.01$ vs low dose ethanol.

creased proMMP-2 activity by about 50% and doxycycline pretreatment reversed the activity almost to control levels for both secretion and synthesis (Figure 2B and D). Doxycycline significantly reduced proMMP-9 activity in gastric tissues both at the level of secretion and synthesis in ethanol-induced ulcerated rats that were doxycycline treated, compared to untreated controls (Figure 3A and C). Doxycycline caused a reduction in secreted proMMP-9 activity to about 5-fold and about 1.5-fold, which was increased about 20-fold and about 7-fold during ulceration by high and low doses of ethanol, respectively (Figure 3B). Similarly, there was a decrease in synthesized proMMP-9 activity by about 18-fold and about 7-fold by doxycycline during protection of ethanol-induced ulceration by low and high doses (Figure 3B). In contrast, doxycycline-pretreated, ethanol-treated rats showed an increase in MMP-2 activity in gastric tissues at the level of secretion and synthesis (Figure 3A and C). Upregulation of MMP-2 activity by doxycycline was more prominent with low-dose compared to high-dose ethanol-induced ulceration (Figure 3D). Thus, MMP-9 activity was downregulated while proMMP-2 activity was

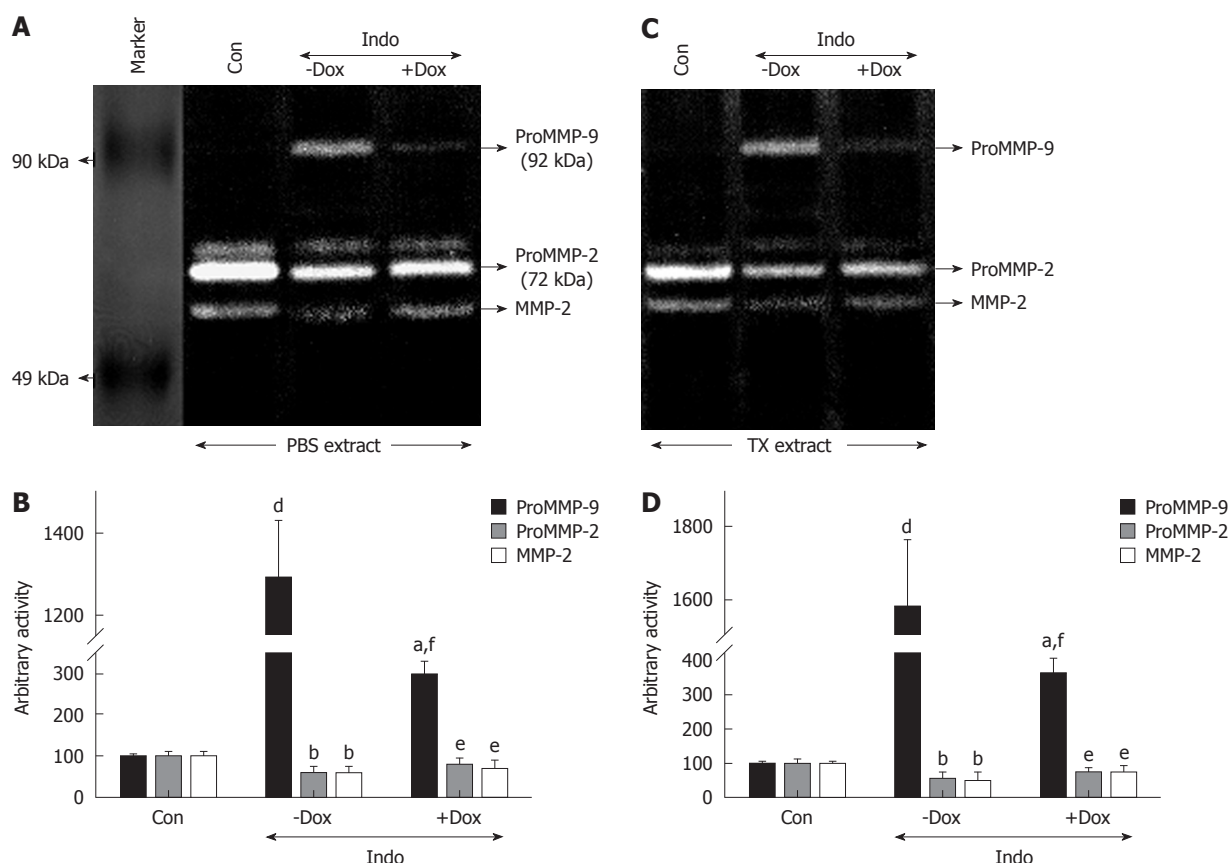


Figure 2 Effect of doxycycline on secreted and synthesized matrix metalloproteinase-2 and -9 activities during protection against indomethacin-induced ulcers. Gastric ulcer was induced by intragastric administration of 48 mg/kg indomethacin in rats, and doxycycline was given 30 min prior to indomethacin for protection studies. Gelatin zymography was performed using phosphate buffered saline (PBS) (A) and Triton X-100 (TX) (C) extracts of gastric tissue from different groups of rats. Histogramic representation of arbitrary activity of pro-matrix metalloproteinase (MMP)-9 (B) and proMMP-2 (D) in PBS and TX extracts of gastric tissues, as measured by Lab Image software using the above zymogram and two other representative zymograms from independent experiments. Data expressed as mean \pm SE. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ vs control; ^d $P < 0.05$, ^e $P < 0.01$ vs indomethacin.

