CHAPTER III

MATERIALS AND METHODS

Chemical substances

Curcumin 95% (Cayman Chemical Company, USA)

Dimethyl Sulfoxide (DMSO) (Sigma Chemical CO., USA)

Streptomycin (General Drug House Co., LTD., Thailand)

Fluoroscein isoFITC-dx-250 (Sigma Chemical CO., USA)

Heparin (Leo Pharmaceutical Products, Denmark)

Sodium Thiopental (Abbott, Italy)

Krebs's solution

Sterile normal saline solution

4% Para-formaldehyde in phosphate buffer

Distilled water

VEGF ELISA kit (R&D systems, Inc., USA)

Xylene

95% alcohol

Absolute alcohol

Citrate buffer pH 6.0

Hydrogen peroxide (Merck)

Phosphate buffer solution pH 7.4

Normal horse serum (Gibco)

NF-KB p65 antibody (sc-109, Santa Cruz Biotechnology Inc.,USA)

Antibody diluent (DAKO, USA)

Anti-rabbit envision (DAKO, USA)

Diaminobenzidine (DAB) (DAKO, USA)

Hematoxylin

Bacterial and animal preparation

H. pylori was originally obtained from peptic ulcer patients who visit at the King Chulalongkorn Memorial hospital. The bacteria were growing in Brucella broth (pH 7.0) supplemented with 10% goat serum for 24 hours at 37 °C in an automatic CO_2-O_2 incubator (85% N_2, 10% CO_2, and 5% O_2). The same conditions were used in the following culture.

25 male Spraque-Dawley rats (Salaya research animal center, Mahidol University, Bangkok, Thailand), weighting about 150-200 grams at the beginning of the experiment, were used. The experimental protocol was approved by the Ethical Committee of Medicine Faculty, Chulalongkorn University, Thailand. The animals were in Macrolon cages (5 animals per cage) and had freely access to food and tap water in a room temperature 18-22 °C, humidity 55%, and 12/12 hours-light/dark cycle. The rats were divided into five groups of five rats each as the following:

Control with vehicle group (Control group)

The animals were received normal saline 1 ml/rat by gavage twice a day at an interval of four hours for three sequential days. Then, they were housed with freely access to water and standard food for two weeks. After that, the animals were treated with 0.1% (v/v) DMSO dissolved in normal saline 1 ml/rat by gavage once daily for one week.

Control with curcumin treatment (600 mg/kg BW) group (curcumin control group)

The animals were received normal saline 1 ml/rat in the same way of previous describe. Instead of 0.1% DMSO dissolved in normal saline, curcumin at a dose of 600 mg/kg BW suspended in 0.1% (v/v) DMSO dissolved in normal saline were treated to the rats by gavage once daily for one week.

H. pylori infection with vehicle group (*H. pylori* infected group)

Rats were inoculated with *H. pylori* by using model of Thong-Ngam *et al.* [29]. Briefly, the *H. pylori* suspension (5×10^8 to 5×10^{10} CFU/ml) in normal saline was fed to the rats (1ml/rat) by gavage twice daily at an interval of four hours for three consecutive days. Two weeks after the inoculation, the animals were treated with 0.1% (v/v) DMSO dissolved in normal saline (1 ml/rat) by gavage once daily for one week.

***H. pylori* infection with curcumin treatment (200 mg/kg BW) group (200 mg/kg BW curcumin treated group)**

In order to make the *H. pylori* inoculation, the same protocol was performed as the previous model. After two weeks of *H. pylori* infection, the rats were treated with curcumin at a dose of 200 mg/kg BW suspended in 0.1% (v/v) DMSO dissolved in normal saline (1 ml/rat) by gavage once daily for one week [174].

***H. pylori* infection with curcumin treatment (600 mg/kg BW) group (600 mg/kg BW curcumin treated group)**

The identical protocol of *H. pylori* inoculation was performed. After two weeks, the rats were treated with curcumin at a dose of 600 mg/kg BW suspended in 0.1% DMSO (v/v) dissolved in normal saline (1 ml/rat) by gavage once daily for one week [174].

