CHAPTER II

LITERATURE REVIEWS

In human, *H. pylori* infection causes inflammation of the gastric mucosa that may develop ulcers or gastric tumors. Its clinical importance brought *H. pylori* to the attention of medical science. However, understanding the complicated pathophysiology of *H. pylori*-associated diseases remains limited by constraints of patient studies and animal models that do not completely mimic all diseases in humans attributed to *H. pylori* infection. Interestingly, researches into *H. pylori* pathogenesis have benefited from studying animal models and some models (mice and rats) are useful in understanding *H. pylori* pathogenesis.

The model used in this study

From many previous studies, understanding *H. pylori* pathogenesis has been limited by the absence of suitable animal models to reproduce the *H. pylori* diseases. Laboratory strains of *H. pylori* does not infect laboratory rodents [15]. Thus, experimental models used animals such as gnotobiotic piglets [16], euthymic germ-free mice [17], athymic nude mice [18], or monkeys [19], but these animals are difficult to handle in large numbers and cannot be used study immune responses. *Helicobacter* species other than *H. pylori*, such as *H. felis* and *H. mustelae*, have been used to infect mice [20] and ferrets [21]. However, these animal models do not mimic human *H. pylori* infection and following pathology because those *Helicobacter* strains are not like *H. pylori* expressing virulent factors required for the induction of ulcers and inflammation [22].

With the well-known limitation, most investigators have adopted the mutant mouse model, defected in immune system, of *H. pylori* infection using the Sydney strain (SS1 *H. pylori*) [23-25]. However, this model is too expensive and it is hard to handle the organisms. Despite its high infectivity rate, the degree of gastritis observed in C57BL/6 mice either 1 or 4 weeks after inoculation was moderate. Very few neutrophils were identified in the mucosa or sub-mucosa of *H. pylori*-infected mice at either time point [26].

H. pylori induced gastritis in rat model has been done in several studies but they used the difficult, expensive, and long-period methods [27, 28]. Besides, those previous studies have been

designed with the aim of establishing histological gastritis that would observe in human, but have not been study in gastric microcirculatory changes.

In this study, we used *H. pylori*-infected rat model [29] that is a simple, but effective model to examined the *H. pylori*-induced gastric mucosal inflammation. In briefly, Sprague-Dawley rats were inoculated by *H. pylori* that was obtained from *H. pylori*-infected patients. Two weeks after inoculation of *H. pylori*, the mild to moderate gastric inflammation were developed as confirmed by hematoxylin and eosin (H&E) staining [29]. In addition, it has been found that the histopathology did not become more progressive along with the time after two weeks of *H. pylori* inoculation [28]. The damaging histology of *H. pylori*-induced gastritis was infiltrated by inflammatory cells into lamina propria, enhanced proinflammatory cytokine releases and generated reactive oxygen species (ROS) [30]. This infected rat model is considered to be an experimental model that closed resemblance to human gastritis.

The stomach areas used in this study

H. pylori can live along the surface of the stomach, gastric pits [31], and induce gastric mucosal inflammation. In this experiment, we chose the mucosal postcapillary venules (PCVs) on the body part of stomach to study the macromolecular leakage and the antrum to examine *H. pylori* infection.

Stomach

The stomach (figure 1) is a J-shaped tube, with two openings, lining between the esophagus and the duodenum. The lesser curvature of the stomach extends the short distance from the esophagus to duodenum along the medial to superior aspect, while the greater curvature extends the longer distance on the lateral to inferior aspect. The stomach has distinct anatomical subdivisions that have functional differences. First, the cardia is a small area presently inside the cardiac opening. Next, the fundus and body (corpus) are the main parts of the stomach. The fundus consists of the dome of the stomach. They secrete hydrochloric acid and enzymes involved in the digestion of food such as pepsinogen. Last, the pyloric region is subdivided into antrum, pyloric cannal, and pyloric sphincter. This region occupies the distal end of the stomach is responsible for mucus, gastrin, and pepsinogen secretion. [32]. The antrum contains cells that sense pH and secrete hormones (such as somatostatin and gastrin) that regulate acid production in the fundus.

H. pylori grows predominantly in the antral region of the stomach which produced acid less than corpus and body region [33]. However, *H. pylori*-induced inflammation is occurred overall stomach. Remarkably, the limiting ridge lining across the lesser and greater curvature separates the forestomach from the more distal stomach [34]. This line was the landmark in this experiment.

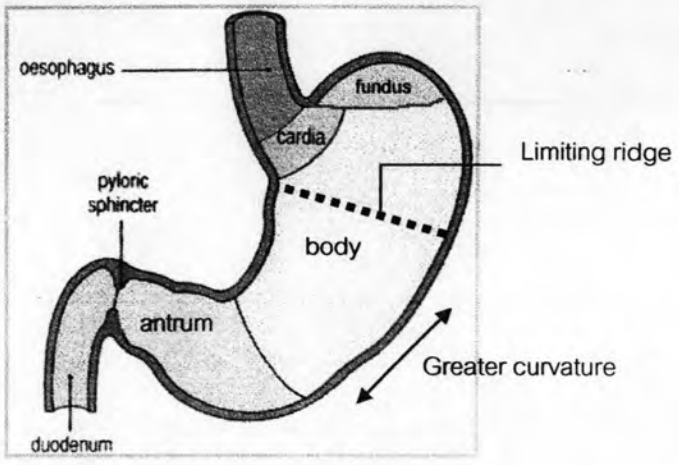


Figure 1 Anatomy of stomach: The body part of the stomach was used to observe the macromolecular leakage. The antrum was used to examine *H. pylori* infection.

The wall of the stomach has four layers (figure 2) consisting of mucosa, submucosa, muscularis propria, and serosa.

The mucosa, surface of the stomach, is made up of epithelium and surrounding connective tissues called the lamina propria. The epithelium contains different types of cells which secrete compounds to aid digestion and stomach protection. The lamina propria contains the supporting framework, such as capillary network and nerve fibers, for the epithelial cells.

Epithelial cells attached by *H. pylori* are disrupted. Therefore, the inflammation can occur locally in the mucosa.

The submucosa is consists of the loose connective tissues in which are embedded lymphatic vessels, blood vessels, and scattered mononuclear cells including mast cells.

The muscularis propria is composed of three layers of smooth muscles: the outer-lumen longitudinal muscles, middle circular muscles, and inner-lumen oblique muscles. The nerves and ganglion cells are located between the outer longitudinal and middle circular muscle layers.

The serosa is a thin covering of loose connective tissues with blood vessels, lymphatic vessels, and nerve fibers. This layer is contiguous with the omentum and ligaments attaching the stomach to spleen and liver.