upregulated in rat gastric tissues during gastroprotection by doxycycline. Expression of MMP-2 was also examined by Western blot analysis (Figure 3E) in doxycycline-pretreated, ethanol-treated gastric tissues. Figure 3F reveals that upregulation of proMMP-2 reached control levels in doxycycline-pretreated, ethanol-treated (low dose) rat gastric tissues. Similarly, upregulated expression of MMP-2 was also reflected in doxycycline-pretreated, ethanol-treated (high dose) rat gastric tissues. β -tubulin was run in parallel to confirm equal protein loading.

Effect of doxycycline on expression MMPs and TIMPs during gastroprotection

To investigate the ability of doxycycline to regulate MMP-3 during prevention of ulcers, indomethacin- and ethanol-induced ulceration were both examined. Figure 4 suggests that indomethacin increased proMMP-3 activity significantly in ulcerated gastric tissues. Figure 4A and C shows that doxycycline inhibited proMMP-3 activity very significantly in PBS- and TX-extracted gastric tissues during protection against indomethacin-induced ulcer. ProMMP-3 inhibitory activity of doxycycline was at a magnitude of about 75% and about 66% for secreted and synthesized protein, respectively, during protection

of indomethacin-induced gastric ulcer (Figure 4B and D). Doxycycline inhibited the activity of secreted proMMP-3 while preventing both severe and moderate gastric ulceration induced by ethanol (Figure 5A). Doxycycline downregulated the activity by about 2-fold while protecting ethanol-treated gastric tissues, and the efficacy was greater for low-dose ethanol-treated tissues (Figure 5B). In other words, doxycycline exhibited an inhibitory effect on proMMP-3 activity by about 50% and about 70% for high-dose and low-dose ethanol-treated gastric tissues, respectively, as depicted in Figure 5B. The effect of doxycycline on MMP-9 and MMP-3 expression in rats was analyzed by Western blotting (Figure 5C). Both high and low doses of ethanol increased the expression of MMP-9 by about 21-fold and about 7-fold, respectively, in rat gastric tissues compared to controls, while treatment with doxycycline inhibited MMP-9 expression by 95% and 85%, respectively. Similarly, MMP-3 expression increased by about 6.5-fold and about 3.5-fold for high- and low-dose ethanol treatment, respectively, and doxycycline reduced their expression by 60% and 80%, respectively (Figure 5C). Moreover, the effect of doxycycline was more pronounced when ulcer was moderate rather than severe. To ascertain the regulatory role of TIMPs in ethanol-induced gastric ulcer,

