

## CHAPTER 2

### RESEARCH METHODOLOGY

#### 1. Medicinal plant

Dried powder (1 kg) of *C. longa* rhizome (voucher specimen no. 31, 1410, 1458; Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand) was macerated in hexane (3 l) followed by ethyl acetate (3 l x 3), respectively, to produce hexane and ethyl acetate (EtOAc) extracts. The EtOAc extract was taken to dryness under reduced pressure, and was then submitted to vacuum silica gel column chromatography using chloroform-methanol mixture as eluent followed by permeation Sephadex LH-20 using methanol as eluent. According to these procedures, 45.5 g of curcumin was obtained (yield; 4.55%). The purity of curcumin was confirmed by comparison of its spectroscopic data and m.p. with literature values (Kosuge, *et al.*, 1985; Roughley and Whiting, 1973) and co-chromatography with a standard sample of curcumin.

#### 2. Animals

Male Wistar-strain rats weighing 180–220 g and male Swiss mice weighing 25–30 g were provided by the Animal House, Faculty of Science, Prince of Songkla University, Thailand, and by the Animal House, Faculty of Pharmaceutical Sciences, Chiba University, Japan. They were housed under normal laboratory condition at  $25 \pm 1^\circ\text{C}$  with a controlled 12-h light-dark cycle and maintained on standard rodent chow and tap water *ad libitum*. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of Prince of Songkla University, Thailand, and the “Principles of laboratory animal care” (NIH publication number 85-23, revised 1985) and guidelines of the Animal Investigation Committee, Chiba University, Japan. When necessary, the rats were deprived of food for 24 h with access to water *ad libitum* before the experiments.

The animal studies were approved by the Committee on Animal Care and in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Prince of Songkla University, Thailand, and the "Principles of laboratory animal care" (NIH publication number 85-23, revised 1985) and guidelines of the Animal Investigation Committee, Chiba University, Japan.

### 3. Chemicals

Chemicals purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.) were 100% acetic acid, aminoguanidine hydrochloride, cimetidine, compound 48/80, dimethyl sulfoxide (DMSO), lansoprazole, mesalazine, pentobarbital sodium, serotonin, sulfasalazine and vanillin. Carboxymethylcellulose 1200 was purchased from Vidhyasom Co., Ltd. (Bangkok, Thailand). All chemicals were of analytical grade. Tetrahydrocurcumin was kindly gifted from Dr. Isobe, Showa Pharmaceutical University (Tokyo, Japan)

#### 3.1 Chemicals and reagents used for cell culture

Mouse macrophage RAW 264.7 cell line was purchased from JCRB Cell Bank (Tokyo, Japan). RPMI 1640 medium was purchased from Kojin Bio (Saitama, Japan). Fetal bovine serum (FBS) was purchased from Gemini Bio Products (CA, USA). Antibiotics solution (penicillin and streptomycin sulfate) was purchased from Invitrogen Co. (CA, USA). Lipopolysaccharide (LPS) from *Escherichia coli* and Trypsin-EDTA (0.05-0.02%) in Hank's Balanced Salt Solution (HBSS), without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.).

#### 3.2 Reagents used for Western blot analysis

Chemicals and reagents purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.) included acrylamide/bis-acrylamide, ammonium peroxodisulfate, bovine serum albumin (BSA), monoclonal mouse anti- $\beta$ -actin clone AC-15, precision plus dual protein standards, sodium orthovanadate, protease inhibitor cocktail and nitrocellulose membrane.

Chemicals and reagents purchased from Wako Pure Chemicals (Osaka, Japan) included methanol, phenylmethylsulfonylfluoride (PMSF), sodium dodecyl sulfate (SDS), sodium chloride, N,N,N',N'-tetramethylethylenediamine (TEMED) and Triton-X 100

Reagents purchased from Cell Signaling Technology, Inc. (Danvers, MA., USA) included monoclonal mouse anti-human IL-1 $\beta$ , anti-iNOS, anti-TNF- $\alpha$  antibodies, and COX-2 antibody.

Reagents purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA., U.S.A.) included goat anti-rabbit IgG, HRP-conjugate immunoglobulin for iNOS and COX-2, rabbit anti-goat mouse IgG, HRP-conjugate immunoglobulin for TNF- $\alpha$ , and goat anti-mouse IgG, HRP-conjugate immunoglobulin for IL-1 $\beta$  and  $\beta$ -actin

Bio-Rad Dc protein assay reagent and Immune-Star™ chemiluminescent reagent were purchased from Bio-Rad Laboratories Inc. (CA., USA).