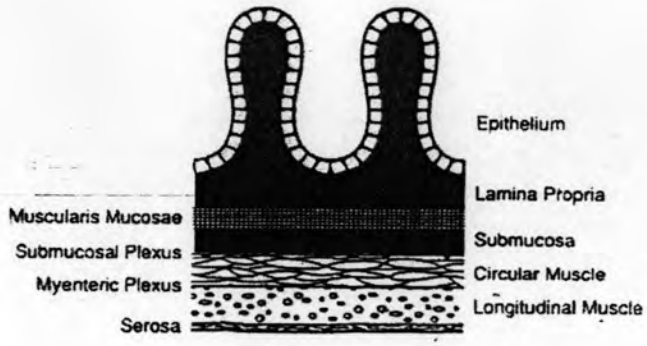


Figure 2 Structure of gastric wall [35]: The mucosa composed of epithelium and lamina propria was exposed to examine the *H. pylori*-induced gastric microvascular leakage.

The stomach vessels used in this study

The postcapillary venules (PCVs) of the stomach selected in this study are located on the mucosa. They collect blood from the, special feature, hexagonal capillary beds and drain it to collecting veins (CVs).

The stomach has rich network of anastomosing vessels derived from the various branches of the celiac trunk and originated from the anterior surface of the thoracic aorta just below the aortic hiatus. The celiac trunk is a short vessel that divides into three arteries (figure 3): the left gastric artery, the splenic artery and the common hepatic artery. Each of these parts supplies a portion of the stomach. The left gastric artery supplies the fundus and left superior portion of the lesser curvature. The splenic artery gives rise to the short gastric arteries that serve to the fundus and body along the greater curvature of the stomach. Moreover, the right and left gastroepiploic arteries also form an anastomosis along the greater curvature. Last supplier, the common hepatic artery gives rise to the gastroduodenal artery and the right gastric artery that contribute the inferior lesser curvature of the stomach.

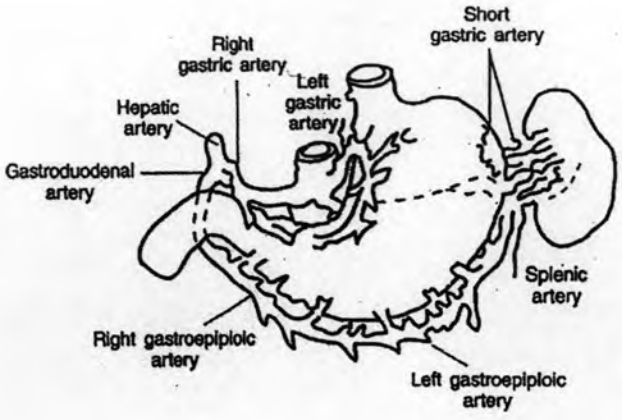


Figure 3 Blood supply of the stomach [32]

Gastric circulation (submucosal and deeper mucosal vasculature)

The circulation of the gastric wall has arrangement, which has branching hierarchy and relative dimensions. The posterior branch of the left gastric artery gives rise to series of long vessels supplying the posterior corpus. These vessels pierce the external muscle layers at the lesser curvature near the cardia and run under the muscle coat in the superficial submucosa radially toward the greater curvature. These small arteries (SA) form the main submucosal arteriole-arterial anastomotic plexus or primary arcade of submucosal arterioles (SMA1). In turn, these vessels form smaller and smaller branches, which interconnect with each other and the parent vessels forming a secondary (SMA2) and a tertiary (SMA3) arcade of submucsal arterioles. The SMA3 gives rise to small mucosal terminal arterioles (MTA) which run perpendicularly through the muscularis mucosae and, on entering the mucosa, divide into the hexagonal mucosal capillary plexus. Collecting veins (CVs) run perpendicularly through the mucosa. Within the deeper mucosa they drain into the venous anastomosis, which, on entering the muscularis mucosae, gives rise to the secondary arcade of submucosal venules (SMV2). Interestingly, CVs were larger in diameter (36.4 ± 1.1 μm) than the deeper mucosal venous anastomosis (31.5 ± 1.3 μm) or the initial part of SMV2. The SMV2 enter the primary arcade of submucosal venules (SMV1), which follow the same course as the primary arterioles and return blood to the small veins (SV). The SV run parallel with SA, penetrated the external muscle layers, and leave the superficial submucosa [36].

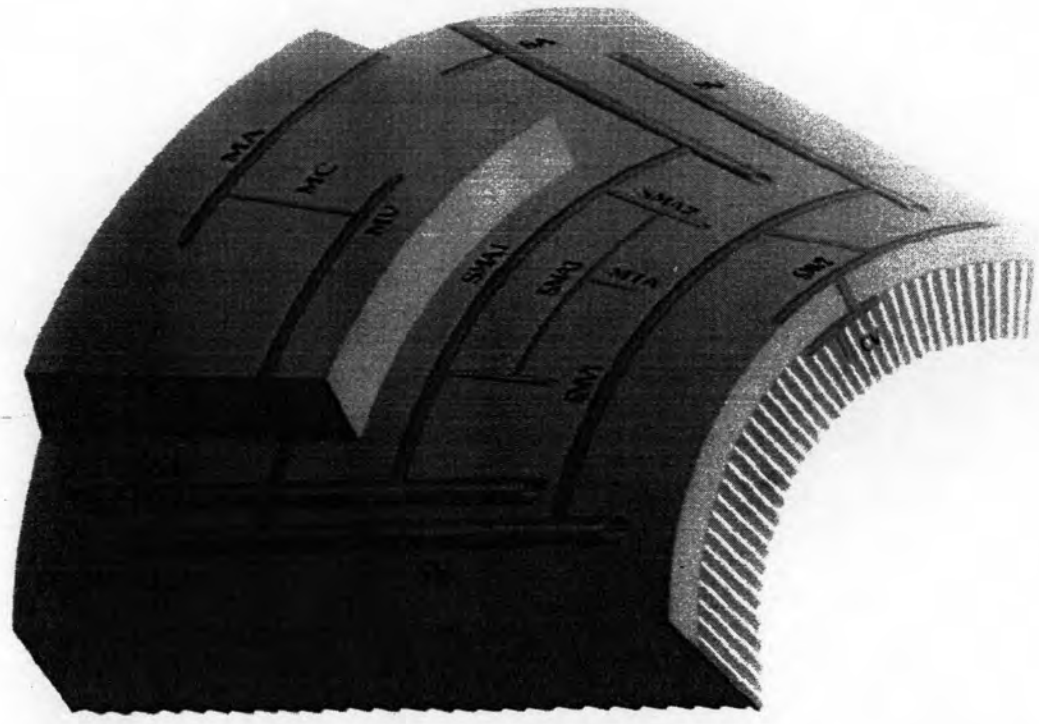


Figure 4 Schematic representation of microvasculature in gastric wall: Blood vessels were identified numerically according to their branching order and vascular hierarchy: small artery (SA), submucosal primary (SMA1), secondary (SMA2), and tertiary (SMA3) arterioles, mucosal terminal arteriole (MTA), collecting venule (CV), submucosal secondary (SMV2) and primary (SMV1) venules, muscle arteriole (MA), capillary (MC), and venule (MV), and submucosal small vein (SV). Common input and output of muscle and mucosal circulations are SMA1 and SMV1 [36].