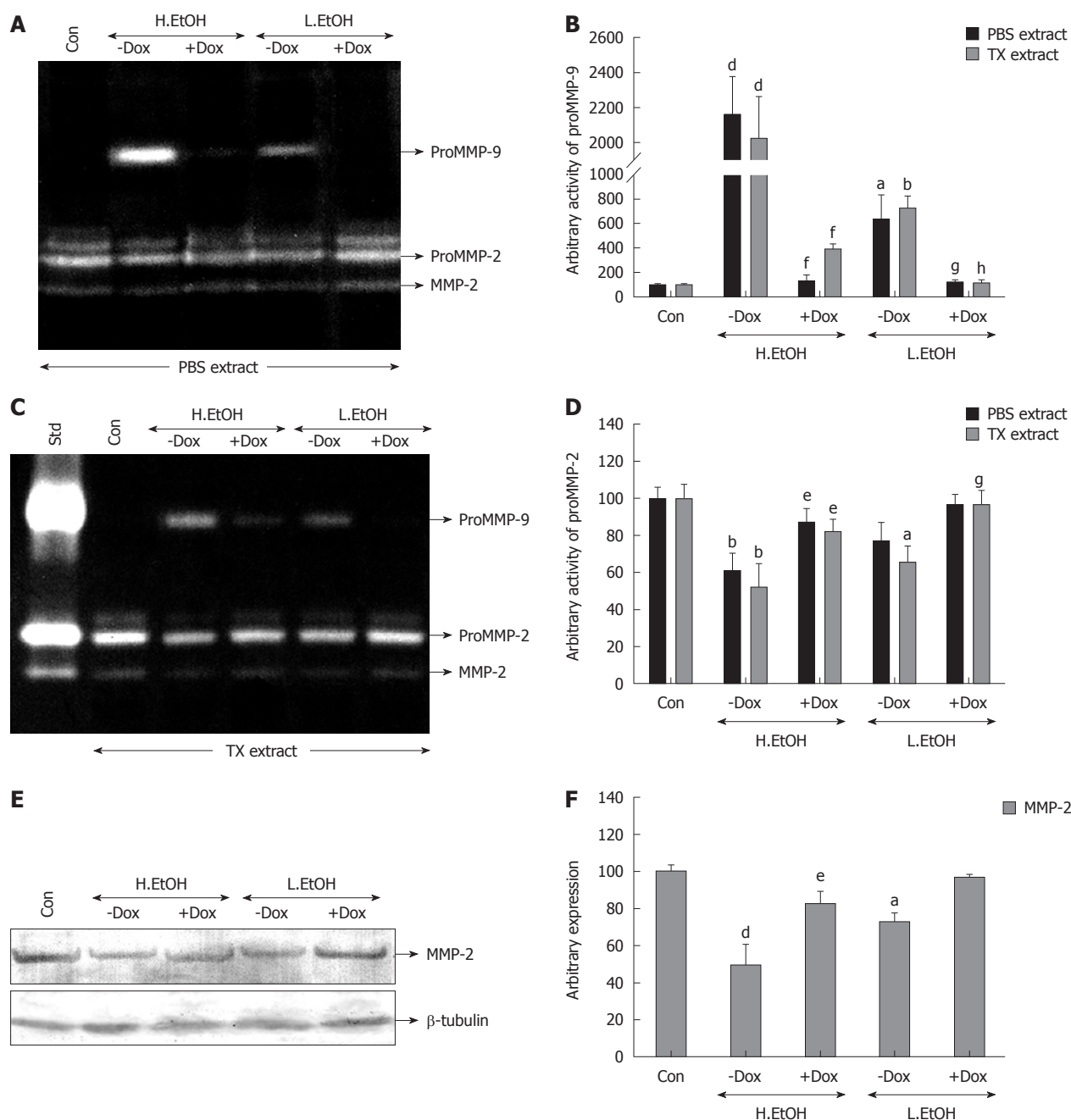


Figure 3 Effect of doxycycline on secreted and synthesized matrix metalloproteinase-2 and -9 activities during protection of ethanol-treated gastric ulcer. Low (0.4 mL/rat) and high (0.8 mL/rat) doses of 70% ethanol were administered intragastrically to induce moderate and maximum gastric ulcers, and doxycycline was administered 30 min prior to ethanol. Gelatin zymography was performed using phosphate buffered saline (PBS) (A) and Triton X-100 (TX) (C) extracts of gastric tissues from different group of rats. Histogramic representation of arbitrary activity of pro-matrix metalloproteinase (MMP)-9 (B) and proMMP-2 and MMP-2 (D) in PBS and TX extracts of gastric tissues as measured by Lab Image software using the above zymogram and two other representative zymograms from independent experiments. PBS extracts were subjected to Western blotting (E) and probed with polyclonal anti-MMP-2 and β -tubulin antibody. Fold changes of MMP-2 (F) are represented in a histogram as measured by Lab Image software using the above western blot and two other representative blots from independent experiments. Data expressed as mean \pm SE. ^a P < 0.05, ^b P < 0.01, ^d P < 0.001, vs control; ^e P < 0.05, ^f P < 0.001 vs high-dose ethanol; ^g P < 0.05, ^h P < 0.01 vs low-dose ethanol of respective PBS and TX extracts. L.EtOH: Low dose ethanol; H.EtOH: High dose ethanol.

TIMP-1 and TIMP-2 expression was checked by Western blot analysis. TIMP-1 expression was reduced by 40% and 60% for high- and low-dose ethanol, respectively, and doxycycline reversed TIMP-1 expression almost to control values. On the other hand, TIMP-2 expression followed an opposite trend. It increased in rat gastric tissue after ethanol treatment. TIMP-2 expression induced by 1.6-fold

and 1.3-fold, respectively, and doxycycline reduced TIMP-2 expression near to control levels. β -tubulin was run in parallel to confirm equal protein loading.