The procedures of animal preparation were concluded in the diagram as shown below

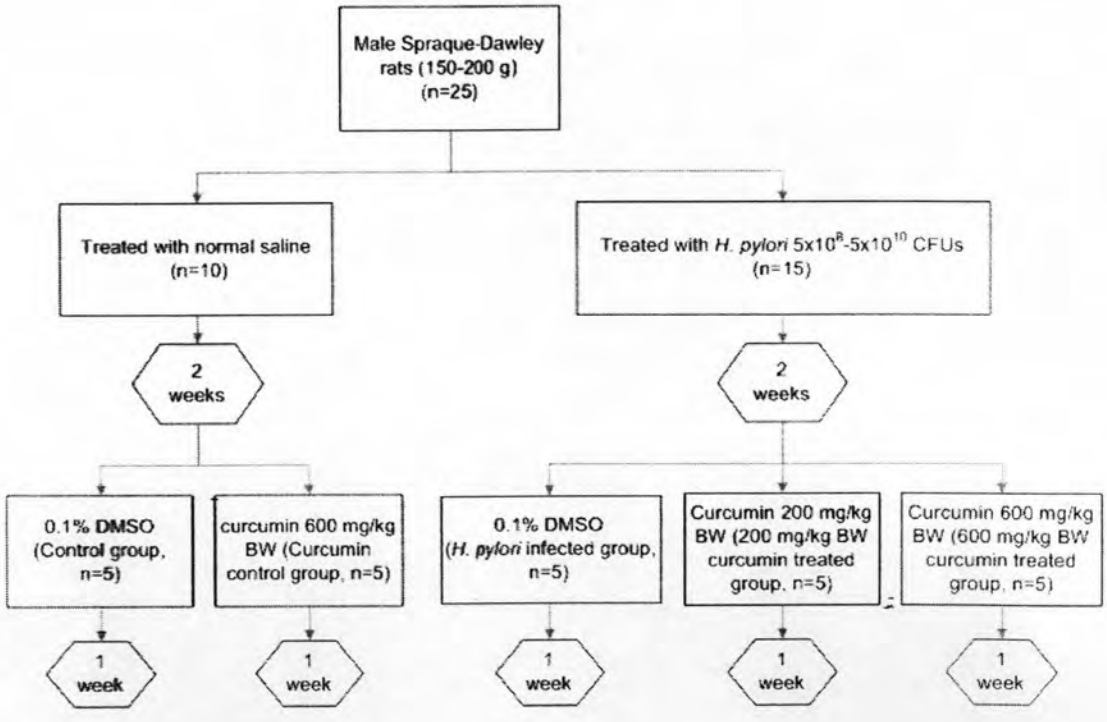

Figure 10 Diagram of experimental groups

Methods

H. pylori inoculation in the rat stomach

Streptomycin suspended in tap water (5 mg/ml) was pre-treated for three days before *H. pylori* inoculation. Then, the animals were fasted for 18 hours but can freely access to tap water. The *H. pylori* suspension (5×10^8 to 5×10^{10} CFU/ml) in normal saline was given to the rats (1 ml/rat) by gavage twice daily at an interval of four hours for three consecutive days. Before the next day of inoculation, the rats were fed with laboratory food for 2 hours. After the last inoculation, these animals were housed with freely access to tap and standard food for two weeks. It had reported that *H. pylori* could induced mild to moderate gastritis, dominated by infiltration of macrophages and lymphocytes into lamina propria of the stomach after two weeks of *H. pylori* infection [28, 29].

Experimental protocol for intravital fluorescent microscopic study of gastric microcirculatory change

The animal was anesthetized by intraperitoneal injection (ip) of Thiopental (60 mg/ml) at a volume of 0.1 ml/100 g BW with further anesthetic administered as required. A tracheotomy was performed. Then, a fine polyethylene catheter (PE 10, inner diameter 0.28 mm) was inserted into the left common carotid artery (inserted 1.0 cm). The carotid catheter was maintained with heparinized saline. The mean arterial pressure (MAP) was monitored via this catheter by using pressure transducer (Nihon Kohden). The another catheter was inserted into jugular vein for injection of fluorescence at a volume of 30 µl (FITC-dx-250, 5 mg/100 µl of normal saline) [176].

The surgical procedure was modified from Kalia *et al.* [89]. After laparotomy, a 1.0 cm incision was made with an electric microcautery (Hyfrecator plus®, Conmed CO., USA) in the posterior wall of the exteriorized stomach parallel to the "limiting ridge", (separates the upper stomach from the lower stomach). Microvascular observations were made from the mucosa of the body part of stomach. Care was taken to avoid burning through blood vessels. Next, the stomach was gently extended and held in place by a stay suture. The incision in the posterior wall was opened using microclamps to allow visualization of the anterior mucosal surface and covered with Saran wrap as shown in figure 11. During the surgery, the area was kept by allowing drops of warmed (37°C) Krebs's solution on the incision. The body temperature of the animal was kept constant at 36-37°C by mean of heating pad. Then, the animal was taken place on microscopic stage of the fluorescent microscope equipped with trans-illumination and epi-illumination optics