Therefore, PCVs (15-30 μm) collect blood from hexagonal mucosal capillary plexus and drain it into CVs. Like other veins, there are leucocyte adhesion and fenestration during inflammation. These structures were selected to investigate the macromolecular leakage induced by *H. pylori* infection.

Helicobacter pylori (*H. pylori*)

The discovery of *H. pylori* was first reported in 1984 by two Australian investigators, Barry Marshall and Robin Warren [1], who isolated the bacteria from mucosal biopsies of patients with chronic active gastritis. Their name was changed from *Campylobacter pyloridis*, *Campylobacter pylori*, and *Campylobacter*-like organism when biochemical and genetic characterization of them shown that it is into the genus *Helicobacter* [37].

H. pylori is a noninvasive, nonspore-forming, and spiral shaped Gram-negative bacterium measuring approximately 3.5 × 0.5 μm. In addition, it has four to six sheathed flagella at one pole as shown in figure 5. It slowly grow in microaerophilic condition, 5% oxygen, 50% carbon dioxide at 37°C [38]. *H. pylori* is unusual organism with remarkably high level of genetic diversity [39] in that it can survive in the human stomach and also multiply in violence-acid environment of the stomach. In *H. pylori*-infected human, *H. pylori* adheres on the gastric epithelial cells and induces gastritis, peptic ulcer, mucosal-associated lymphoid tissue (MALT) lymphoma and gastric cancer.

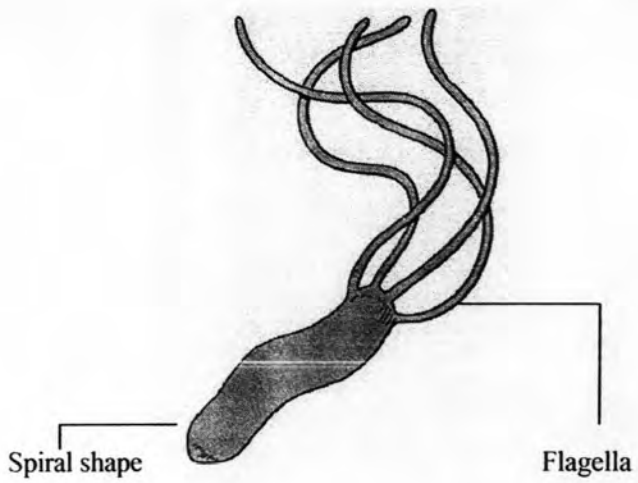


Figure 5 Characteristic of *H. pylori*: *H. pylori* is a Gram-negative bacterium which has flagella on the one pole. These flagella and spiral shape of *H. pylori* help the bacterial movement into the mucus of stomach.

Pathogenesis of *H. pylori* infection

H. pylori is highly adapted to the stomach environment, with a unique array of features that permit entry into the mucus, swimming and spatial orientation in the mucus, attachment to epithelial cells, evasion of the host immune response, and, as a result, persistent colonization and virulent factors transmission.

H. pylori is transmitted from person to person by the fecal-oral route. The first barrier that bacteria must exceed is the acidity in the stomach lumen. To overcome this, *H. pylori* produces a potent urease enzyme. By catalyzing urea breakdown, urease generates carbon dioxide

and ammonia, which potentially buffer the surrounding microenvironment and the bacterial cytosol [40]. In addition, urea is an important source of nitrogen for the bacteria.

Powerful flagella help the bacteria to swim through the viscous mucous layer covering the gastric epithelium, where bacterial adhesion proteins mediate a close interaction with the host cells [41]. *H. pylori* can bind tightly to epithelial cells by multiple bacterial surface components. The outer-membrane protein (Hop), such as BabA, binds to the fucosylated Lewis B blood-group antigen on the gastric epithelial cells [42]. Several Hop protein families also mediate adhesion to epithelial cells.

When *H. pylori* adheres on gastric epithelial cells, it releases virulent factors to immune subversion. The main features of *H. pylori* virulence factors are summarized below:

The *cag* Pathogenicity Island (*cag* PAI) gene

The *cag* PAI gene is an approximate 40 kilobases (kb) genetic unit. The intact *cag* PAI, which is associated with severe disease (reviewed in Ref. [43]), encodes 31 proteins, which form a type IV secretion system (T4SS) capable of directly transferring bacterial proteins to the cytoplasm of target cells (reviewed in Ref. [44]). In addition, CagA also encoded from *cag* PAI is delivered by the T4SS to host cells, where it is phosphorylated on tyrosine residues by Src family protein tyrosine kinases. Then, the recruitment of adaptor proteins involved in nucleation of actin polymerization and cytoskeletal rearrangements is occurred [45, 46]. Among these, the tyrosine phosphatase SHP-2 might also couple CagA to cell signaling in leading to a growth factor–like cellular response and cytokine production by the host cell [47]. Furthermore, the *cag* PAI has been implicated in the transcription factor NF-KB activation and subsequent activation of genes encoding proinflammatory cytokines [48].

Vacuolating cytotoxin (VacA)

VacA is a secreted 87 kDa protein toxin, which causes vacuolar degeneration of epithelial cells *in vitro* and gastric epithelial erosion *in vivo*. Following proteolytic cleavage of a pro-toxin, VacA is released into the extracellular space as homo-oligomers consisting of 6–7 or 12–14 VacA monomers. Low or high pH treatment results in disassembly of the oligomers into membrane-inserting monomers and is associated with enhanced VacA cytotoxicity. VacA binds to a specific cell surface receptor and then inserts and oligomerizes in cell membranes to form anion-selective and voltage-dependent channels. In addition, VacA has loosened tight junctions in monolayers of polarized epithelial cells (review in Ref [49]).

Neutrophil-activating protein (NAP)

NAP is related to bacterioferritins. It is probable that the primary function of NAP in the bacteria is to ensure adequate iron uptake and that its inflammatory activity is a secondary acquired or fortuitous activity (reviewed in Ref. [50]). NAP, a cytosolic protein, is released by bacterial lysis and interacts directly with neutrophils, monocytes and mast cells, resulting in the activation of their inflammatory functions (reviewed in Ref. [51]).

These virulent factors destroy gastric epithelial cells and induce host innate and adaptive responses.

