CHAPTER V

DISCUSSION

H. pylori infection induces gastritis and gastroduodenal mucosal ulceration, and is associated with an increase risk of gastric adenocarcinoma. Moreover, *H. pylori* virulent factors and host's inflammatory responses are involved in the pathogenesis. Our study aims to investigate the effects of curcumin, an anti-inflammatory plant-derived agent, attenuate *H. pylori*-induced gastritis in rats. In this study, Sprague-Dawley rats were used for *H. pylori*-induced gastritis and their mechanisms of gastric inflammation were partly examined as well.

Rat model of *H. pylori* infection

The *H. pylori* rat model in this study followed previous study [29] that has 69.84% successful infected rate. By using either rapid usease or histology testing criteria, our *H. pylori*-infected gastritis model showed the successful rate at 85%. The increase of successful rate may be involved in the pathogenesis of *H. pylori* toxic strains or host's immune response.

Strains of *H. pylori* are associated with disease outcomes. *H. pylori* strains that have virulent factors including the cytotoxin VacA, the adhesion BabA, or *cag PAI* gene encoded protein CagA induce peptic ulcer and gastric adenocarcinoma in long term of infection [41, 183-188]. Furthermore, lysates of toxic strain expressing VacA and CagA, but not those of non-toxic strain that do not express VacA or CagA, cause gastric damage in mice and that purified VacA cytotoxin causes gastric injuries when administered orally [22]. The virulent *H. pylori* strain from peptic ulcer patients were inoculated to rats that can induce gastritis [28]. *H. pylori* in this study was isolated from peptic ulcer patients, although we did not examine the *H. pylori* strains [29]. Nevertheless, the variation of individual immune-responses may be the cause of uninfected rats.

Interestingly, *H. pylori* causes gastric inflammation but not ulceration in rat gastric mucosa. By histological examination, we did not found ulceration in rat gastric mucosa. Furthermore, mild to moderate gastritis were developed with neutrophil extravasation into gastric lamina propria as shown in figure 19 (b). Similar to other models, *H. pylori*-infected rats induce only mild and moderate gastritis [26-28, 189]. As coexistence with humans, *H. pylori* has evolved complex strategies to maintain a mild inflammation of the gastric epithelium while limiting the

extent of immune effector activity. Several mechanisms and the bacterial factors involved in immune subversion have, in recent years, been elucidated (review in Ref [65]).

Physiologic characters of animals

The physiologic characters; MAP, SBP, DBP, and HR; may change in some pathogenic conditions. *H. pylori* infection may alter these parameters, so we measured them as a reference. In this study, we found that there were no significantly difference in all groups as shown in the table 2.

There are many reports found that *H. pylori* can disturb gastric endothelial cells and locally induce vasoconstriction. Kalia *et al.* [34] demonstrated that *H. pylori* extract application on gastric mucosa induced transient vasoconstriction of PCVs within five minutes. However, *H. pylori* infection is able to modify the gastric mucosal contents of nitric oxide (NO) [190], a potent second messenger and vasodilating agent of paramount importance in the regulation of splanchnic circulation [191]. By application of *H. pylori* extracts on gastric mucosa, many studies suggested that *H. pylori* caused gastric blood flow reduction [30, 192]. By *H. pylori* inoculation to mouse stomachs, Elizalde *et al.* [26] showed that *H. pylori* infected mice exhibited a significant increase in gastric mucosal blood flow and NO production one week after infection, but those parameters returned to basal levels by four weeks. These studies did not report the changes of systemic blood circulation, but local changes may have little effect on systemic blood circulation.

In this result, we showed that curcumin treatment did not affect on overall hemodynamic blood circulation at base line and *H. pylori* infection condition. Although curcumin has anti-inflamation activities that involved in regulation of activated immune cells and endothelial cells [193], curcumin did not change systemic blood circulation. Furthermore, Srimal and Dhawan [150] found that curcumin inhibited the carrageenan-induced edema in rats, and blood pressure and respiration of anaesthetized rats were not affected by curcumin.

Effects of *H. pylori* infection on gastric microvascular leakage, serum VEGF level, and NF-ΚB p65 expression

In this study, we used *H. pylori*-infected rat model [29] and examined the gastric inflammation that is generally accompanied by alterations in vascular function manifested by enhanced gastric microvascular leakage. Besides, serum VEGF level and NF-ΚB p65 expression in gastric epithelial cells were also monitored.