(Nikon Optiphot-2). After intravenous application of FITC-dx-250, epi-illumination was achieved with a 50 W, mercury lamp (Nikon HB-10101AF) with a 488 nm attached to excitation filter and 515 emission barrier filters. An interval microscope with a 20x long working distance objective (Nikon) were used to observe microvessels in the stomach mucosa. A video camera (Nikon MTT-SIT 68) that was mounted on the microscope projected the image onto a black and white monitor (Sony PVM-145E). The time lapse videocassette recorder (Sony SVT-124p) was used to record the images of microvessels on videotape (Sony super DX) for further analysis. The instruments are shown in figure 12

The leakage of endogenous factors selectively enhanced the permeability of PCVs (diameter 15-30 μm) more than that of capillaries (diameter 4-9 μm) [177-179]. In modified observation of the macromolecular leakage from PCVs from Kalia *et al.* and Somboonwong *et al.* [89, 180], the intravital microscope with a 20x objective lens was used. Anatomical of blood vessels that supply the body region of stomach were selected as a landmark in all animals [36]. Under circumstances resulting in increased microvascular permeability to macromolecules, the leakage of FITC-dx-250 can be observed as a ratio of intensity of the fluorescence between outside (I_{out}) and inside (I_{in}) vessel. The digital image analysis of intensity ratio (I_{out} / I_{in}) was measured at zero time and thirty minutes after the intravenous injection of FITC-dx-250 by GLOBAL LAB® image/2 program. Then, the intensity ratio was calculated using the equation: [(I_{out}/I_{in}) at 30 minute – (I_{out}/I_{in}) at 0 minute] × [100 / (I_{out}/I_{in}) at 0 minute].

At baseline of 0 minute, we measured from the record of selected regions 5 minutes after FITC-dx-250 injection [89], and we measured at 30 minute on the same view after the baseline. The sample of images is shown in figure 13 and 14