Inhibitory effect of doxycycline on MMP-9, -2 and -3 activities *in vitro*

The effect of doxycycline on MMP activity *in vitro* was

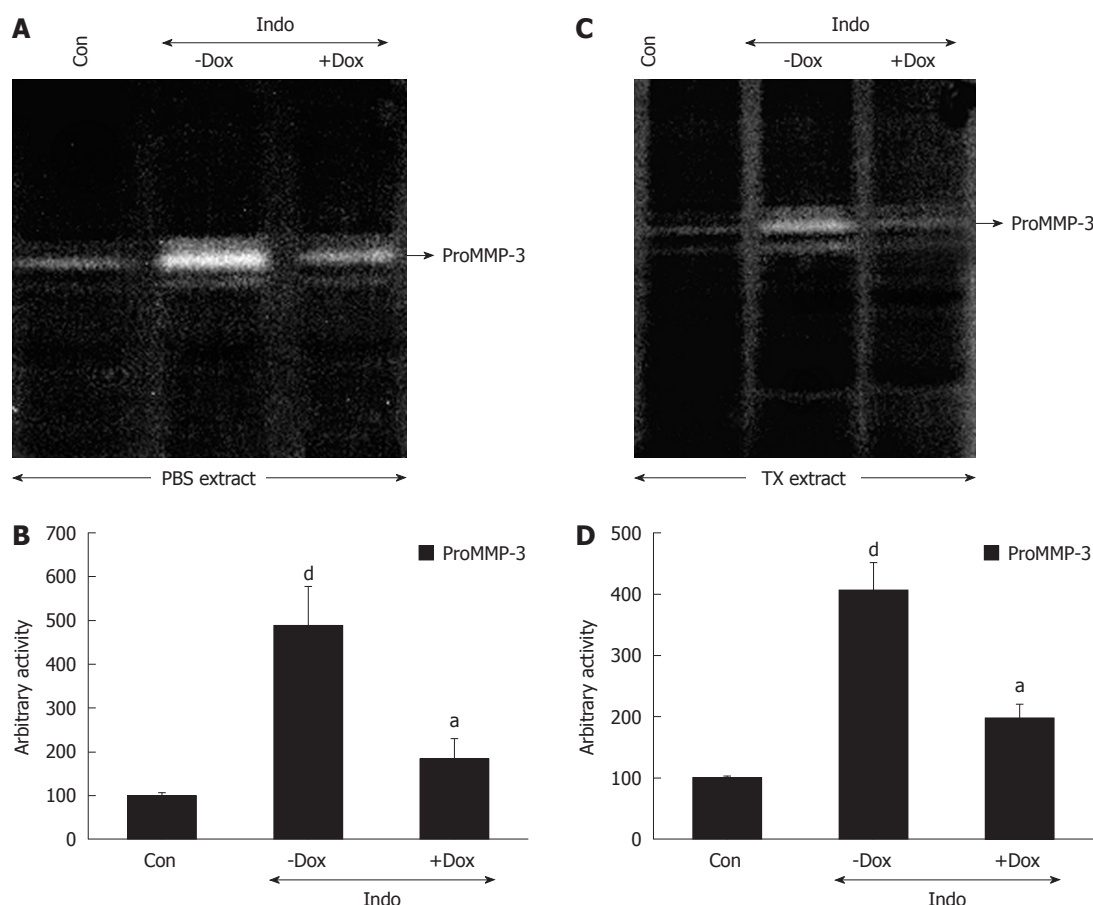


Figure 4 Effect of doxycycline on matrix metalloproteinase-3 activity during protection against indomethacin-induced gastric ulcer. Gastric ulcer was induced by intragastric administration of 48 mg/kg indomethacin in rats, and doxycycline was given 30 min prior to indomethacin for protection studies. Casein zymography was performed using phosphate buffered saline (PBS) (A) and Triton X-100 (TX) (C) extracts of gastric tissue from different groups of rats. Histogrammic representation of arbitrary activity of pro-matrix metalloproteinase (MMP)-3 in PBS (B) and TX (D) extracts of gastric tissues as measured by Lab Image software designed using the above zymogram and two other representative zymograms from independent experiments. Data expressed as mean \pm SE. ^a $P < 0.05$, vs indomethacin; ^d $P < 0.001$ vs control.

evaluated by casein as well as gelatin zymography. Gastric tissue extracts with detectable MMP-2, MMP-3 and MMP-9 activities were used as controls for the inhibition experiment. Doxycycline inhibited MMP-2, MMP-3 and MMP-9 activities dose dependently, as judged by zymography (Figure 6A). Doxycycline at a dose of 100 $\mu\text{mol/L}$ and 200 $\mu\text{mol/L}$ inhibited MMP-9 activity by 30% and 50%, respectively, while MMP-2 and MMP-3 activities were inhibited by 65% in each case. *In vitro* inhibition studies of MMP activity were also performed in parallel using zinc chelator (1, 10 phenanthroline) to confirm the metalloprotease property. Phenanthroline at a dose of 100 $\mu\text{mol/L}$ inhibited MMP-2, MMP-3 and MMP-9 by about 60%, about 62% and about 55%, respectively. In addition, the activity of MMP-2, MMP-3 and MMP-9 were further decreased by 10-15% more at high doses of phenanthroline (200 $\mu\text{mol/L}$) in each case (Figure 6B).

Reduction of oxidative stress by doxycycline *in vivo* and *in vitro*

Oxidative damage of cellular lipid and protein is directly associated with gastric ulceration. To determine the effect of doxycycline on oxidative damage of gastric tissues

due to indomethacin, we measured protein oxidation and lipid peroxidation in control, indomethacin-treated, and indomethacin-treated doxycycline-pretreated gastric tissue homogenates. Indomethacin caused excessive oxidative damage that was associated with a about 2.5-fold and about 2-fold increase in lipid peroxidation and protein oxidation, respectively, while doxycycline treatment reduced them by about 80% and about 93%, respectively (Table 1). Moreover, the antioxidant, glutathione was reduced by about 36% compared to controls, and doxycycline recovered the glutathione content up to 92% during gastroprotection. In order to assess the possible antiulcer mechanism of doxycycline, we examined its protective effect on H_2O_2 -mediated suppression of proMMP-2 activity *in vitro*. PBS-extracted gastric tissues from control rats were incubated with 200 $\mu\text{mol/L}$ doxycycline followed by H_2O_2 treatment for 1 h at 37°C, and gelatin zymography was performed. The activity of MMP-2 in the zymogram (Figure 7A) was decreased by about 45% compared to the controls by H_2O_2 , and doxycycline rescued H_2O_2 -mediated suppression of MMP-2 activity by about 80% (Figure 7B). As expected when H_2O_2 treated samples were incubated with catalase, it failed to suppress MMP-2