Gastric microvascular leakage

This study determined gastric microvascular leakage on PCVs by measuring the leak of macromolecules; dextran (molecular weight 250,000 Daltons) labelled fluorescent dye. With intravital fluorescent microscopy and image-intensity analysis, we measured the percentage of macromolecular leakage. The PCVs on gastric mucosa were selected from these following criteria: vessel diameter range 15-30 μm, blood flow collected from branches of hexagonal capillaries, and the vessels that supplied body region of stomach. Although the antrum is *H. pylori* predominate, the inflammation is occurred overall stomach. The body region is appropriate for studying gastric microcirculation because of the large area. In addition, the landmark, limiting ridge that separates the upper stomach from the lower stomach is lining near the body region. Therefore, PCVs on body region were selected to examine the change of gastric microcirculation. 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. From the experiment, there was increase intensity of leakage along the time course. However, the long time (45 minutes) caused too high interstitial intensity to identify the vessel border and to measure. In this study, the appropriate time to measure increase leakage is at 30 minute after FITC-dx-250 injection. Moreover, the results of macromolecular leak were shown in percentages of the increase leakage calculated by the equation: $[(I_{out}/I_{in})$ at 30 minute $- (I_{out}/I_{in})$ at 0 minute$] \times [100 / (I_{out}/I_{in})$ at 0 minute$]$. The intensity ratio (I_{out}/I_{in}) measurement is used to reduce the difference of initiated intensity among animals.

In this study, *H. pylori* infected group presented significant increase of macromolecular leakage (15.41% ± 2.83) compared with control group (10.69% ± 1.43) as shown in the table 3 and figure 21. The *H. pylori* infection significantly increased macromolecular leakage by several ways of host-immune response.

First of all, by activation of neutrophils, *H. pylori* causes neutrophil infiltration via the NF-KB-induced IL-8 production that generate a gradient along which neutrophils are recruited. In addition, *H. pylori* causes expression of adhesive molecules on endothelial cells that interact with neutrophil surface protein (CD11a and CD11b) [4, 67, 74]. Neutrophil transmigration is associated with increased permeability of vascular endothelial cells that may also allow macromolecules to leak [68, 194]. Moreover, activated neutrophils also have a specific way to regulate endothelial permeability that provide macromolecular leakage [70].

Furthermore, during *H. pylori*-induced inflammation, macrophages and leukocytes were also activated and produced reactive oxygen or nitrogen species or inflammatory cytokines such as TNF-α or IL-1β that influence endothelial cells [115, 171]. Besides, Kurose *et al.* [75] and Kalia *et al.* [76] showed that *H. pylori*-activated mast cells released proinflammatory mediators such as PAF and histamine that may increase microvascular permeability. From previous studies, these inflammatory mediators induce the expression of adhesion molecules on endothelial cells [195] and open gaps in the endothelium of the PCVs [196]. Therefore, these mediators disturb endothelial function and increase microvascular permeability that causes macromolecular leakage.

In addition, the vascular hyperpermeability allowing extravasation of macromolecular proteins, inflammatory cells, and fluid is determined through the influence of pro-angiogenic factors such as VEGF [93, 194, 197]. *H. pylori* infection may also be characterized by activation of neoangiogenic process [74, 198]. Therefore, *H. pylori* infection induces VEGF production that may increase macromolecular leakage by VEGF receptor-mediated signaling pathways that induce endothelial fenestration. For example, VEGF receptor Flk associated with cadherins at cell-cell junctions [88] may facilitate the vascular permeability response. Furthermore, downstream signaling by VEGF is quite diverse, and includes transduction molecules such as (PI(3)K)/Akt, Ras/Raf/MEK/Erk, Src, and PLC-γ/eNOS. A wide array of factors has been implicated in the VEGF-induced permeability response (reviewed in Ref [79]).

Serum VEGF level

In this study, we measured serum VEGF level by using ELISA technique. The serum samples collected from blood were diluted with the kit calibrator diluent at 4 times dilution. This dilution was appropriate for interpreting the values from the standard curve. Besides, the diluted samples were duplicates in the assay. The presented results were read from the standard curve and already multiplied by 4.