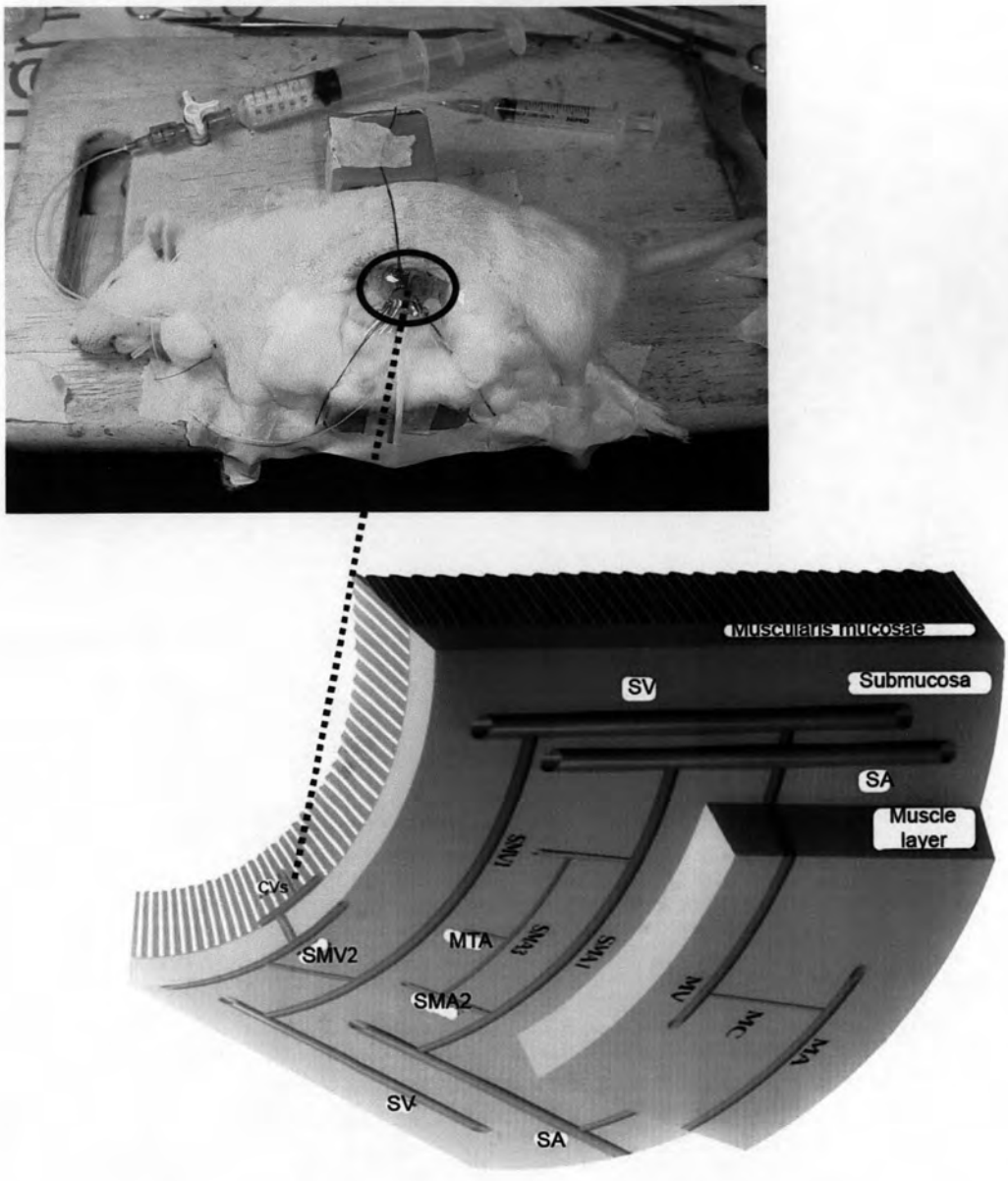

Figure 11 Preparation model for study macromolecular leakage on gastric PCVs in anterior mucosa at body region of the stomach: PCVs in the gastric mucosa that collecting blood into CVs were selected for studying macromolecular leakage.

The PCVs were selected to study including:

1. Diameters of PCVs are 15-30 μm [36].
2. Blood in PCVs must collectively flow from branches of hexagonal mucosal capillary to CV [36].
3. PCVs are located at the body region of stomach [89].

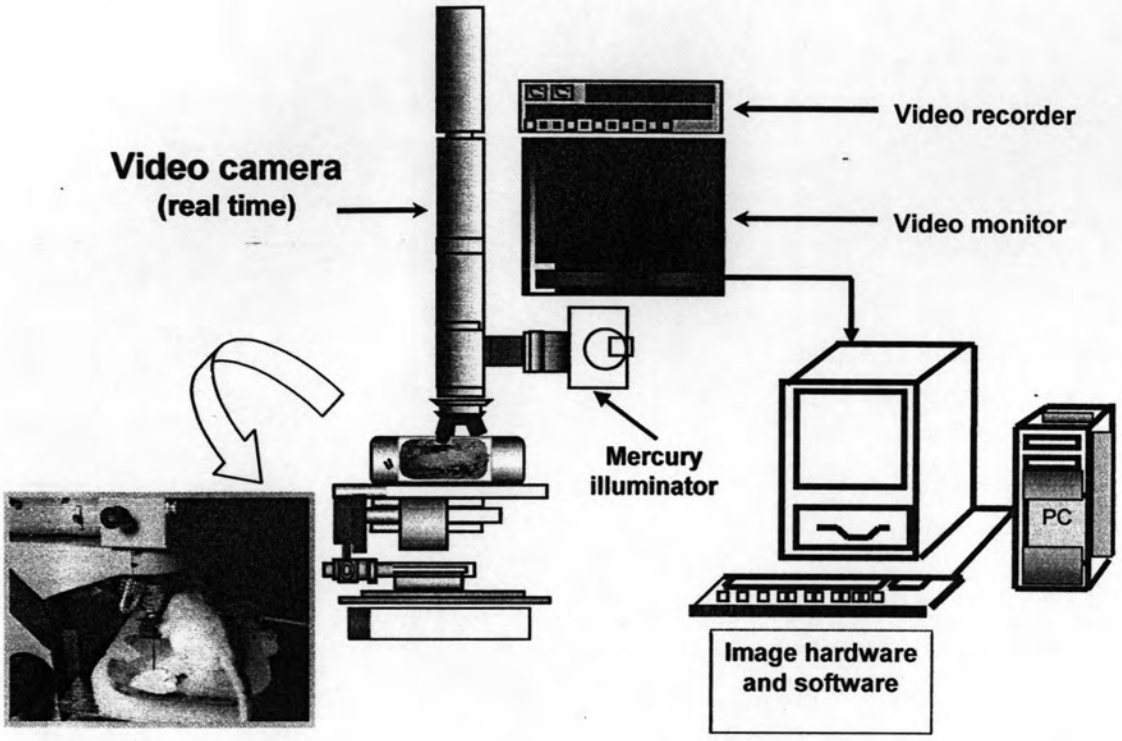

Figure 12 Intravital fluorescent microscopy and instruments are used for studying of macromolecular leakage.