Host inflammatory response

The host response to *H. pylori* involves in the damage to the gastric epithelium and therefore has an integral role in *H. pylori* pathogenesis.

Host inflammatory response consists of the recruitment of neutrophils, followed by T and B lymphocyte, plasma cell, and macrophage, along with epithelial-cell damage [52]. Initially, induction of interleukin (IL)-8 expressions by gastric epithelial cells depends on CagA injection by *H. pylori*. This chemokine secreted by epithelial cells bind to the proteoglycan scaffolding, generating a gradient along which neutrophils are recruited. Interestingly, *H. pylori* strains carrying the *cag* PAI induce a far stronger IL-8 response than *cag*-negative strains, and this response depends on activation of nuclear factor-KB (NF-KB) [53] and the early-response transcription factor activator protein 1 (AP-1) [54, 55]. Besides, *H. pylori* urease and porins may contribute to extravasation and chemotaxis of neutrophils, phagocytes, and mast cells [56, 57]. However, activation of these immune cells by *H. pylori* virulence factors results in the release of reactive oxygen species (ROS) [58], which damage gastric epithelial cells and endothelial cells.

H. pylori infection induces a vigorous systemic and mucosal humoral response [59]. The chronic phase involves in an adaptive lymphocyte response. Lymphocyte recruitment is facilitated by chemokine-mediated expression of vascular addressins such as vascular-cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). With these adhesion molecules, antigen-presenting cells (APCs) and T cells are also attracted to the lamina propria. Basically, T helper (Th) 1 cells are induced mostly in response to intracellular pathogens and Th2 cells stimulate B cells in response to extracellular pathogens. Because *H. pylori* is noninvasive and induces a strong humoral response, a Th2-cell response would be expected. Paradoxically, *H. pylori*–specific gastric mucosal T cells generally present a Th1 phenotype [60]. Studies in gene-targeted mice have further showed that Th1 cytokines promote gastritis, whereas Th2 cytokines

are protective against gastric inflammation [61]. This biased Th1 response, combined with Fas-mediated apoptosis of *H. pylori*–specific T-cell clones [62], may favor the persistence of *H. pylori*. Furthermore, B cells reach the damaged epithelium in later stages of infection, where they form follicles of proliferating cells, which can develop into a MALT lymphoma. Some *H. pylori*–infected patients have an autoantibody response directed against the H^+/K^+–ATPase of gastric parietal cells that correlates with increased atrophy of the corpus [63].

To maintain extended colonization of the human gastric mucosa, *H. pylori* must avoid both innate and adaptive immune responses. By avoidance of immune response, *H. pylori* virulent factors and enzymes provide this function. For example, *H. pylori* is more resistant to phagocytic killing than other Gram-negative bacteria. This feature is unique to Cag and Vac positive (type I) *H. pylori* strains. *In vitro* studies demonstrate that phagocytosis of type I *H. pylori* by macrophages is delayed and that viable bacteria accumulate in larger than normal phagosomes, referred to as megasomes, which result from homotypic phagosome fusion [64]. In addition, VacA inhibits induction of adaptive immune responses by blocking antigen processing and suppressing T-cell activation [65]. Moreover, to avoid the negative effects of reactive oxygen intermediates (ROIs), *H. pylori* produces enzymes involved in ROI scavenging, such as catalase and superoxide dismutase (reviewed in Ref. [66]).

Therefore, the host immune response does not lead to eradication of the infection but may contribute to tissue damages.

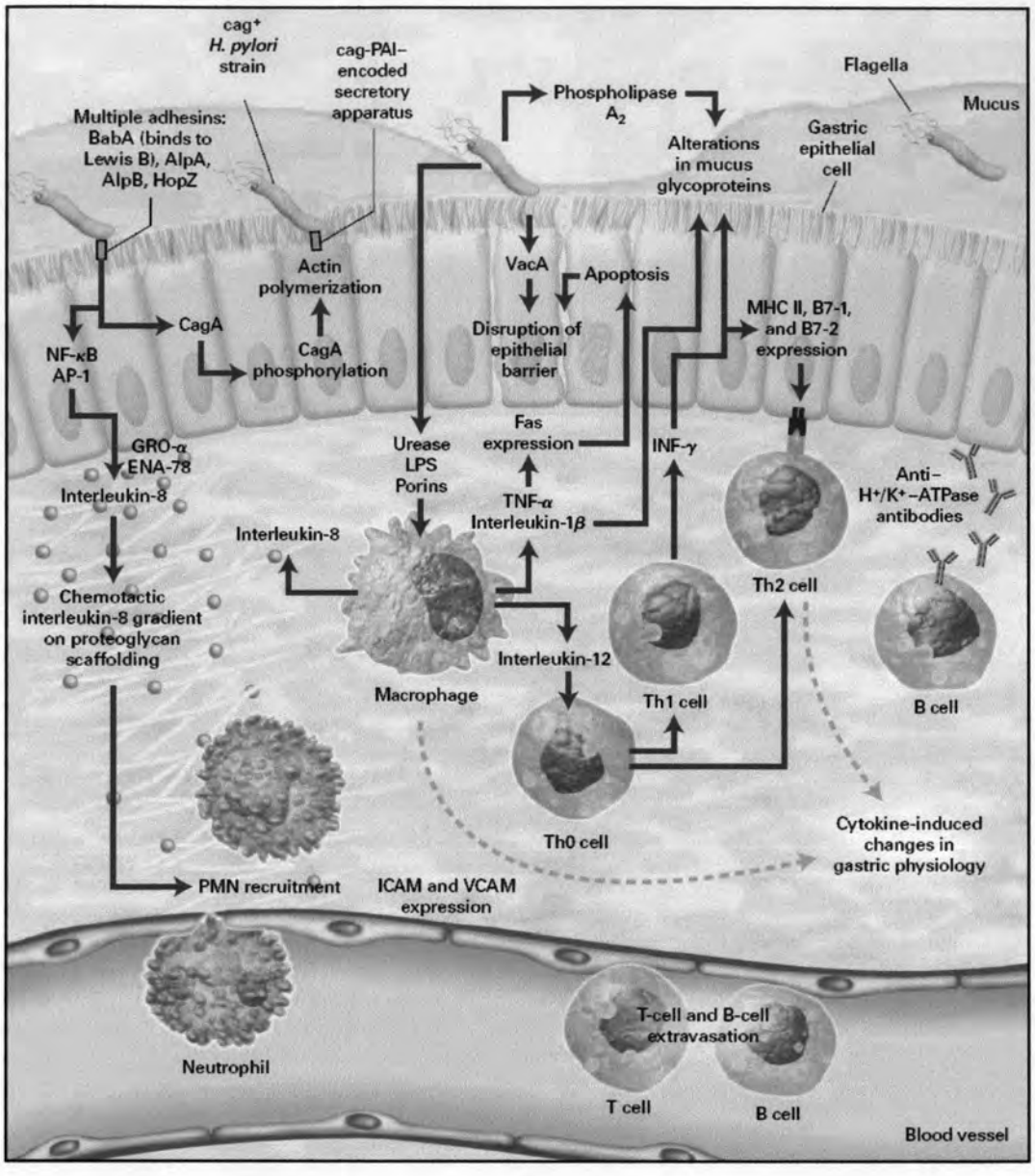


Figure 6 Pathogenesis of *H. pylori* infection [4]: The host response to *H. pylori* participates in the induction of gastric epithelial damage and therefore has an integral role in *H. pylori* pathogenesis. *H. pylori* adheres on the gastric epithelial cells by bacterial adhesion proteins. Then, virulent factors are delivered into host cells. Especially, CagA induces many pathological conditions. For example, activation of NF-ΚB that causes production of many inflammatory mediators inducing gastric inflammation.