As the results, it showed that *H. pylori* infection led significant increase of serum VEGF level (619.43 pg/ml ± 145.68) compared with control group (228.57 pg/ml ± 40.41) as shown in the table 4 and figure 27. This observation confirms previous studies obtained from *in vitro* and *in vivo* studies. In *in vitro* study, the increase of VEGF expression could be linked to the VacA of *H. pylori* [77]. Moreover, *H. pylori* infection stimulated *vegf-A* gene expression in gastric epithelial cells [78]. In *in vivo* study, by using laser microdissected, *H. pylori*-infected mice showed VEGF expression that peaked during the intermediate time points (2 weeks after *H. pylori* infection) in gastric epithelial cells [114]. In our study, we did not localize the expression of VEGF. However, the significant increase of serum VEGF level in *H. pylori*-infected rats may be from the increase of VEGF in gastric epithelial cells as shown in many studies [77, 78, 114].

The significantly increased VEGF may involve in increase of macromolecular leakage or induction of neoangiogenesis in *H. pylori*-infected mucosa. By VEGF receptor-mediated signaling pathways, VEGF receptor Flk associated with cadherins at cell-cell junctions [88] may facilitate the vascular permeability response, whereas Flk associated with αv integrins [102] ($\alpha v\beta 3$ and $\alpha v\beta 5$) at the cell-matrix interface may influence permeability as well as angiogenesis.

The VEGF gene expression can be influenced by extracellular growrh factors, cytokines, oxidative stress, hypoxia, and genetic alteration [93, 111]. *H. pylori* infection causes VEGF expression on gastric epithelial cells by several pathways of transcriptional level. First, *H. pylori* potently up-regulates VEGF gene expression in gastric epithelial cells by cis-acting promoter elements, including a hypoxia responsive site, proximal GC-rich elements as well as Egr-1, and AP2 recognition motifs [111, 112, 199, 200]. In addition, *H. pylori* activated zinc-figure transcription factors Sp1 and Sp3 as molecular mediators of *vegf-A* expression [78]. Moreover, the expression is also regulated through transcription factor NF-KB [110, 115, 163].

NF-KB p65 expression

NF-KB is a nuclear transcription factor required for the expression of genes involved in cell proliferation and immune responses. In resting cells, NF-KB is coupled IKB and resides in the cytosol as an inactive form. In response to inflammatory stimuli NF-KB translocates into the nucleus to function as an active transcription factor.

In present study, we observed the expression of NF-KB subunit p65 in gastric epithelial cells by immunohistochemistry and counterstained with hematoxylin for nuclear detection. Besides, one thousand cells of gastric epithelium were counted to indicate as a percentage of NF-KB p65 expression.

As shown in the table 5 and figure 28 of the results, expression of NF-KB p65 was significantly increased in *H. pylori* infected group (44.20% ± 5.24) compared with control group (28.58% ± 2.82). This observation confirmed many previous results obtained from *H. pylori*-induced gastritis in patients, cultured epithelial cells upon *H. pylori* infection, and rodent stomach. *H. pylori* infection activates NF-KB in gastric epithelial cells and immune cells in lamina propria of patients with *H. pylori*–induced gastritis [129-131]. Moreover, *in vitro* study indicated that *H. pylori* induced NF-KB activation [164]. In addition, NF-KB p65 expression in rodent stomach was studied. In 2001, Takahashi and colleges [134] found that NF-KB p65 was activated in ulcerated tissue but not in normal mucosa. Beside, the recently study found that *H. pylori*-infected mice induced NF-KB p65 expression on gastric epithelial cells [135].

Interestingly, in our results, we found that NF-KB p65 expression was mostly in the cytoplasm of gastric epithelial cells. There are many evidences indicating that the cytoplasmic strained of NF-KB p65 was considered as activated NF-KB.

First of all, the p65 antibody used in this study was rabbit polyclonal affinity purified antibody against a peptide mapping within the N-terminus of NF-KB p65 of human origin. The N-terminal domain called the RH domain contains sequences responsible for dimerization, DNA binding, nuclear localization and IKB binding [119]. Therefore, localization of activated NF-KB p65 was assessed with the antibody that reacts only to activated NF-KB.

In addition, other studies have demonstrated a predominat cytoplasmic and focal nuclear localization with both polyclonal and monoclonal antibodies. Using polyclonal anti-p65 antibodies, results have shown a predominant cytoplamic straining in tumor cells in gastric [128] and oral carcinomas [201]. Using monoclonal antibody with high selectivity and specificity,

results on differentiating B cells have demonstrated that only 10-20% of NF-KB protein was detectable in the nucleus, whereas the remaining were remained in the cytoplasm [202, 203].

Moreover, using p65 antibody from Santa Cruz company was labelled NF-KB localization in gastric tumor cells [133] and prostate tumor cells [204]. The expression of NF-KB p65 was only in the cytoplasm with scattered nuclear positivity.