RNA extraction reagent (TRIzol™) and RNA extraction kit (RNAeasy mini™) were purchased from Qiagen (CA., USA). Glycine was purchased from MP Biochemicals (Ohio, USA). Tris-HCl was purchased from Invitrogen (Carlsbad, CA., USA)

### **3.3 Chemicals and reagents used for reverse-transcribed polymerase chain reaction (RT-PCR)**

GeneRuler 50bp DNA Ladder, 10X Loading dye was purchased from Fermentas Inc. (Hanover, MD., USA)

One step reverse-transcribed polymerase chain reaction kit was purchased from Qiagen, (CA., USA)

Molecular biology agarose was purchased from Bio-rad Laboratories Inc. (CA., USA)

The sense and antisense primers used for mouse iNOS, COX-2, TNF- $\alpha$  and  $\beta$ -actin were purchased from Sigma-Aldrich Japan (Tokyo, Japan).

### **3.4 Chemicals and reagents used for real-time reverse-transcribed polymerase chain reaction (real-time RT-PCR)**

Reverse transcription reagents: Oligo(dT)<sub>12-18</sub> primer, 5X First strand buffer, 0.1M

dithiothreitol (DTT), 10 mM dNTP, and Superscript II reverse transcriptase were purchased from Invitrogen (Carlsbad, CA., USA)

Pre-Developped TaqMan™ assay reagents/Human 18S rRNA mix; TaqMan™ universal PCR Master Mix; Mouse iNOS, COX-2, TNF- $\alpha$  and - $\beta$ -actin oligonucleotide primers; and rat iNOS primers were purchased from Applied Biosystem Inc. (CA., USA)

### 3.5 Chemicals and reagents used for immunohistochemistry

$\alpha$  Rabbit IgG Alexa 488 was purchased from Invitrogen (Carlsbad, CA., USA)

Mounting solution (Tissue-Tek OCT™) was purchased from International Medical Equipment Inc., Japan.

Other reagents use in cell culture techniques purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.) included acetone, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), picric acid, paraformaldehyde, NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O and Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O.

Curcumin used in *in vivo* study and cimetidine were suspended in 1% carboxymethyl-cellulose (CMC), while aminoguanidine was dissolved in saline. Curcumin was also dissolved in dimethyl sulfoxide (DMSO) immediately before intraperitoneal administration at a dose of 20 mg/ml DMSO/kg body weight. DMSO was administered in a volume of 1 ml/kg bodyweight. All drugs were prepared immediately before use and administered in a volume of 0.5 ml/100 g body weight.

Curcumin, vanillin and tetrahydrocurcumin used in *in vitro* study were dissolved in DMSO. The concentration of DMSO in all assays did not exceed 0.1%.

## 4. Methods

Since many reports showed that curcumin, when given at a dose of 10-200 mg/kg intraperitoneally or orally, exhibits various biological effects such as anti-tumor and anti-inflammatory in many organs (Awasthi, *et al.*, 1996; Razga and Gabor, 1995; Reddy and Lokesh, 1994, 1996; Singletary, *et al.*, 1996; Susan and Rao, 1992), the dose range of 5-80 mg/kg curcumin is used in the present study.

#### 4.1 Determination of gastric acid and pepsin secretion

A pylorus ligation was carefully performed on fasted rats under anesthesia with pentobarbital sodium (50 mg/kg, *i.p.*). The rats received either 1% CMC (5 ml/kg, *i.d.*), curcumin (5-40 mg/kg, *i.d.*), cimetidine, (100 mg/kg, *i.d.*) or aminoguanidine, (30 mg/kg, *s.c.*) immediately after the ligation. Four hours later, the rat was killed under deep-ether anesthesia, and the gastric juice was collected and centrifuged at 1300 g for 10 min. After measuring the volume of the supernatant, the total acid output was then analyzed by titration with 2 mM NaOH using 2% phenolphthalein as an indicator and expressed as  $\mu\text{Eq/ml}$  or  $\mu\text{Eq/h}$ . Further, pepsin activity was determined by a slight modification of Anson (Anson, 1938), using bovine hemoglobin as a substrate.

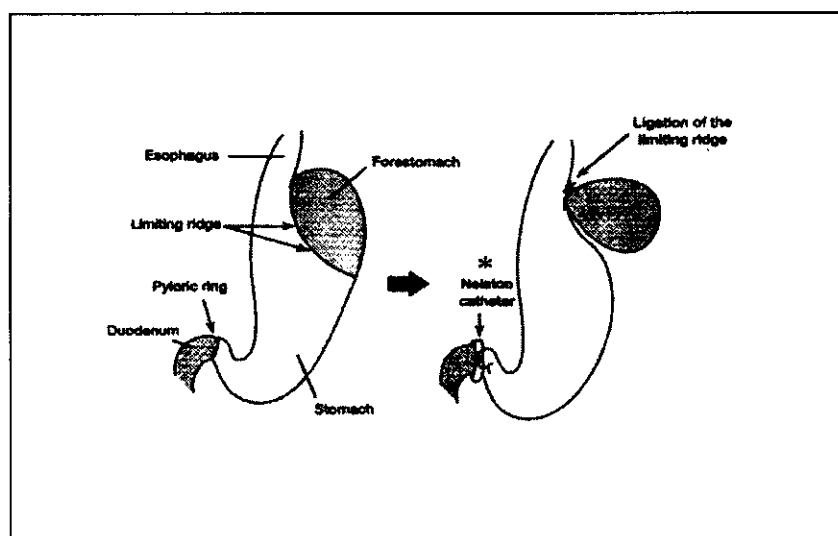
#### 4.2 Effect of curcumin on acute acid reflux esophagitis