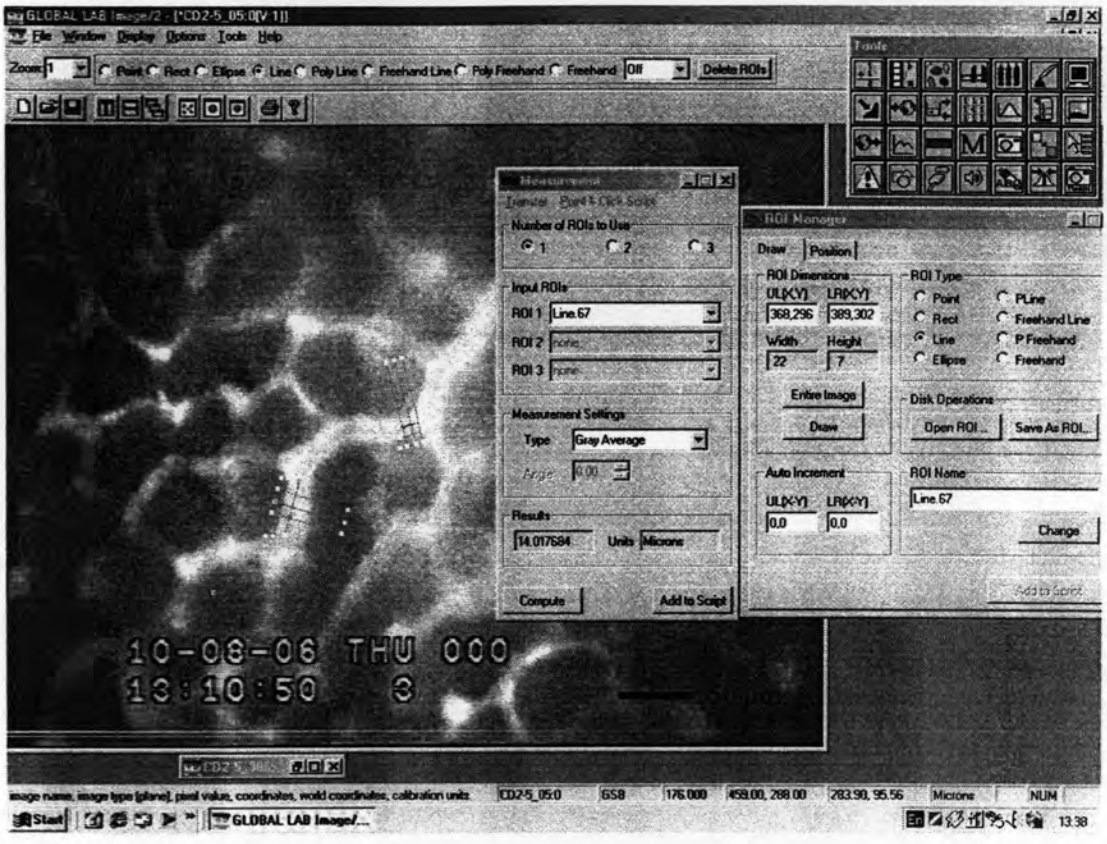

Figure 13 GLOBAL LAB® image/2, an image analysis program is used for determining intensity ratio. The percentage of the increase leakage was calculated by the equation: [(I_{out}/I_{in}) at 30 minute – (I_{out}/I_{in}) at 0 minute] × [100 / (I_{out}/I_{in}) at 0 minute]. By the criteria of selected PCVs, at least 3 PCVs were selected to measure. At 0 minute of FITC-dx-250 injection, the intensity from 3 points in each vessel was averaged.