In this experiment, host inflammatory responses were measured as the following parameters: leakage of macromolecules from gastric postcapillary venules (PCVs), serum level of vascular endothelial growth factor (VEGF), and expression of NF-ΚB subunit p65.

Vascular permeability and macromolecular leakage

H. pylori induces gastric mucosal inflammation that leads to increase of gastric microvascular permeability and leak, which result in macromolecular leakage.

Regulation of vascular permeability (the movement of fluids and molecules between the vascular and extravascular compartments)

During inflammation, many factors involve in vascular hyperpermeability. Initiately, neutrophils and other leukocytes transmigrate from the vascular compartment into the inflamed tissue space. This process is initiated by leukocytes rolling along the vessel wall, followed by firm adhesion to the wall and migration across it. The interaction between neutrophil surface protein (CD11a and CD11b) and endothelial adhesion molecules such as ICAM-1 [67] promotes neutrophil recruitment. At first glance, it may come as no surprise that neutrophil transmigration is associated with increased vascular permeability, because the migrating leukocytes leave microvessels through holes in the wall, which may also allow plasma and macromolecules to leak [68]. However, careful investigations over the last two decades have unequivocally shown that the two processes can be dissociated. Neutrophil adhesion and transendothelial migration is possible with or without plasma leakage, and plasma leakage is possible with or without transmigration. Specifically, the permeability-increasing activity of neutrophils can be inhibited by polyanions like dextran sulfate [69]. This means that the concept of leukocytes drilling holes through which plasma then escapes is incorrect; rather, neutrophils seem to have a specific way of regulating endothelial permeability. Recently, Gautam *et al.* [70] identified neutrophil heparin-binding protein (HBP) as the key and possibly only mediator involved in the permeability-enhancing activity control process as shown in the following figure.

In addition, increase microvascular permeability may be induced by other factors. For example, platelet-activating factor (PAF) [71]. Moreover, VEGF also potently induces vascular leak. Although VEGF-induced angiogenesis is often accompanied by a vascular permeability response, VEGF-induced vascular leak is not require for angiogenesis [72]. Interestingly, vascular permeability can be regulated by VEGF as well as a wide array of inflammatory mediators.

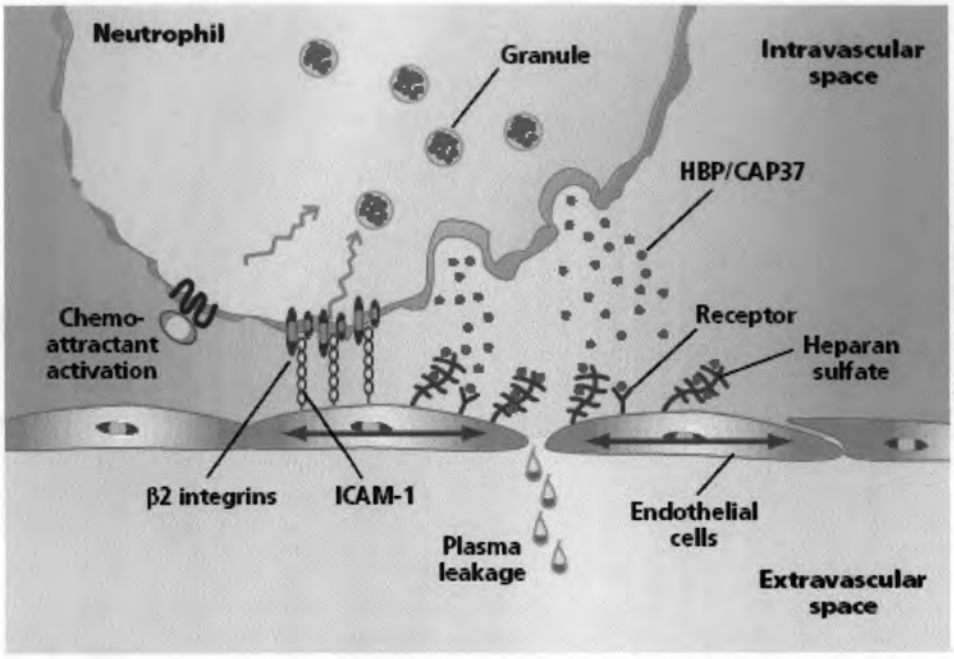


Figure 7 Macromolecular leakage induced by neutrophil extravasations: At a site of inflammation, a neutrophil is activated by a chemoattractant, and adheres to an endothelial cell through $\beta 2$ integrins (heterodimers on neutrophil surface; for example, leukocyte function associated molecule-1 (LFA-1)) becoming engaged to endothelial ligands such as ICAM-1. The engagement of $\beta 2$ integrins triggers the release of granules containing HBP/CAP37 (dots), a molecule that binds to heparan sulfate (antennalike structures on endothelial cells) and potentially to an unknown receptor. HBP/CAP37 causes endothelial cells to undergo cytoskeletal rearrangements (arrows) that lead to interendothelial gaps which allow plasma (drops) to leak into the extravascular space [73].

H. pylori-induced macromolecular leakage

Recruitments of neutrophils and leukocytes in gastric lamina propria are influenced by *H. pylori* infection. From the previous study, the expression of adhesion molecules on endothelial cells is increased from *H. pylori* infection [74]. Moreover, 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. In addition, *in vitro* examination [71] suggested that PAF increased macromolecular leakage without neutrophils. Furthermore, *H. pylori* stimulated host VEGF expression *in vitro* [77, 78] through several signalling cascades.

Therefore, because of these vascular hyperpermeability factors induced by *H. pylori* infection, the macromolecular leakage would be observed from gastric mucosal vessels.