Rats were laparotomized under light ether anesthesia to ligate the pylorus and the junction between the forestomach and the corpus (the limiting ridge) (Figure 13) (Nakamura, *et al.*, 1982). Either 1% CMC (5 ml/kg, *i.d.*), curcumin (20-40 mg/kg, *i.d.*), lansoprazole (1 mg/kg, *i.d.*), or aminoguanidine (30 mg/kg, *s.c.*) was administered immediately after the ligation. The rats were then further deprived of food and water. Six hours later, the rats were killed under deep-ether anesthesia and the gastroesophageal portion of the digestive tract was excised. The ulcers in the thoracic esophagus were scored macroscopically, using an ulcer index according to the following criteria: 0, no injury; 1, erosion of mucosal epithelium; 2, the length of hemorrhagic ulcer area < 20 mm; 3, the length of hemorrhagic ulcer area 20-30 mm; 4, the length of hemorrhagic ulcer area 30-40 mm; 5, the length of hemorrhagic ulcer area >40 mm or perforation.

#### 4.3 Effect of curcumin on chronic acid reflux esophagitis

After the rats had been anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), esophagitis was produced by ligating the limiting ridge with a 2-0 silk thread and covering the duodenum near the pylorus ring with a 18 Fr Nelaton catheter which was fixed to the serosa of the pylorus with a 5-0 nylon thread to prevent dislodgement of the catheter (Figure 13) (Omura, *et al.*, 1999). The rats were

further deprived of food for 48 h but allowed free access to drinking water after the surgery. Either 1% CMC (5 ml/kg, *p.o.*), curcumin (20-80 mg/kg, *p.o.*), DMSO (1 ml/kg, *i.p.*), lansoprazole (1 mg/kg, *p.o.*), aminoguanidine (30 mg/kg, *s.c.*) or curcumin dissolved in DMSO (40 mg/kg, *i.p.*) was given to the rat once daily at 10.00 *a.m.* for two or three consecutive weeks after the surgery. Then the rats were killed under deep ether anaesthesia 24 h after the final administration of the test drugs and the gastroesophageal portion of the digestive tract was excised and photographed. The major and minor axes of each site showing signs of esophagitis were measured and scored macroscopically, using a ulcer index according to the following criteria: 0, no ulcer; 1, ulcer area  $\leq 10 \text{ mm}^2$ ; 2, ulcer area  $\leq 20 \text{ mm}^2$ ; 3, ulcer area  $\leq 30 \text{ mm}^2$ ; 4, ulcer area  $\leq 40 \text{ mm}^2$ ; 5, ulcer area  $\leq 50 \text{ mm}^2$ ; 6 ulcer area  $> 50 \text{ mm}^2$  or with complications (perforation or severe esophageal constriction or severe hyperplasia). The entire area of damage was then fixed in 10% formalin for histological evaluation. From paraffin block, 5  $\mu\text{m}$  thick sections were obtained, stained with Hematoxylin and Eosin (H&E) and Massontrichrome, and determined for the incidence and severity of histologic changes. If there was no evidence of macroscopic esophagitis, the middle part of the esophagus was used instead.

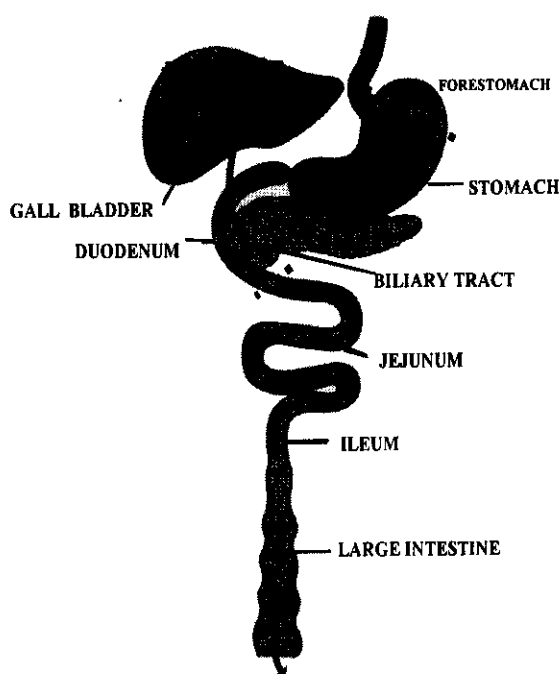


**Figure 13 Induction of acid reflux esophagitis in rat**

\* The pylorus ring was ligated tightly with a 2-0 silk thread in an acute acid reflux esophagitis model, but was covered with a 18 Fr Nelaton catheter in a chronic acid reflux esophagitis.