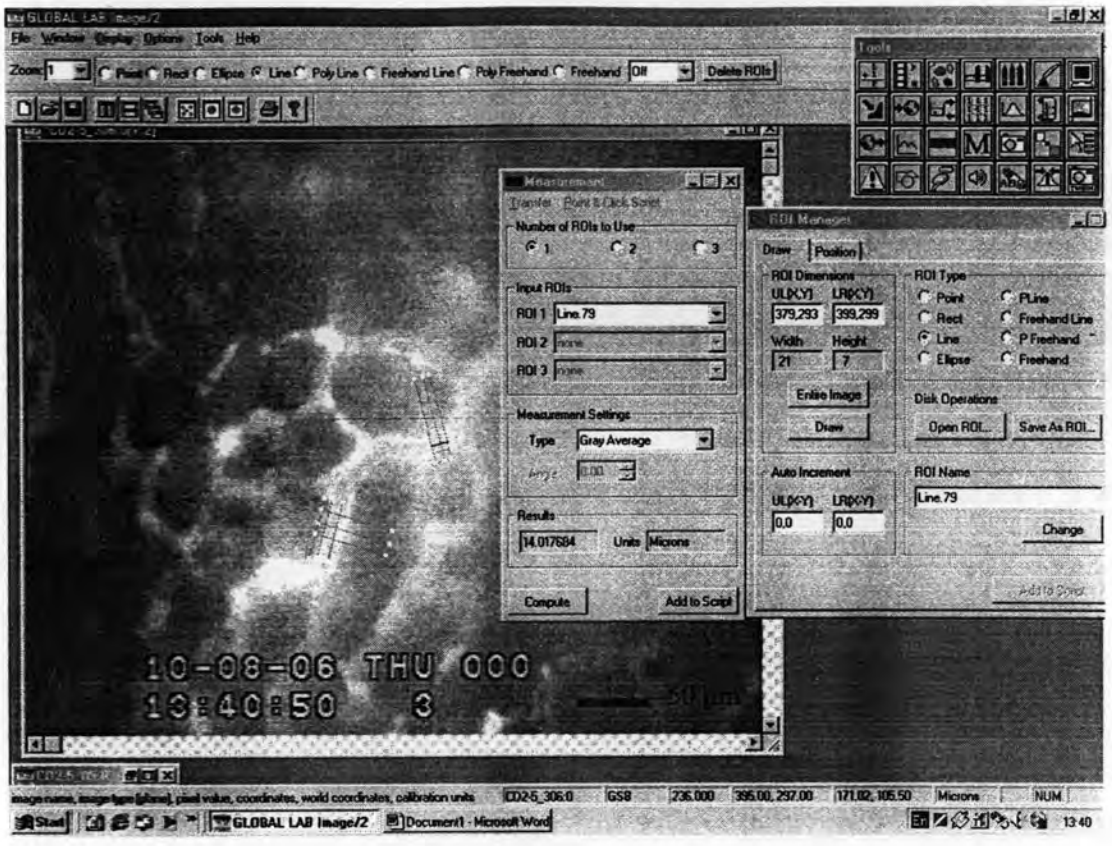

Figure 14 GLOBAL LAB® image/2, an image analysis program is used for determining intensity ratio. The percentage of the increase leakage was calculated by the equation: [(I_{out}/I_{in}) at 30 minute – (I_{out}/I_{in}) at 0 minute] × [100 / (I_{out}/I_{in}) at 0 minute]. By the criteria of selected PCVs, at least 3 PCVs were selected to measure. At 30 minute of FITC-dx-250 injection, the intensity from the same area was measured.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a useful method in evaluating the concentration of proteins in a sample such as cell culture supernatant, serum, or plasma. ELISA kit is performed in 96 well plates which allow high throughput results. A monoclonal antibody has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any antigen present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of color measured is in

proportion to the amount of antigen bound in the initial step. The sample values are then read off the standard curve.

VEGF assay

To measure VEGF level in serum, blood samples were collected by cardiac puncture after the finish of intravital fluorescent procedure. Blood samples were allowed to clot for two hours at room temperature before centrifuging for twenty minutes at approximately 1000 times of gravity (G). Then, the serum was stored in the freezer at -80°C until the day of analysis. The samples were not allowed to repeat freeze-thaw cycles.

At the day of study, serum samples were used to produce 4-fold dilutions with the kit calibrator diluent prior assay. The Quantikine® rat VEGF (R&D systems, USA) was used to determine level of VEGF in rat serum. The assay procedures were performed as protocol descriptions from the company. In briefly, all samples, standards, and control were prepared as in the reagent preparation part of the protocol and assayed in duplicate. Assay diluent was added to each well and followed by standards, control, or samples to each well. Incubate for two hours at room temperature on horizontal orbital microplate shaker set at 500 ± 50 round per minute (rpm). Next, each well was aspirated and washed for five times. Then, VEGF conjugate was added to each well and incubated for one hour on the shaker. After one hour, each well was aspirated and washed for five times. Then, substrate solution was added to each well. Incubation was performed for thirty minutes and must protect from light. After that, stop solution was added to each well and gently tapped the plate to ensure through mixing. The last step, the optical density (O.D.) was determined within thirty minutes by using a microplate reader (Anthos 2010, Universal Systems CO., LTD., Thailand) set to 450 nm and wavelength correlation to 540 nm.

To calculate results, the duplicated readings for each standard, control, and samples were averaged and subtracted the average of zero standard optical density. Then, the standard curve was created by using computer software of generating a linear curve-fit. The measured O.D. of each unknown was converted to its corresponding concentration by standard curve. The below table and figure showed the calculation of results.