In vivo studies of *H. pylori*-induced gastric microcircular leakage

A number of methods have been used to evaluate the process of vascular permeability in response to a variety of permeability-inducing agents [79]. The fundamental differences between the various *in vitro* assays are evident in their ability to detect the earliest significant response to a particular permeability factor. For example, vascular leak response to thrombin and VEGF ranges from 5 minutes to 2 hours [80] and 2-6 hours [81], respectively. *In vivo* assay is the Miles assay [82], which is often used to quantify vascular leak in the skin or the ear, and more recently in the lung [83]. However, the Miles assay is limited because the measured solute flux is influenced by diffusive permeability, surface area, hydraulic conductivity, capillary pressure and interstitial pressure [84].

Microscopy techniques have commonly been used to localize extravasation of macromolecular tracers of different sizes, which are labeled with heavy metals, radioisotopes, fluorescence or horseradish peroxidase. These techniques use intravital microscopy to monitor the leakage from single, isolated vessels or intact vascular beds from exteriorized thin tissues. These methods have been used to illustrate vascular leak and oedema in the brain [85], inflammation [86], and tumour-associated blood vessels [87]. In particular, transmission electron microscopy has been used to evaluate endothelial barrier properties and extravasation of ferritin in blood vessels after VEGF administration [88].

The intravital microscopy was used to detect microcirculatory changes affected from *H. pylori*. In 1993, the effects of topical administration of *H. pylori* extracts on changes in mesenteric microcirculation were detected by using intravital microscopic technique [67]. The exposed mesentery that was applied by *H. pylori* extracts resulted in increase leukocyte adhesion and emigration in venules. From those results, *H. pylori* extracts exhibited changes in rat mesenteric microcirculation. However, *H. pylori* infection was localized on the stomach, and the leukocyte involvement demonstrated within the mesentery may not be mirrored in the gastric mucosa. In 1997, Kalia *et al.* [89] studied on the gastric mucosal microcirculation changes with *H. pylori* extracts by intravital fluorescent *in vivo* microscopy. They applied *H. pylori* water extracts on rat gastric mucosa and examined the macromolecular leakage, leukocyte adherence, leukocyte rolling, and platelet activity for 90 minutes. They found that *H. pylori* induced increases in macromolecular leakage occurred after 5 minutes, and induced adherent platelet thrombi and

circulating platelet emboli after 5 and 15 minutes, respectively. Leukocyte adherence did not occur although early transient rolling was observed. However, the activated leukocytes were observed after chronic administration of *H. pylori* extracts by gavage three times daily at three-day intervals [90]. Furthermore, Kalia and co-workers [76] found that *H. pylori* water extracts activated mast cells to release proinflammatory mediators such as PAF and histamine that may increase microvascular permeability. These activated mast cells may contribute to the development of gastric mucosal damage. In 2002, the acute effects of genotypically different *H. pylori* strains on the rat gastric mucosal microcirculation were studied by intravital fluorescent *in vivo* microscopy [34]. The CagA positive strain induced significant and sustained macromolecular leakage by 5 minutes. Transient and less leakage was observed with its isogenic VacA negative mutant and other nontoxigenic strains regardless of CagA status. In addition, the significant increase in leukocyte adhesion, platelet aggregation, and postcapillary venule vasoconstriction were only observed with the CagA positive and toxigenic strain.

The microscopy technique, a useful technique, has been used to monitor and localized the leakage of macromolecules from single, isolated vessels or intact vascular beds from exteriorized thin tissues. Therefore, in this study, the intravital fluorescent microscopy technique was use to monitor the macromolecular leakage from gastric mucosal postcapillary venules (PCVs) of rats.

Vascular endothelial growth factor (VEGF)

During *H. pylori* infection, there is a strong VEGF mRNA expression. The VEGF may control the vascular permeability or long-term neoangiogenesis of *H. pylori* infection.

The originally characterized form of VEGF (referred to VEGF-A) is an approximately 34–46-kD a homodimeric glycoprotein. Two homologous VEGF receptors, fetal liver kinase (Flk-1) and *fms*-like tyrosine kinase (Flt-1), are expressed by vascular endothelial cells *in vitro* and *in vivo* beginning during early vascular embryonic development.

VEGF is a survival factor for endothelial cells (ECs), both *in vitro* and *in vivo* [91, 92]. In addition, VEGF is known also as vascular permeability factor, based on its ability to induce vascular hyperpermeability [93, 94]. It is now well established that such permeability-enhancing activity underlies significant roles of VEGF in inflammation and other pathological circumstances. VEGF induces an increase in hydraulic conductivity of isolated microvessels; this effect is mediated by increased calcium influx [95]. Furthermore, an intradermal injection of VEGF can induce vascular leakage in 5 min that is largely eliminated within 20–30 min.

Endogenous paracrine expression of VEGF adjacent to fenestrated endothelium could contribute to a persistent increase in vascular permeability such as is observed in kidney and brain choroid plexus [96]. It has been proposed that members of the Src family activating pathway mediate VEGF-dependent vascular permeability [72].

The experimental evidence suggests that the vascular leak induced by VEGF and other inflammatory mediators occurs via distinct molecular processes [85]. In any events, the process of vascular leak is quite complex, and depends on a number of variables, including the physical properties of the fluid or molecule being transported (size, charge, configuration), gradients between compartments (pressure or concentration), and the mode of transports (through channels or vesicles within an individual cells or through inter-endothelial junctions between adjacent cells) [79]. Molecules of various sizes may extravasate by distinct means, with different kinetics, and with varying degrees of clearance by lymphatic vessels [97].

Endothelial cells exposed to VEGF allow passage of particles of different sizes by a variety of physical mechanisms. VEGF can induce the formation of fenestrations, or small pores, in the thin endothelial cytoplasm, allowing leak of small solutes [98]. VEGF also induces formation of caveolae, small plasmalemmal invaginations, which allow vesicular transport of small proteins through the cytoplasm of a single endothelial cell [99]. Feng *et al.* [100] coined the term vesiculo-vacuolar organelles (VVOs) to describe the fusion of vesicles to form a channel through the endothelial cytoplasm. These mechanisms may provide a physical pathway for very small solutes to pass through a single endothelial cell, but the leak of larger proteins probably occurs at the intercellular junctions between adjacent endothelial cells. The size and numbers of these junctional gaps and pores are affected by VEGF in a dose-dependent manner that varies between vascular beds, suggesting different dose thresholds for VEGF in different tissues [101]. VEGF induces endothelial fenestration by VEGF receptor-mediated signaling pathways. To do that, VEGF receptor Flk localized in endothelial cells may determine specific signaling events after VEGF stimulation. For example, 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 [79]. Downstream signaling by VEGF is quite diverse, and includes transduction molecules such as phosphatidylinositol-3-OH kinase (PI(3)K)/Akt, Ras/Raf/MEK/Erk, Src and phospholipase Cγ (PLC-γ)/endothelial nitric oxide synthase (eNOS).