#### 4.4 Effect of curcumin on acute mixed reflux esophagitis

Rats were laparotomized under light ether anesthesia to ligate the lower duodenal part below the bile duct and the limiting ridge with 2-0 silk thread (Figure 14). Either 1% CMC (5 ml/kg, *ij.*), curcumin (20-80 mg/kg, *ij.*), lansoprazole (1 mg/kg, *ij.*), or aminoguanidine (30 mg/kg, *s.c.*) was administered immediately after the ligation and the rats were further deprived of food and water. Six hours later, the rats were killed under deep ether anesthesia and the gastroesophageal portion of the digestive tract was excised and examined macroscopically. The gross esophageal damage appeared as erosions, haemorrhage, or ulcerations were expressed as using the same criteria as describe in acute acid esophagitis model. The entire area of damage was then fixed in 10% formalin for histological evaluation. From paraffin block, 5  $\mu$ m thick sections were obtained, stained with H&E and Massontrichrome, and determined for the incidence and severity of histologic changes. If there was no evidence of macroscopic esophagitis, the middle part of the esophagus was used instead.



**Figure 14 Induction of acute mixed reflux esophagitis in rat**

The dashed line (---) means the ligation with 2-0 silk thread.

#### **4.5 Preventive effect of curcumin on acute gastric mucosal lesions induced by ethanol**

Thirty minutes after the administration of either 1% CMC (5 ml/kg, *p.o.*), curcumin (5-40 mg/kg, *p.o.*), cimetidine (100 mg/kg, *p.o.*) or aminoguanidine (30 mg/kg, *i.p.*), 80% ethanol (1 ml/200 g body weight) was orally administered to the fasted rat. One hour later, the rat was killed under deep-ether anesthesia, and the stomach was excised and fixed with 2% formalin. The sum of the length (mm) of all lesions for each stomach was used as a lesion index.

#### **4.6 Preventive effect of curcumin on acute gastric mucosal lesions induced by serotonin**

Gastric mucosal lesions were induced by serotonin treatment in rats according to the method of Yasuhiro, *et al.*, 1997. The rat was given serotonin (20 mg/kg, *s.c.*) once daily for 4 days. Either 1%CMC (5 ml/kg, *p.o.*), curcumin (20-80 mg/kg, *p.o.*), cimetidine (100 mg/kg, *p.o.*) or aminoguanidine (30 mg/kg, *i.p.*) was given once daily for 4 days, 30 min before the administration of serotonin. The rat was killed under deep-ether anesthesia 24 h after the final administration of serotonin, and the stomach was removed, inflated by injecting 10 ml of 2% formalin, immersed in the same solution for 10 min to fix the tissue walls, and opened along the greater curvature. The area (mm<sup>2</sup>) of macroscopic lesions was measured under a dissecting microscope (10x), summed per stomach, and used as a lesion index.

#### **4.7 Preventive effect of curcumin on acute gastric mucosal lesions induced by compound 48/80**

Gastric mucosal lesions were induced in fasted rats by a single injection of compound 48/80 (0.75 mg/kg, *i.p.*), a mast-cell degranulator. Either 1% CMC (5 ml/kg, *p.o.*), curcumin (20-80 mg/kg, *p.o.*), cimetidine (100 mg/kg, *p.o.*) or aminoguanidine (30 mg/kg, *s.c.*) was administered 30 min before compound 48/80 injection. Three hours later, the rat was killed under deep-ether anesthesia, and the severity of the damaged area was



then examined microscopically (10x) and quantified by the scoring system (Ohta, *et al.*, 1999).