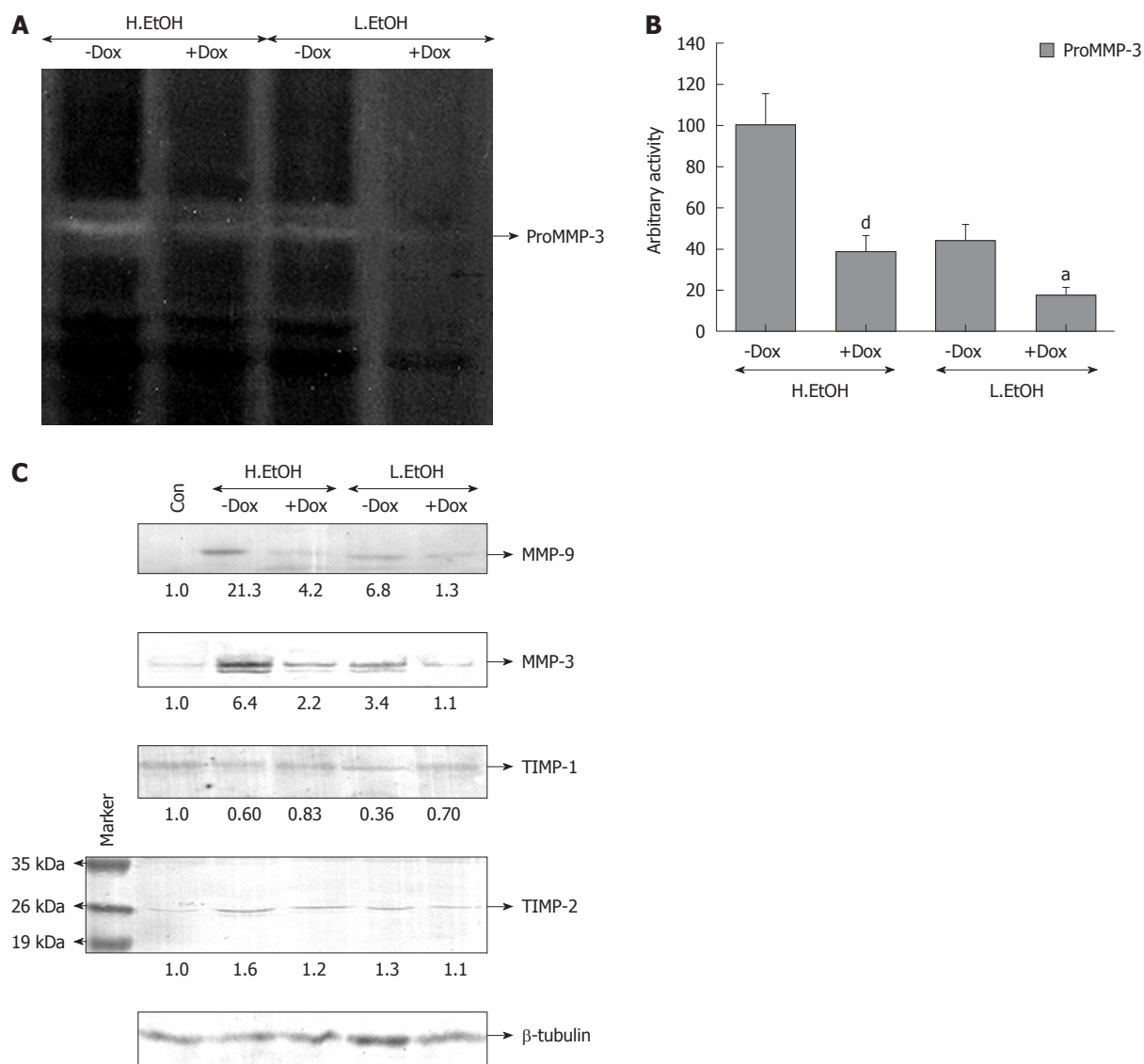


Figure 5 Effect of doxycycline on matrix metalloproteinase and tissue inhibitors of metalloproteinase activity and expression during protection against ethanol-treated gastric ulcer. Ulcers were induced in rat using 70% ethanol at low and high doses, and protection by doxycycline was performed. Casein zymography (A) was performed using phosphate buffered saline (PBS) extract of different gastric tissues. Histogramic representation (B) of arbitrary activity of pro-matrix metalloproteinase (MMP)-3 as measured by Lab Image software using the above zymograms and two other representative zymograms from independent experiments. PBS extracts of gastric tissues from different experimental groups were subjected to Western blot blotting (C) and probed with polyclonal anti-MMP-9 and -3, tissue inhibitors of metalloproteinase (TIMP)-1 and -2, and anti- β -tubulin antibodies. Fold changes of proteins as represented below each band were measured by Lab Image software using the above western blots and two other blots from independent experiments. Data expressed as mean \pm SE. ^a $P < 0.05$ vs low-dose ethanol; ^d $P < 0.01$ vs high-dose ethanol. L.EtOH: Low dose ethanol; H.EtOH: High dose ethanol.

activity. Indomethacin-induced ulceration is associated with generation of H_2O_2 , which in turn, damages tissues by interacting with protein and lipid. Thus, neutralizing H_2O_2 -mediated damage by doxycycline suggests the antioxidant property of doxycycline.