Besides, there is a difference of the pattern of immunoreactive cells between control group and *H. pylori* infected group as shown in figure 29 and 30. In control group, NF-KB p65 was indicated in the gastric epithelial cells on the base of gastric mucosa. These cells functionally proliferate to replace epithelial cells, suggesting that NF-KB activation is associated with cell proliferation. In the other hand, NF-KB p65 indicated in the gastric epithelial cells was increased in *H. pylori* infected group.

Therefore, predominately cytoplasmic detection in our study may result from the type of antibody using. This anti-p65 antibody could not detect nuclear-localized NF-KB p65.

Role of curcumin on gastric microvascular leakage, serum VEGF level, and NF-KB p65 expression

One of the main goals of this study was to analyse the anti-inflammatory property of curcumin-mediated inhibition of *H. pylori*-induced gastritis in rats.

Curcumin is a phytophenolic compound extracted from tumeric rhizomes. This active ingredient shows a great of biological properties. In this study, anti-inflammatory property was focused on. Curcumin at doses of 200 mg/kg BW and 600 mg/kg BW were used. These concentrations may be potently effect on gastric inflammation in rat. Despite rapid metabolism of curcumin, it was directly treated once a day into the stomach. Control rats were also treated with 600 mg/kg BW (high dose) curcumin to investigate effects of curcumin on control rats. By safety of curcumin, curcumin control group showed no change of parameters compared with control group. DMSO was used as a solvent of curcumin, and 0.1% (v/v) DMSO used in this study has no effect on cell viability [166].

However, the *in vitro* study [9] suggested that high dose of curcumin can inhibit *H. pylori* growth in cell culture. In this study, it was observed that both of rapid urease test and histopathology in *H. pylori* infection followed by curcumin treated groups still had the positive

results. It is indicated that the dose of curcumin had rarely effects to eradicate *H. pylori* in this *in vivo* study.

The anti-inflammatory property of curcumin-mediated inhibition of *H. pylori*-induced gastritis in rats was monitored by reduced gastric microvascular leakage. Besides, serum VEGF level and NF-KB p65 expression in gastric epithelial cells were also monitored

Gastric microvascular leakage

This study showed that the curcumin significantly reduced macromolecular leakage from PCVs in *H. pylori* infection, but there was no difference between 200 mg/kg BW curcumin treated groups (12.32% ± 2.13) and 600 mg/kg BW curcumin treated groups (12.14% ± 1.86) as shown in the table 3 and figure 21. Curcumin suppressed macromolecular leakage via several mechanisms.

First, curcumin inhibits neutrophil infiltration followed by macromolecular leakage. *H. pylori* causes neutrophil infiltration via the NF-KB-induced IL-8 production that was blocked by curcumin [164].

Second, curcumin blocked macromolecular leakage by suppressing recruitment of leukocytes. Curcumin inhibited LOX, the enzyme transforms arachidonic acid in leukotrienes, which take part in leukocytes recruiting and play a role in inflammation [165, 167]. Moreover, curcumin inhibited the attachment of monocytes to endothelial cells by suppressing the expression of endothelial cell adhesion molecules ICAM-1, VCAM-1, and ELAM-1 [168]. In addition, this yellow substance can suppress inflammatory cytokines TNF-α and IL-1 [169, 170] that induced adhesion molecules expression. It also has been reported that curcumin repressed PAF and arachidonic acid mediated platelet aggregation [205]. Furthermore, curcumin is an antioxidant substance. Curcumin is capable of scavenging oxygen free radicals such as superoxide anions and hydroxyl radicals [206].

In this study, *H. pylori*-stimulated increased macromolecular leakage was blocked by both of curcumin doses and reduced to control level as shown in the table 3 and figure 21. The possible mechanism may be that curcumin inhibited *H. pylori*-stimulated neutrophil infiltration via the suppression of NF-KB-induced IL-8 production.

Serum VEGF level

Although macromolecular leakage was suppressed by curcumin, VEGF level did not decrease when curcumin applications. In this study, both of curcumin doses did not significantly decrease the high level of serum VEGF from *H. pylori* infection as shown in the table 4 and the figure 27. Rising serum VEGF level may imply effect of *H. pylori* infection, but VEGF may not directly involve in macromolecular leakage. In the other hand, high level of serum VEGF in *H. pylori* infection may indicate the angiogenesis.