#### 4.8 Curative effect of curcumin on chronic gastric ulcer induced by topical application of acetic acid

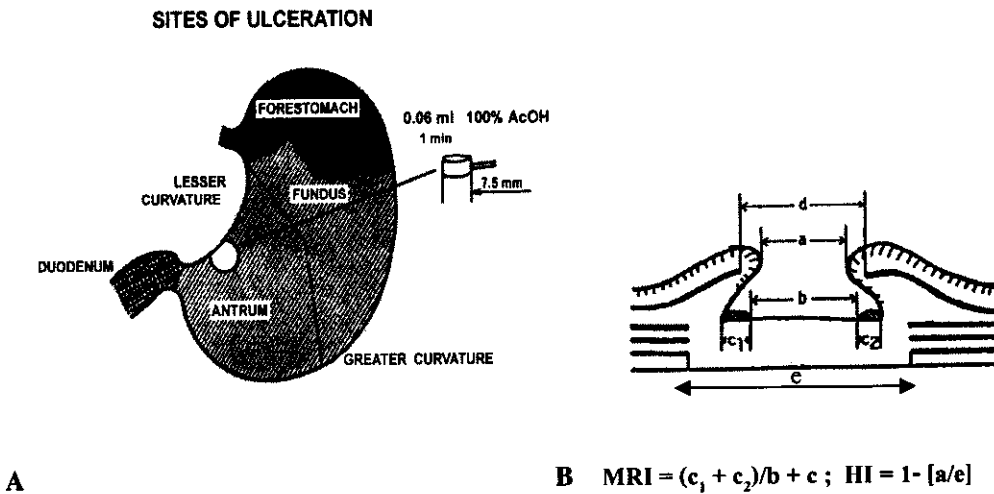
Gastric ulcer was induced by acetic acid treatment in rats according to the method of Okabe, *et al.*, 1971 (Figure 15 A). The abdomen of a rat anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*), was opened and a cylindrical plastic mold (6 mm diameter) was tightly placed upon the anterior serosal surface of the stomach wall (antrum). Acetic acid (100%, 0.06 ml) was then poured into the mold and allowed to remain for 60 s. After removal of the acetic acid solution, the abdomen was closed and the rat was fed normally. Either 1% CMC (5 ml/kg, *p.o.* bid), curcumin (20-80 mg/kg, *p.o.* bid), cimetidine (100 mg/kg, *p.o.* bid) or aminoguanidine (30 mg/kg, *s.c.* od) was administered to the rats for 10 consecutive days, beginning on the 4<sup>th</sup> day after the operation. The rat was killed on the 14<sup>th</sup> day after the operation, and the ulcer was examined macroscopically and histologically for the ulcer index (UI), % curation, % mucosal regeneration index (MRI), and % healing index (HI) (Figure 15 B).

$$\text{UI (mm}^2\text{)} = \text{the length (mm)} \times \text{the width of the ulcer (mm)}$$

$$\% \text{ curation} = \left[ \frac{(\text{UI}_{\text{control at 4th day}} - \text{UI}_{\text{treatment at 14th day}})}{\text{UI}_{\text{control at 4th day}}} \right] \times 100$$

$$\% \text{ MRI} = \left[ \frac{\text{regeneration of the mucosal layer}}{\text{defect of the mucosa} + \text{regeneration of the mucosal layer}} \right] \times 100$$

$$\% \text{ HI} = 1 - \left[ \frac{\text{defect of the mucosa}}{\text{distance of ruptured muscularis mucosa}} \right] \times 100$$



**Figure 15 Induction of chronic gastric ulcer in rat by topical application of acetic acid (A) and histological measurement of the ulcer (B)**

#### 4.9 Effects of curcumin on ulcerative colitis

Colitis is induced experimentally in mice by the oral administration of various concentrations (wt/vol) of dextran sulfate sodium (DSS) in distilled water provided *ad libitum* for one week (Okayasu, *et al.*, 1990). To evaluate the preventive effect of curcumin on ulcerative colitis, either 1% CMC (5 ml/kg, *p.o.*), curcumin (20-80 mg/kg, *p.o.*), mesalazine (100 mg/kg), or sulfasalazine (100 mg/kg, *p.o.*) is given once daily for seven consecutive days during the induction of colitis period. To evaluate the curative effect of curcumin on ulcerative colitis, either of 1% CMC (5 ml/kg, *p.o.*), curcumin (20-80 mg/kg, *p.o.*), mesalazine (100 mg/kg), or sulfasalazine (100 mg/kg, *p.o.*) is given once daily for seven consecutive days starting from the 8<sup>th</sup> day after the replacement of DSS with distilled water. Clinical assessments including water drinking volume, body weight, stool consistency (evaluated by naked eyes), and presence of occult blood in the stools [evaluated by a guaiac paper test (ColoScreen<sup>®</sup>, Helena Laboratories, Beaumont, USA)] are measured every two days. Blood clotting around the anus is considered as the gross blood per rectum. The mice are killed with an overdose of intraperitoneal injection of pentobarbital sodium (100 mg/kg) on the 8<sup>th</sup> day after the induction of colitis in preventive study, and on the 15<sup>th</sup> day after the induction of colitis in curative study. The abdomen is

opened along the median line, and the colon is rapidly excised by longitudinal incision and then straightened on a wax block. The total length of the colon is calculated from the border of the cecum and ascending colon to the rectum at the symphysis bone. Then the incised colon is rinsed carefully to evacuate the fecal matter in iced saline solution as soon as possible. The weight of spleen and the incised colon are measured. The gross morphology of colonic mucosal damage is immediately examined under a stereomicroscope and any visible damage is quantified by the scoring system: grade 0, no damage; grade 1, localized hyperemia, but no ulcers; grade 2, linear ulcers with no significant inflammation; grade 3, linear ulcers with inflammation at one site; grade 4, two or more sites of ulceration and/or inflammation; grade 5, two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending > 1 cm along the length of the colon; grade 6, an area of ulceration and inflammation extending > 2 cm along the length of the colon (Wallace, *et al.* 2000).