When endothelial cell-cell junction integrity is disrupted *in vivo*, the consequence is leak of serum/plasma proteins and circulating cells from the blood vessels [79]. Sites of vascular leak where underlying basement membrane is exposed can attract platelets [88] or even tumour cells [103] to a potential site for adhesion or transendothelial migration. Platelets attracted to these sites of disruption bind to von Willebrand factor, fibronectin or underlying collagen, leading to their activation. These activated platelets often deposit high levels of VEGF [104], thus creating more leaks and attracting more platelets.

Regulation of VEGF gene expression

VEGF mRNA expression is induced by exposure to low oxygen tension under a variety of pathological circumstances [105]. It is now well established that hypoxia-inducible factor (HIF)-1 is a key mediator of hypoxic responses [106]. In addition, many molecules have been implicated as positive regulators of angiogenesis, including fibroblast growth factor (FGF), transforming growth factor (TGF)-α, TGF-β, hepatocyte growth factor (HGF, or scatter factor), keratinocyte growth factor, insulin-like growth factor-1, and platelet-derived growth factor, up-regulate VEGF mRNA expression, suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment [107, 108].

A wide array of factors has been implicated in the VEGF-induced permeability response (reviewed in Ref [79]). Interestingly, inflammatory cytokines such as IL-1β, IL-6 TNF-α, angiogenin, IL-8, and angiopoietins induce expression of VEGF in several cell types. This observation is in agreement with the hypothesis that VEGF may be a mediator of angiogenesis and permeability in inflammation disorders [108, 109].

A VEGF gene expression has been showed to be regulated through several signaling cascades, including MAP kinase, c-Jun N-terminal kinase (JNK), and Extracellular signal-regulated kinase (ERK) as well as NF-KB pathways [110-112].

H. pylori-induced VEGF expression

H. pylori infection induces VEGF production in gastric epithelial cells. Strowski and co-workers [78] found that *H. pylori* potently up-regulates production and release of VEGF-A as well as *vegf-A* mRNA levels via Mitogen-activated protein kinase/Extracellular signal-regulated kinase kinase (MEK)>ERK-1/-2 kinase cascade, and they provided strong evidence that enhanced recruitment of Sp1 and Sp3 transcription factors to two proximal GC-rich *vegf-A* promoter elements mediates *H. pylori*-triggered *vegf-A* gene expression. Furthermore, amplification of Ras

lead to VEGF up-regulation [113]. In 2005, study of Brandt *et al.* [53] indicated that *H. pylori* CagA caused activation of Ras signalling pathway that activate NF-KB. VEGF is also produced by NF-KB activation [110-112]. In addition, Mueller *et al.* [114] found that gastric epithelial cells, especially mucus-producing cells, of *H. pylori*-infected mice expressed VEGF mRNA that peaked on fourteen days after infection. The following figure shows the regulation of host VEGF expression by *H. pylori*.

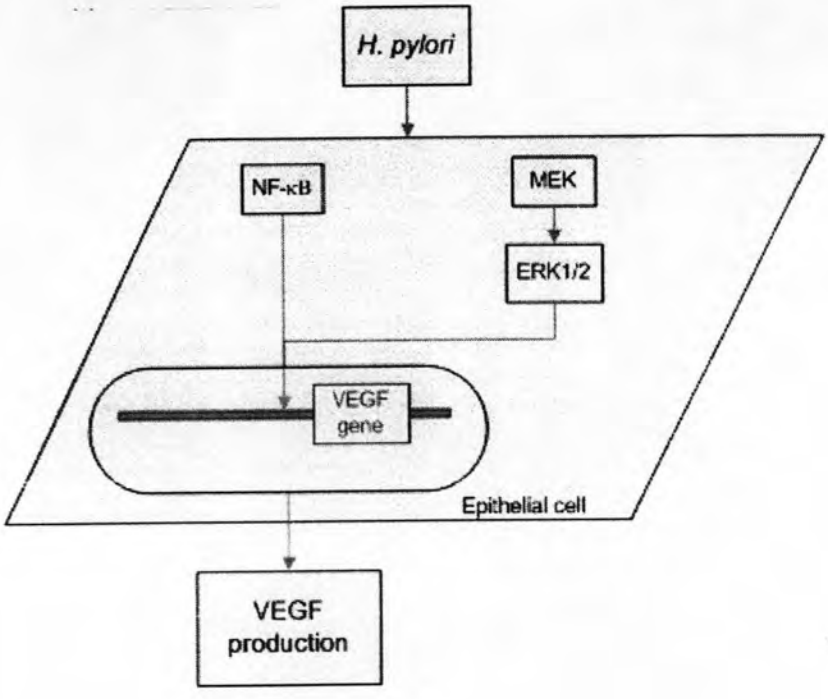


Figure 8 Regulation of VEGF expression: *H. pylori* stimulates signal transduction in gastric epithelial cell that evokes NF-KB as well as MEK>ERK1/2 activation. These are crucial for transmission of the *H. pylori*-dependent effects to VEGF gene.

Enzyme-linked immunosorbant assay (ELISA) studies of VEGF level

The VEGF level was commonly monitored by using ELISA technique. Caputo *et al.* [77] used ELISA technique to show that *H. pylori*-VacA strain led to VEGF expression in *in vitro* study through an epidermal growth factor receptor. They measured VEGF by using a commercially available sandwich VEGF ELISA kit from R&D Systems Company. In addition, this commercial kit was used for monitoring VEGF level in cancer cell line of IL-1β-induced VEGF production [115].

Moreover, serum VEGF level, by ELISA technique, of acetic acid-induced ulcer was studied in *in vivo* experiment [116, 117]. The researchers found that serum VEGF significantly increased in acid-induced ulcer group compared with no ulcer group. They suggested that the serum VEGF increase is consistent with a need for increased angiogenesis in an effort to heal the ulcer.

By the ELISA technique, VEGF level was monitored in the rat serum. Therefore, in this study, we used ELISA technique to quantified rats serum VEGF level.

Nuclear factor-kappa B (NF-KB)

H. pylori infection immediately induces NF-KB up-regulation as shown in the figure 6. This transcription factor leads to transcriptional regulation of many proinflammatory genes.

NF-KB transcription factors form homodimers and heterodimers with DNA binding site, called KB sites, to regulate the expression of many genes. Various intracellular pathways induced by a many of biological factors and environmental conditions can activate the NF-KB dimer by signaling degradation of the NF-KB inhibitor, IKB proteins. IKB degradation uncover a nuclear localization sequence (NLS) in each subunit of the NF-KB dimer and allow the dimer to translocate from cytoplasmic location into the nucleus. In the nucleus, NF-KB modulates the expression of many genes including cytokines, growth factors, acute phase response proteins, immunoreceptors, other transcription factors, cell adhesion molecules, viral proteins, and regulators of apoptosis [118].