Recently, Tuccillo *et al.* [207] found that VEGF is over-expressed in gastric mucosa of *H. pylori*-infected dyspeptic patients and that this effect paralleled the increase in a number of blood vessels suggesting the involvement of VEGF expression in the process of neo-angiogenesis. In particular, VEGF contributes to the restoration of normal mucosa architecture after injury through stimulation of angiogenesis in the ulcer [208].

Moreover, several evidences suggest that VEGF expression influences the long-term outcomes of *H. pylori* infection [114]. For example, gastric adenocarcinoma is usually defined by high levels of VEGF expression accompanied by an increased intratumoral microvessel density [209, 210]. The functionality of VEGF in gastric cancer was proven in a xenotransplantation model in which growth of tumor could be reduced by administration of antibodies against circulating VEGF [211].

As shown in the table 4 and the figure 27, both of curcumin doses did not significantly decreased mean of serum VEGF in *H. pylori*-infected rats. It is indicated that curcumin did not completely inhibit VEGF production.

VEGF gene expression can be regulated by several signaling pathways, including MAP kinase ERK and JNK as well as NF-KB pathways [110-112]. *H. pylori*-induced activation of JNK and NF-KB activity is inhibited by curcumin [164, 212]. In contrast, curcumin does not affect *H. pylori*-induced activation of ERK1/2 and p38-MAP kinase indicating that curcumin selectively inhibits protein kinase-dependent signalling pathways [164, 213, 214]. Therefore, VEGF level did not significantly reduce after using curcumin for treating *H. pylori*.

NF-KB p65 expression

Curcumin is an anti-inflammation substance because it can suppress the central mediator of inflammation, NF-KB. We also indicated that curcumin significantly reduced NF-KB p65 expression in gastric epithelial cells as shown in the table 5 and the figure 28. In our results, curcumin showed significantly decrease percentage of immunoreactive cells in *H. pylori* infection followed by 200 mg/kg BW curcumin treatment (33.99% ± 4.83) and 600 mg/kg BW curcumin treatment (37.11% ± 4.34) compared with *H. pylori* infected group (44.20% ± 5.24).

Curcumin is assumed to be an inhibitor effective against a broad spectrum of protein kinases. As demonstrated in the earlier study, the block of *H. pylori*-induced NF-KB activation in human epithelial cells by curcumin is based upon an IKBα stabilization due to inhibition of IKK activity. Since the inhibitor was added directly to the kinase reaction, the conclusion can be drawn that curcumin directly blocks IKK activity [164]. By, blockage of IKK, the phosphorylation and degradation of IKB will be not occurred resulting in inhibition of NF-KB activation. Furthermore, it is possible that this may be due to the inhibitory effect of curcumin on protein kinase C resulting in inhibition of NF-KB activation [215].

In this study, NF-KB activated by *H. pylori* infection was detected by immunohistochemistry with anti-p65 antibody. One target of NF-KB is the gene encoding IL-8, the chemoattractant substance that is induced in gastric epithelial cells upon *H. pylori* infection (reviewed in Ref [4]). The IL-8 is produced as well as adhesion molecules such as ICAM-1, VCAM-1, and cadherin on endothelial cells that functionally induce neutrophil extravasations during gastric inflammation. The neutrophil extravasations induce macromolecular leakage from gastric microvessels that was detected by intravital fluorescence microscopy with FITC-dx-250 labelled macromolecules. It is implicated that *H. pylori*-induced macromolecular leakage was evoked by NF-KB expression. Moreover, as shown in this study, curcumin in high and low dose can significantly diminish *H. pylori*-induced NF-KB activation and macromolecular leakage.

This experiment showed no difference between doses of curcumin because it is possible that curcumin is metabolized rapidly. The fact that curcumin inhibits both NF-KB activation as well as the gastric microvascular leakage makes this pharmacologically safe compound as a possible candidate for treatment of *H. pylori*-associated gastritis.

In consider to the this study, it suggested that *H. pylori*, a gastrointestinal pathogen, induced gastric inflammation by activating transcription factor NF-KB in gastric epithelial cells. Then, the production of inflammatory mediators, especially IL-8 and VEGF, has been occurred. The macromolecular leakage is evoked by IL-8-induced neutrophil infiltration. Moreover, increased VEGF may involve in the macromolecular leakage by stimulating disruption of endothelial barrier function. Interestingly, curcumin, an anti-inflammatory substance, at dose of 200 mg/kg BW and 600 mg/kg BW improved *H. pylori*-induced gastric inflammation by diminishing NF-KB activation. With this idea, curcumin could prevent the macromolecular leakage as well as VEGF production. However, many molecular pathways are involved in VEGF production. In addition, curcumin did not completely inhibit VEGF production.