#### **4.10 *In vivo* study on the effect of curcumin on inflammatory cytokines on the gastric ulcer healing in rats**

The rats treated with topical application of acetic acid were killed on the 14<sup>th</sup> day after the operation and the ulcerated area was then divided longitudinally along the greater curvature into two equal parts. One part was subdivided into two equal parts for Western blot and quantitative real-time RT-PCR analysis. The other part was used for immunohistochemistry.

Tissue sample for either Western blot analysis or quantitative real-time RT-PCR was kept at -80°C until assay.

Tissue sample for immunohistochemistry was immersed in Zamboni (picric acid–paraformaldehyde) fixative for 3 h at 4°C, followed by rinsing with 8, 15, 20 and 30% sucrose in 0.1 M phosphate buffer at 4°C. The sample was then embedded in the aluminum mold containing mounting solution, Tissue-Tek OCT™ and frozen immediately in liquid nitrogen. The sample was stored at -80°C until assay.

#### 4.10.1 Western blot analysis for iNOS, COX-2, TNF- $\alpha$ and IL-1 $\beta$

Each gastric mucosal sample was scraped off the underlying muscularis externa and serosa, and placed in a homogenate buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1 mM sodium orthovanadate, 1% Triton X-100, 1% protease inhibitor cocktail and 1 mM PMSF) (100 mg sample/1 ml buffer). The sample was homogenized for two 30-second bursts of a Polytron homogenizer under ice-cold condition and then centrifuged at 11,000 g for 10 min at 4°C. After determination of the protein concentration in supernatant using the Bio-Rad Dc protein assay reagent according to the manufacturer's instruction (Bio-Rad Laboratories Inc., CA., USA), 50  $\mu$ g of protein per sample was subjected to 8-15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane by electric blotting with 200 milliampere for 30-60 minutes. The immunoblot was incubated for 30 min with blocking solution [Tris buffer saline-Tween 20 (TBS-T) (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM KCl, 10% Tween 20) containing 5% skim milk powder]. Blots were then washed three times (5 min each) with TBS-T solution, followed by incubation overnight at 4°C with a 1/1,000 dilution of monoclonal anti-iNOS, COX-2, TNF- $\alpha$  and IL-1 $\beta$  antibodies, and 1/10,000 dilution of anti- $\beta$ -actin in TBS-T solution containing 1% BSA and 0.5% FBS. After three washing steps (30 min each) with TBS-T solution, the membranes were incubated for 1 h at room temperature with secondary antibodies: goat anti-rabbit IgG, HRP-conjugate immunoglobulin for iNOS and COX-2; rabbit anti-goat mouse IgG, HRP-conjugate immunoglobulin for TNF- $\alpha$ ; and goat anti-mouse IgG, HRP-conjugate immunoglobulin for IL-1 $\beta$  and  $\beta$ -actin, followed by three more washing steps with TBS-T solution. Detection was performed using the enhanced chemiluminescence detecting reagent (Bio-Rad Laboratories Inc., CA., USA) on the image scanner, LAS 1000 Plus (Fuji Film Ltd., Tokyo, Japan) according to the manufacturer's instructions.

#### 4.10.2 Analysis of iNOS Messenger RNA (m RNA) by real-time RT-PCR

Total RNA was extracted from scrapings of each gastric mucosal sample by homogenizing the sample in TRIzol™-reagent (100 mg sample/1 ml reagent) with two 30-second bursts of a Polytron homogenizer under ice-cold condition, followed by the

centrifugation for 10 min at 11,000 g, 4°C. The extracted RNA was homogenized by passing through a 0.9 mm diameter needle and purified by using a RNeasy mini kit. The concentration and purity of total RNA were determined by spectrophotometrically at 260 and 280 nm. The ratio of absorbance at 260 to 280 nm was 1.8-2.0.

cDNA synthesis was performed by the reverse transcription system using Oligo dT<sub>12-18</sub>, 5x First strand buffer, 0.1 M DTT, 10mM dNTP mix, and superscript II reverse transcriptase. The reactions were incubated in 4 µg RNA/20 µL RNase free water with 2 µL Oligo dT<sub>12-18</sub> for 10 min at 70°C, 8 µL 5x first strand buffer, 4 µL 0.1 M DTT, and 2 µL 10mM dNTP mix were then added and the mixture was incubated for 2 min at 42°C, followed by the adding of 2 µL Superscript II reverse transcriptase and further incubation for 50 min at 42°C. Finally, the reaction was inactivated by incubating the mixture for 15 min at 70°C. PCR amplifications were performed in a total volume of 25 µL, containing 1 µL cDNA sample, 12.5 µL Taqman<sup>®</sup> Universal PCR master mix, 1.25 µL Pre-Developped Taqman<sup>®</sup> assay reagents/Human 18S rRNA mix as an internal standard, 1.25 µL of each primer, and 9 µL Milli Q water. Every sample was measured in duplicate. Negative control (a no template control) was added to the PCR to screen for possible contamination and genomic amplification. Each assembled plate was then capped and run in the ABI PRISM 7000 Sequence Detector (Applied Bio Inc., CA., USA) using the following cycling conditions : 50°C, 2 min; 95°C, 10 min; 50 cycles of 95°C, 15 sec and 60°C, 1 min. The primer used was rat iNOS TATTCCCAGCCCAACAACACAGGAT (Rn00561646\_m1).