Standard VEGF (pg/ml)	Obtical density (O.D.) (450 nm-540 nm)		Average O.D.	Corrected O.D.
	Data 1	Data 2		
0	0.055	0.069	0.062	-
31.2	0.164	0.14	0.152	0.09
62.5	0.103	0.116	0.1095	0.0475
125	0.246	0.214	0.23	0.168
250	0.254	0.285	0.2695	0.2075
500	0.535	0.48	0.5075	0.4455
1000	0.788	0.852	0.82	0.758
2000	1.266	1.383	1.3245	1.2625

Table 1 The corrected O.D. plotted on y-axis of standard curve was calculated from the mean subtract the average of zero standard O.D. Note: pg/ml = picogram per milliliter; nm = nanometer

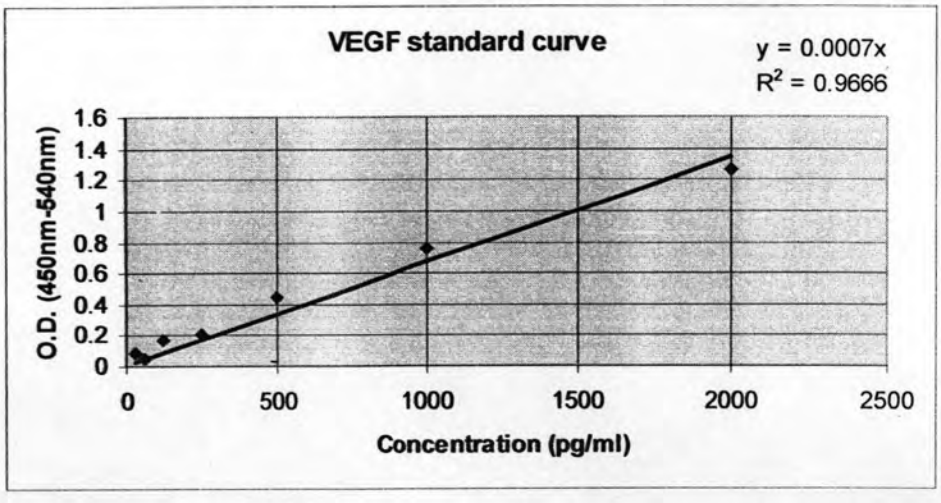

Figure 15 A standard curve of VEGF: The x-axis and y-axis of the standard curve represented standard VEGF concentrations (pg/ml) and O.D., respectively. Strong correlation (R^2 = 0.9666) was able to present by the linear equation of y = 0.0007x.

The data were linearized with computer software and the best fit line can be determined by regression analysis. From the linear equation of standard curve, the sample concentration will be calculated. If samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

Detection of *H. pylori* infection in gastric tissues

After finishing all parameters, antral tissues from the rat stomachs were taken for the *H. pylori*-positive tests. The present of *H. pylori* was determined by either positive rapid urease test or positive histology

Enzymatic test by rapid urease test

The stomach was removed and opened along the greater curvature from the anesthetised rat. The remained food in stomach was gentle removed. The 2 mm^2 of gastric mucosa from antrum, the area that *H. pylori* most colonized [28], was cut and examined the urease enzyme activity. The tissues were put in a gel tube that contained urea and phenol red, a pH indicator. In the case of *H. pylori* infection, the *H. pylori* urease enzyme activity converting urea into ammonia will be occurred. The increase of ammonia causes increase pH, so that the pH indicator in urease test tube will be changed to pink as shown in figure 15. The results of rapid urease tests are read in 24 hours. The urease test has 86% to 97% sensitivity and 86% to 98% specificity [181].

Positive urease test

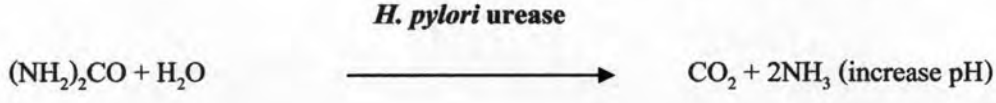

Figure 16 Rapid urease test: The presence of *H. pylori* urease is indicated by ammonia production.

Histopathology

After taking gastric antral tissue for the urease test, the remaining was fixed in 4% paraformaldehyde in 0.2 M sodium phosphate buffer, pH 7.4 at room temperature. Then, gastric

tissue was processed by standard methods, embedded in paraffin, cut at 5 μm thickness, picked up on glass slides, and stained with hematoxylin-eosin (H&E). The slides were observed on light microscopy. However, the presence of *H. pylori* was detected by Warthin-Starry staining in the unclear cases. A grading system was adapted to assess the level of bacteria colonization.

In this study, the colonizing of *H. pylori* and grading inflammation scored by the pathologist were not showed in the results. However, the scores were adapted to assess the level of bacterial colonization in rat stomach. The estimation of gastric inflammation was used the updated Sydney System [29, 182].