DISCUSSION

NSAIDs, along with other aggressive factors like ethanol, *Helicobacter pylori* infection, stress and steroids, increases the risk of gastric ulcer formation^[3,4,11-13,19]. Increased production of ROS and inflammatory cytokines, inhibition of cell proliferation, infiltration of inflammatory cells, reduced production of mucus, growth factors, and

prostaglandins are factors involved in the pathogenesis of gastric ulcers^[9,11]. Research has been focused recently on the importance of ECM and its turnover in the ulcerated margins of gastric tissue. Increased epithelial cell turnover is associated with the repair process without altering the composition and organization of the gastric ECM. Matrix proteins are secreted by different cell types including fibroblasts and epithelial and endothelial cells and assemble into an ECM network in the spaces surrounding the cells^[5,9,10]. Recent studies have described the involvement of MMPs in cleaving and remodeling of ECM. MMPs have been implicated in various processes including tumor growth metastasis, chronic ulceration, fetal tissue development, and bone resorption. MMPs

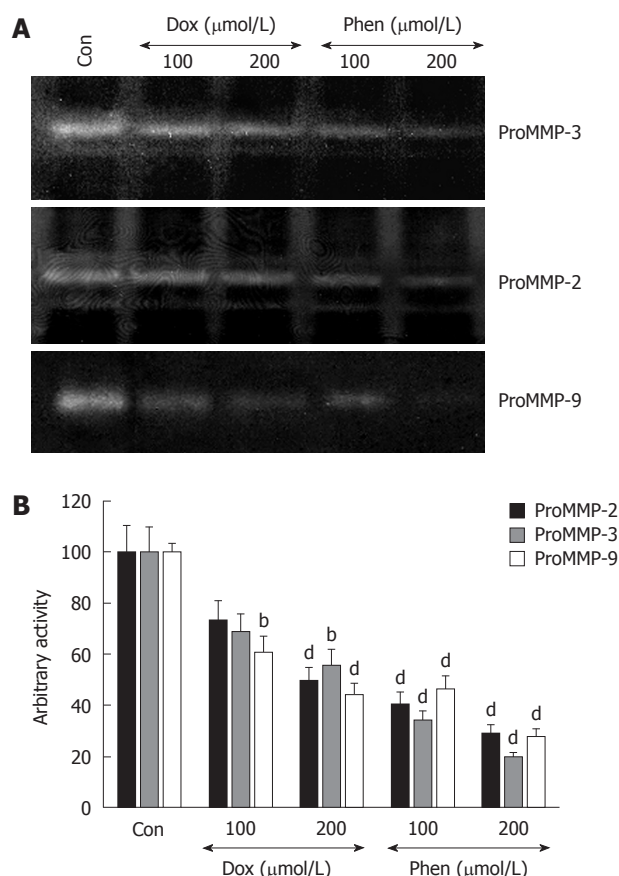


Figure 6 Inhibitory effect of doxycycline on matrix metalloproteinase activity in rat gastric tissue extract *in vitro*. A: Gelatin and casein zymography of gastric tissue extract following treatment with doxycycline or 1, 10-phenanthroline at two different concentrations; B: Histogrammic representation of pro-matrix metalloproteinase (MMP)-3, proMMP-2 and proMMP-9 activities as measured by Lab Image software using the above zymograms and two other representative zymograms from independent experiments. Data expressed as mean \pm SE. ^b $P < 0.01$, ^d $P < 0.001$ vs control phenanthroline was labeled as Phen in the figure.

Table 1 Effect of doxycycline on glutathione, lipid peroxidation and protein oxidation level in indomethacin-induced gastric ulcer

Samples	Glutathione (nmol/g tissue)	Lipid peroxidation (nmolTBARS/mg protein)	Protein oxidation (nmol/mg protein)
Control	182 \pm 6.50	1.13 \pm 0.16	2.66 \pm 0.12
Indomethacin	116.50 \pm 7.72 ^b	2.52 \pm 0.07 ^b	5.14 \pm 0.32 ^b
Doxycycline + indomethacin	167.25 \pm 8 ^d	1.27 \pm 0.06 ^d	3.36 \pm 0.25 ^{a,d}

Doxycycline was administered 30 min before indomethacin in Sprague-Dawley rats. Control animals received vehicle orally. Glutathione content ($n = 18$), lipid peroxidation ($n = 18$), and protein carbonylation ($n = 18$) were measured in gastric tissue homogenate. Results are represented as mean \pm SE. Data expressed as mean \pm SE. ^a $P < 0.05$, ^b $P < 0.001$ vs control; ^d $P < 0.01$ vs indomethacin.

capable of degrading ECM proteins are believed to play a significant role during the pathogenesis and healing of gastric ulcer^[3,4,12]. These enzymes are synthesized as latent proenzymes and their activity is tightly regulated at