#### 4.10.3 Immunohistochemistry of iNOS and TNF- $\alpha$

Immunostaining of iNOS in the gastric mucosa was performed on a paraffin-embedded sample. Briefly, approximately 6-7 µm cryostat section of the sample was deparaffinized with cold 0.1M phosphate buffer (0.2 M NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O and 0.2M Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O). After permeabilization by acetone for 3 minutes at -10°C and 0.1 % Triton-X 100 in 0.1 M phosphate buffer for 10 min at 4°C respectively, section was blocked by 1% BSA-phosphate buffer solution for 1 h at 4°C, which was followed by quenching the endogenous peroxidase activity by immersing the section slide in

3% hydrogen peroxide-phosphate buffer solution for 5 min. Primary antibody for iNOS or TNF- $\alpha$  (the same as used in Western blot analysis) was then added at a dilution of 1:500 and 1:100 respectively in 0.1% BSA-phosphate buffer solution and left overnight at 4°C. After the section was washed with 0.1 M phosphate buffer respectively, secondary antibodies: iNOS, anti-rabbit IgG Alexa Flour 488 and TNF- $\alpha$ , anti-goat IgG Alexa Flour 488), were applied at a dilution of 1:500 in 0.1% BSA-phosphate buffer solution and incubated for 40 min at room temperature. The section was then washed with 0.1 M phosphate buffer and examined using the Carl Zeiss laser scanning system LSM 510 and LSM 5 PASCAL (filter BP 505-530, wavelength 488 nm). Negative control was performed by removing the primary antibody.

#### **4.11 *In vitro* study on the effect of curcumin, vanillin and tetrahydrocurcumin on lipopolysaccharide-induced iNOS, COX-2, and TNF- $\alpha$ expression in the macrophage cell line RAW 264.7.**

##### **4.11.1 Cell culture and sample treatment**

Mouse macrophage RAW 264.7 cell lines were cultured in 24 well plate ( $2 \times 10^5$  cells/well) at 37°C in RPMI 1640 medium containing 10% FBS and 1% penicillin plus streptomycin sulfate in a humidified atmosphere of 5% CO<sub>2</sub>. When the cells reached a density of approximately 80% confluence, they were incubated in medium (using RPMI 1640 without 10% FBS) containing 1 $\mu$ g/ml of LPS from *Escherichia coli*. Various concentrations of curcumin, vanillin and tetrahydrocurcumin dissolved in DMSO were added together with LPS.

Cells used for Western blot analysis were washed with cold phosphate buffered saline (PBS) after 16 h of incubation and then harvested using homogenate buffer. Finally, cell debris was removed by microcentrifugation at 13,000 g, 4°C for 20 min, followed by quick freezing of the supernatants.

Cells used for RT-PCR and real-time RT-PCR analysis were washed with cold PBS after 16 h of incubation and the RNA was then extracted with TRIzol™ reagent. The extracted RNA was homogenized by passing through a 0.9 mm diameter needle and

purified by using a RNeasy mini kit. The concentration and purity of total RNA were determined by spectrophotometrically at 260 and 280 nm. The ratio of absorbance at 260 to 280 nm was 1.8-2.0.

#### **4.11.2 Western blot analysis for the detection of protein expression for iNOS, COX-2, and TNF- $\alpha$**

After determination of the protein concentration in cell lysate using the Bio-Rad Dc protein assay reagent according to the manufacturer's instruction (Bio-Rad Laboratories Inc., CA., USA), 50  $\mu$ g of macrophage cellular protein was subjected to 8-15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane by electric blotting as described in section 4.10.1. Primary antibodies were 1/1,000 dilution of monoclonal anti-iNOS, COX-2 and TNF- $\alpha$  antibodies and 1/10,000 dilution of anti- $\beta$ -actin. Secondary antibodies were goat anti-rabbit IgG, HRP-conjugate immunoglobulin for iNOS and COX-2; rabbit anti-goat mouse IgG, HRP-conjugate immunoglobulin for TNF- $\alpha$ ; and goat anti-mouse IgG, HRP-conjugate immunoglobulin for  $\beta$ -actin. All experiments were performed at least three times.