The percentage of *H. pylori*-infected rate was calculated from following equation: 100 × (number of infected-rats / number of inoculate rats). Number of infected rats was identified by positive test either urease test or histopathology. The positive urease test must detect the color change of pH indicator in 24 hours. The antrum samples that give pink in the urease test tube were *H. pylori* positive. Moreover, the remained stomach samples were detected *H. pylori* colonization by the pathologist as scores. The score0 means no *H. pylori* detection. The stomach samples that were given score1 or more were *H. pylori* positive.

Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of localizing proteins in the cells of tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. The IHC is wildly used in the diagnosis of cancer and in the basic research to understand the distribution and localization of biomarkers in different part of tissues. Visualizing an antibody-antigen interaction can be accomplished in a number of ways. Classically, a secondary antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a color-producing reaction.

Expression of NF-KB p65 reactive cells

The stomach sections were performed to examine NF-KB p65 activation on the epithelial cells by the following processes.

The stomach sections were deparaffinized with xylene and alcohol for ten minutes. After water washing, sections were retrieved the antigen (NF-KB p65) with citrate buffer pH 6.0 in microwave for thirteen minutes. Next, 3% Hydogen peroxide (H_2O_2) and 20% normal horse serum were performed on the slides to block endogenous peroxidase activity for five minutes and block nonspecific binding for twenty minutes, respectively. Then, the primary antibody used for

NF-ΚB p65, a polyclonal antibody against the p65 subunit, was applied at a dilution of 1:100 for one hour at room temperature. After that, the secondary antibody, biotinylated anti-rabbit immunoglobulins, for this protein was used for thirty minutes. When the development of the color with Diaminobenzidine (DAB) was detected, the slides were counterstained with hematoxylin.

Under light microscopy, the expression of NF-ΚB p65 was cytoplasmic with scattered positive nuclei as shown in figure 17. To verify the activation of NF-ΚB p65, the numbers of dark brown-strained cells were counted. One thousand of gastric epithelial cells were counted for each rat. The data were shown as percentage (%) of immunoreactive cells calculating from this equation: the percentage of immunoreactive cells (%) = (number of strained cells × 100) / 1000.

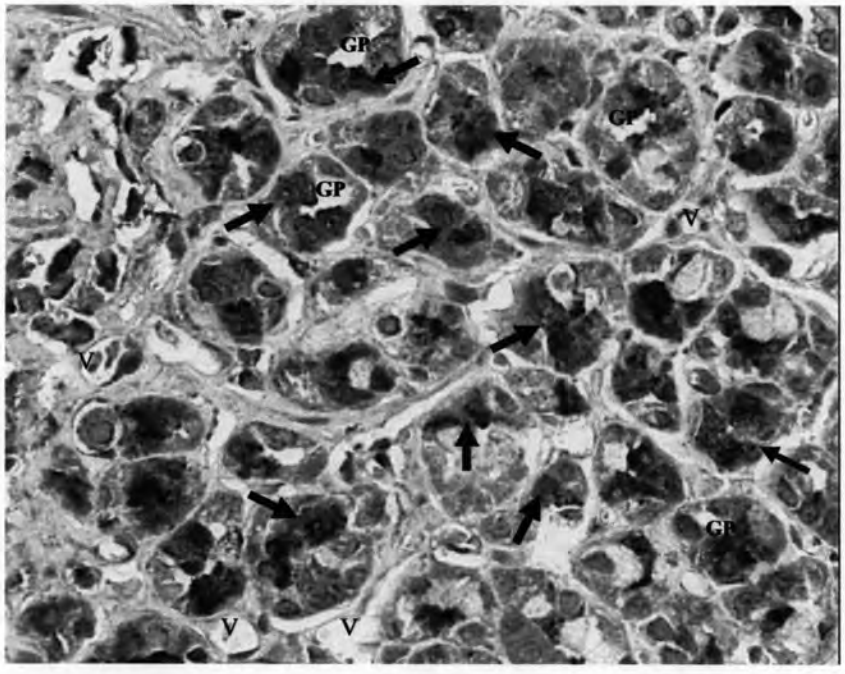

Figure 17 Immunohistochemical staining of NF-ΚB p65 antibody: DAB stained immunoreactive cells (dark brown); nuclear counterstaining was performed with hematoxylin (×40). Cytoplasmic strained in gastric epithelial cells were shown as arrows. This section shows the gastric pit (GP) that formed by gastric epithelium invagination. Moreover, vessels (V) surrounded by endothelial cells are also shown. In this study, only gastric epithelial cells were counted.

Statistic analysis

Data were expressed and analyzed as means ± standard deviation. Each parameter was compare among groups with One-way analysis of variance (One-way ANOVA), LSD, and Duncan ($\alpha = 0.05$) with SPSS program for window version 13.0