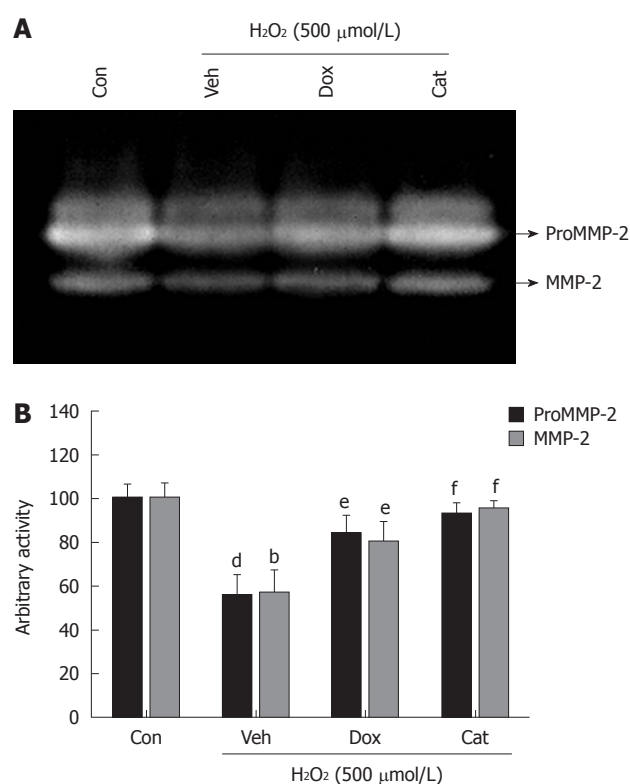


Figure 7 H₂O₂ scavenging activity of doxycycline. A: Gelatin zymography of phosphate buffered saline (PBS) extract from control gastric tissue was incubated with H₂O₂ (500 mmol/L) at 37°C for 1 h. Equal amounts of PBS extract were pretreated with doxycycline (200 μmol/L) before incubation with H₂O₂. In one lane (Cat, lane 4), the extract was pretreated with catalase before reaction with H₂O₂; B: Histogrammic representation of pro-matrix metalloproteinase (MMP)-2 and MMP-2 activities as measured by Lab Image software using the above zymograms and two other representative zymograms from independent experiments. Veh: Vehicle; Cat: Catalase. Data expressed as mean \pm SE. ^b $P < 0.01$, ^d $P < 0.001$ vs control; ^e $P < 0.05$, ^f $P < 0.01$ vs vehicle.

several levels including the post-translational level^[33]. The coordinate action of proteases, in particular the MMPs and antiproteases, including the TIMPs, is thought to play an important role in connective tissue degradation^[5]. In the present study, the authors intended to assess the relationship between MMP-2, MMP-3 and MMP-9 activity due to induction of gastric injury. Herein, doxycycline has been used to examine its gastroprotective potential.

Doxycycline, a widely prescribed antibiotic, is also a non-specific MMP inhibitor. It also possesses antiangiogenic and anti-inflammatory and neuroprotective properties, and is a convenient drug with low cost, and clinical tolerability without serious side effects^[34]. Ryan *et al*^[35] have reported divalent chelating activity of doxycycline. The beneficial effects of doxycycline are mainly mediated by its inhibition of MMP-9 and MMP-2. Doxycycline is safe in treating certain diseases because it reduces the level of pathophysiologically excessive MMPs but not to the level below normal physiological conditions. The effective dose of doxycycline is 100-200 mg/d for adult humans^[36].

In the present study, we primarily showed that doxycycline potentially inhibited MMP-9 and MMP-3 activity

while arresting gastric damage in rats. It is noteworthy that doxycycline upregulated MMP-2 activity and expression during gastroprotection. Our results suggest that doxycycline acts through mechanisms distinctly different from its inhibitory effect on MMPs. Thus, doxycycline has a different action on MMP-2 activity in gastric damage, and indicates its regulatory role in addition to its inhibitory effect on MMPs. The increased MMP-2 activity may be required for preservation of matrix integrity, because function of the gastric epithelial and mucosal layer could be modulated by ECM components in gastric tissues^[3,19]. Herein, an interesting feature of doxycycline is that it reduced MMP-2 activity *in vitro* and increased it *in vivo*. One interpretation is that doxycycline binds to $\text{Ca}^{2+}/\text{Zn}^{2+}$ at catalytic sites of MMP *in vitro* and inhibits the enzymatic activity of MMPs^[22,35]. Another interpretation may be that doxycycline perturbs the signaling pathways related to mitogen-activated protein kinase or Smad^[27], and subsequently leads to beneficial systemic changes. *In vivo* upregulation of MMP-2 activity could be explained as an anti-apoptotic role of doxycycline during gastroprotection^[34]. An alternative interpretation is that doxycycline inhibits oxidative stress and upregulates expression of MMP-2, or decreases antiprotease expression, thus shifting the protease/antiprotease balance towards high enzymatic activity. The beneficial effect on gastric damage may not be solely attributed to MMP-9 and MMP-3 inhibition. Other proteases and antiproteases may play a critical role, and pleiotropic effects of doxycycline may offer a better approach. This study provided convincing data to suggest a new pharmacological approach for the drug doxycycline for prevention of NSAID- or ethanol-induced gastric ulcers.