In this present study, using of curcumin indicated that the macromolecular leakage is regulated via activation of NF-KB in gastric epithelial cells but not serum VEGF level as shown in the following figure.

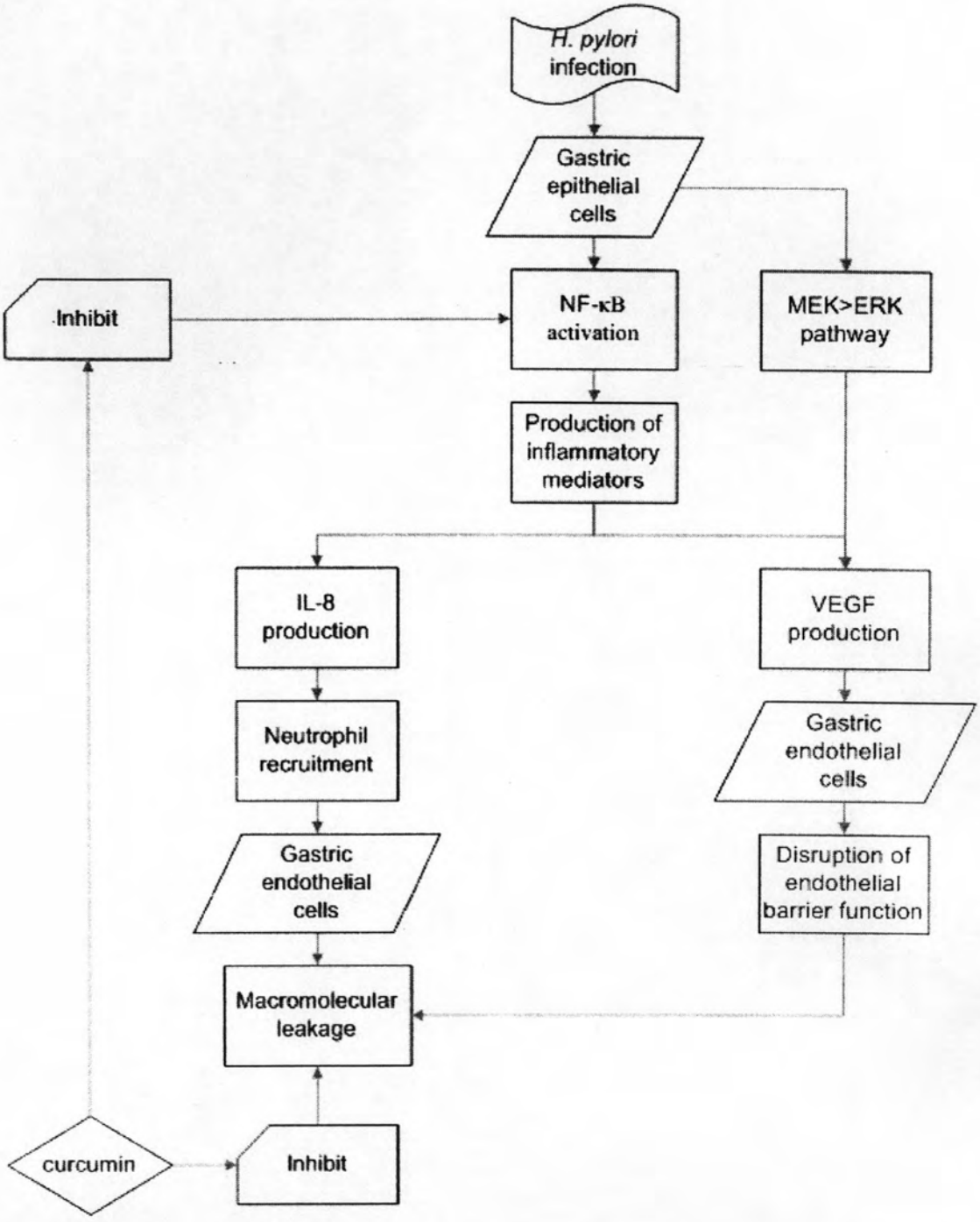

Figure 31 Diagram of this study: Curcumin reduced *H. pylori*-induced gastric inflammation by inhibiting the NF-KB subunit p65 expression in gastric epithelial cells and macromolecular leakage from gastric PCVs.