#### **4.11.3 RT-PCR for the detection of messenger RNA (mRNA) for iNOS, COX-2, and TNF- $\alpha$**

After purification, RNA concentration was determined spectrophotometrically. RT-PCR was performed using the commercially available one step reverse-transcribed polymerase chain reaction kit (Qiagen<sup>®</sup>). Briefly, 2  $\mu$ g of RNA was mixed with 5x one step RT-PCR buffer, one step RT-PCR enzyme mix, dNTP mix, 5x Q solution, and primers according to the manufacturer's recommendation (Qiagen, CA., USA) in a 50  $\mu$ L volume. The amplification was carried out with a GeneAmp PCR system Dice thermal cycler (Takara Bio Inc., Shiga, Japan). The sense and antisense primers used for mouse  $\beta$ -actin as an internal standard were 5'-TCATGAAGTGTGACGTTGACA TCCGT-3' and 5'-CCTAGAAGCATTGCGGTGCACGATG-3', respectively. The primers for iNOS consisted of a 5'-AATGGCAACATCAGGTCGGCCATCACT-3' (sense) and a 5'-GCTG TGTGTACAGAAAGTCTCGAACTC-3' (antisense). The primers for COX-2 consisted of

a 5'-ACTCACTCAGTTTGTGAGTCATTC-3' (sense) and a 5'-TTTGATTAGTACTG TAGGGTTAATG-3' (antisense). The primers for mouse TNF- $\alpha$  consisted of a 5'-ATGAG CACAGAAAGCATGATC-3' (sense) and a 5'-TACAGGCTTGTCCTCGAATT-3' (antisense). After initial denaturation for 15 min at 95°C, 30 amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing, and 1.5 min of 72°C extension), COX-2 (1 min of 94°C denaturation, 1 min of 60°C annealing, and 1 min of 72°C extension) and TNF- $\alpha$  (1 min of 95°C denaturation, 1 min of 55°C annealing, and 1 min of 72°C extension), and followed by the final PCR temperature of 72°C for 10 min. A portion of the PCR mixture was then electrophoresed on a 2% agarose gel containing ethidium bromide in TBE buffer (20 mM Tris.HCl, pH 7.4, 2 mM EDTA, pH 8.0). The bands were visualized by UV irradiation and photographed.

#### **4.11.4 Real-time RT-PCR for the detection of messenger RNA (mRNA) for iNOS, COX-2, and TNF- $\alpha$**

cDNA synthesis was performed by the reverse transcription system using Oligo dT<sub>12-18</sub>, 5X First strand buffer, 0.1 M DTT, 10 mM dNTP mix, and superscript II reverse transcriptase. The reactions were incubated in 4  $\mu$ g RNA/20  $\mu$ L RNase free water with 2  $\mu$ L Oligo dT<sub>12-18</sub> for 10 min at 70°C, 8  $\mu$ L 5X First strand buffer, 4  $\mu$ L 0.1 M DTT, and 2  $\mu$ L 10 mM dNTP mix were then added and the mixture was incubated for 2 min at 42°C, followed by the adding of 2  $\mu$ L Superscript II reverse transcriptase and further incubation for 50 min at 42°C. Finally, the reaction was inactivated by incubating the mixture for 15 min at 70°C. PCR amplifications were performed in a total volume of 25  $\mu$ L, containing 1  $\mu$ L cDNA sample, 12.5  $\mu$ L Taqman Universal PCR master mix, 1.25  $\mu$ L Pre-Developped Taqman assay reagents/Human 18S rRNA mix as an internal standard, 1.25  $\mu$ L of each primer, and 9  $\mu$ L Milli Q water. Every sample was measured in duplicate. Negative control (a no template control) was added to the PCR to screen for possible contamination and genomic amplification. Each assembled plate was then capped and run in the ABI PRISM 7000 Sequence Detector (Applied Bio Inc., CA., USA) using the following cycling conditions: 50°C, 2 min; 95°C, 10 min; 50 cycles of 95°C, 15 sec and 60°C, 1 min. The primers used were mouse iNOS GGCCACATCGGATTC



ACTTGCAAG (Mm00440485\_m1), mouse TNF- $\alpha$  AAGGGATGAGAAGTTCCCAAA  
TGG (Mm00443258\_m1), and mouse COX-2 GGGCCATGGAGTGGACTTAAATCAC  
(Mm00478374\_m1). All experiments were performed at least three times.

## 5. Statistics

The result are expressed as mean  $\pm$  S.E.M. Statistical analysis was performed using one-way analysis of variance followed by the LSD or Dunnett's test for multiple comparisons. Statistical analyses of the severity of gastric mucosal lesions were carried out using the Kruskal-Wallis test. Statistical analyses were performed using the Mann-Whitney U test for nonparametric data. Value of  $p < 0.05$  was regarded as significant.