While the exact mechanism of gastric ulceration is still not completely understood, various cellular stresses including oxidative stress play a central role in the progression of gastric ulcers. Inflammatory reactions induced by indomethacin are a significant source of ROS, for example, H_2O_2 and the superoxide radical, which play a causative role in gastric injury^[19]. ROS generally modulate MMP activity either indirectly through redox-dependent regulation of MMP gene transcription or directly through modification of MMP structure^[2]. However, the direct association of ROS with the regulation of MMPs during gastric ulceration is not well studied. Several *in vivo* and *in vitro* cell culture studies have hypothesized activation of gelatinases through ROS or ROS generators when applied time and dose dependently. Herein, incubation of pro-MMP-2 in an *ex vivo* system with H_2O_2 resulted in suppression of enzyme activity, which suggests that ROS play a significant role in modulating MMP-2 activity. We also showed that doxycycline possessed inhibitory activity against H_2O_2 -mediated suppression of MMP-2 activity, which indicated the antioxidant action of doxycycline. Doxycycline offered gastroprotection by preventing oxidative damage caused by lipid peroxidation and protein oxidation. Ulcerated samples exhibited reduced thiol content, whereas that was almost completely restored to

control levels by doxycycline during gastroprotection. Although doxycycline has been reported as a potent inhibitor of MMPs, the antioxidant activity is worth mentioning in arresting gastric injury.

The major observations of the present study highlight that: (1) gastric injury is associated with upregulation of proMMP-3 and -9 activity, as well as expression in rat gastric tissues; (2) doxycycline is very effective in preventing gastric damage, and the mechanism of gastroprotection is attributable to its inhibitory effect on proMMP-3 and -9, while enhancing MMP-2 *in vivo*; and (3) doxycycline has a modulatory role with TIMP-1 and TIMP-2 during protection against gastric ulcers. The regulatory role of proMMP-2 activity may be dependent on the antioxidant action of doxycycline. We showed that doxycycline provides protection against gastric damage *via* inhibition of protein oxidation, lipid peroxidation and glutathione depletion. Moreover, suppression of MMP-2 activity by oxidative stress is rescued by doxycycline. Overall, this study is believed to be the first to demonstrate effectiveness of doxycycline in prevention of gastric damage *via* regulation of MMPs. Doxycycline therefore, may be considered as a candidate towards a therapeutic strategy for gastric ulcers. Also, proMMP-3 and -9 overexpression in gastric injury may be exploited as therapeutic targets, and doxycycline may be used as target-based therapy.

COMMENTS

Background

A class of zinc-dependent enzyme named matrix metalloproteinases (MMPs) is capable of degrading proteins of cellular and extracellular origin of various tissues. Their function is regulated by endogenous inhibitors such as tissue inhibitor of metalloproteinases and α -2 macroglobulin, which helps to maintain the protease-antiprotease balance. Imbalance in MMPs action is implicated in different diseases including gastric ulcer. Various factors mainly alcohol, non-steroidal anti-inflammatory drugs and infection with *Helicobacter pylori* cause gastric injury. In recent years, there has been a paradigm shift in understanding the mechanism of gastric ulcers, which includes regulation of MMPs and remodeling of the matrix surrounding the cells. Healing of gastric injury is a complicated process that comprises a series of cell-matrix interactions, and cell-cell signaling. Existing drugs against gastric injury have some limitation and side effects. Researchers are looking for novel drugs that have fewer side effects, easy availability, and low cost. A research group in India has examined the effect of doxycycline on gastric injury *via* regulation of MMPs.

Research frontiers

Doxycycline (derivative of tetracycline) is used commonly as an antibiotic. However, the antiulcer mechanism of doxycycline has not yet been explored fully. This study is believed to be the first to investigate the regulation of MMP-2 expression by doxycycline while preventing gastric injury.

Innovations and breakthroughs

The gastroprotective effect of doxycycline was tested against ethanol- or indomethacin-induced gastric mucosal injury in rats. Doxycycline prevented oxidative damage caused by lipid peroxidation and protein oxidation. Ulcerated samples exhibited reduced glutathione content, and it was almost completely restored to control levels by doxycycline during gastroprotection. Injured tissues exhibited significantly higher MMP-3 and MMP-9 activities, while doxycycline treatment markedly decreased both enzymes during gastroprotection. In contrast, the reduced MMP-2 activity in injured tissues was reversed by doxycycline during ulcer prevention. In addition, *in vitro*, doxycycline inhibited significantly all MMPs and proMMP-2, -3 and -9. Swarnakar *et al* have demonstrated a novel role of doxycycline in the upregulation of MMP-2 activity and expression during protection against gastric ulcers.

Applications

Altogether, the work established the use of doxycycline as an antiulcer drug that may lead to effective therapy for gastric ulcer. Combination therapy of doxycycline along with other existing antiulcer drugs such as famotidine, ranitidine or omeprazole may be better for treatment of gastric inflammation.

Peer review

This is believed to be the first demonstration of the dual action of doxycycline, namely, upregulation of MMP-2 activity and reduction of oxidative stress, in arresting gastric injury. Long-term use of doxycycline may provide therapeutic intervention for chronic gastric ulceration that poses a risk of progression to carcinoma.

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