The mammalian NF-KB protein family members, including p50, p52, c-Rel, RelA (p65), and RelB, share an N-terminal domain called the Rel homology (RH) domain. The RH domain contains sequences responsible for dimerization, DNA binding nuclear localization and IKB binding. The classic form of activated NF-KB is a heterodimer consisting of one p50 and one p65 subunit [119].

Regulation of NF-KB

NF-KB regulation can occur at multiple levels as following; (1) dimerization, (2) inhibition by IKB (and thus control of its nuclear translocation), (3) DNA binding, (4) interaction with other transcriptional co-activators, and (5) interaction with the basal transcriptional machinery [119].

At the level of inhibition by IKB, most agents that induce IKB degradation, including tumor necrosis factor alpha (TNF-α), IL-1, bacterial lipopolysaccharide (LPS), viral double stranded RNA, and ionizing radiation, accomplish complicate degradation via an IKB-kinase (IKK) enzyme. The IKK is an unusual kinase in which contains two related kinases, IKK-α and IKK-β, that are active as a dimer. Activation of IKK leads to the phosphorylation of two specific serines near the N terminus of IKB-α, which targets IKB-α for ubiquitination and degradation by the proteasome [120]. The IKB-α contains both an NLS and a nuclear export sequence (NES) such that the signal can be abrogated by newly-synthesized IKB-α entering the nucleus, removing NF-KB dimers from DNA, and causing their exporting-mediated transport to the cytoplasm [121, 122].

The active NF-KB transcription factor promotes the expression of over 150 target genes. The majority of proteins that are encoded by NF-KB participate in the host immune response. For example, cytokines and chemokines, as well as receptors required for immune recognition, such as MHC molecules, proteins involved in antigen presentation and receptors required for neutrophil adhesion and transmigration across blood vessel walls [118].

H. pylori–induced NF-KB activation

In fact, several studies have shown that *H. pylori* can induce the activation of NF-KB in human gastric cancer cell lines using an electrophoretic mobility shift assay (EMSA) [123-125]. Moreover, Keates *et al.* [124] demonstrated that *H. pylori* activates NF-KB in the epithelium, followed by increased levels of IL-8 mRNA and protein. It is well documented that cagA of *H. pylori* can activate NF-KB and *H. pylori* cagA infection often causes chronic atrophic gastritis accompanied by intestinal metaplasia and dysplasia [126-128]. In addition, Mueller and co-workers [114] used laser microdissection and cDNA microarray technique, and found that NF-KB gene expressed in gastric epithelial cells of *H. pylori* infected mice at early and throughout time course (28 days) of infection.

Immunohistochemical studies of NF-KB p65 expression

The microbial lipopolysaccharide (LPS) and cytokines such as TNF and IL-1 result in translocation of the NF-KB (p50-p65) dimer into the nucleus and activation of inflammatory and cell survival responses.

Many reports [124, 129-131] studied NF-KB p65 expression in *H. pylori* infected patient by incubating stomach sections with NF-KB p65 subunit antibody from Santa Cruz

Biotechnology, INC. In immunohistochemistry, this antibody binds to an epitope on the p65 subunit of NF-ΚB, which is exposed only after IΚB dissociation. Thus, a p65 antibody will only recognize NF-ΚB in its activated state [124]. They found immunoreactivity signal, that mean activated NF-ΚB p65 expression, with brown color in nucleus and cytoplasm of gastric epithelial cells and in nucleus of lamina propria immune cells. After antimicrobial therapy, staining was greatly diminished in epithelial.

In the study of Zang and co-workers [129], they examined NF-ΚB p65 expression (used sc-109, NF-ΚB p65 antibody, Santa Cruz) before and after antibiotic treatment on *H. pylori* infected patients. They found the strong reduced expression of p65 subunit in gastric epithelial cells but yet retained expression in lamina propria immune cell. Moreover, the uses of sc-109 antibody were reported in many studies of gastric cancer patients [128, 132, 133].

Takahashi and his team [134] studied NF-ΚB p65 expression in gastric fibroblast cells isolated from acid-induced ulcer in rats. Immunohistochemistry showed that NF-ΚB p65 activation in the nucleus of fibroblast, macrophage/monocyte, and neutrophil. In the recently report [135], the researcher evaluated NF-ΚB p65 activation in the *H. pylori*-infected gastric mucosa of mice by immunofluorescent staining using anti p65 (sc-109). Furthermore, they examined the expressions of proinflammatory cytokines with inhibition of NF-ΚB pathway by using phosphorothioate antisense and sense oligonucleotide against the NF-ΚB p65. In their results, they found that pretreatment with NF-ΚB p65 antisense oligonucleotide inhibited the activation of NF-ΚB and the expressions of TNF-α and IL-1 in *H. pylori*-infected gastric mucosa. Moreover, sense oligonucleotide did not influence on the expression of proinflammatory cytokines.

By immunohistochemistry technique, NF-ΚB p65 expression has been monitored in the tissues. In addition, this technique can identify the activation of NF-ΚB by monitoring the expression of NF-ΚB p65. Therefore, in this study, immunohistochemistry was used to examine the expression of NF-ΚB p65 in the rat gastric epithelial cells.

Curcumin (diferuloylmethane)

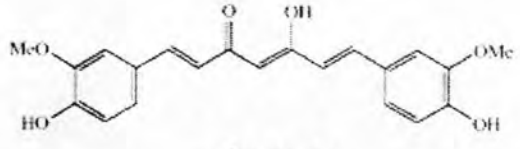
Curcumin (diferuloylmethane), the natural yellow pigment in tumeric, is isolated from the rhizomes of the plant *Curcuma longa* Linn. (*C. longa* L.).

C. longa

C. longa belongs to the Zingiberaceae family, a perennial herb that measures up to 1 meter high with a short stem, and is distributed throughout tropical and subtropical regions of the world. *C. longa* is widely cultivated in Asian countries, mainly in India and China. Its rhizomes are oblong, ovate, pyriform, and often short-branched. The rhizomes are a household remedy in Nepal [136]. As a powder, called turmeric, it is bright yellow and has been used as a coloring agent in food in the United States. In India, it has been used for centuries as a spice and a food preservative, and also for its various medicinal properties. The current traditional Indian medicine claims the usage of tumeric against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism, and sinusitis [137].

Active ingradients of tumeric

In the 19th century, there has been considerable interested in the active compounds in tumeric called curcuminoids. The major curcuminoid is called curcumin (diferuloylmethane), which makes up approximately 90% of the curcuminoid content in tumeric, followed by demethoxycurcumin and bisdemethoxycurcumin. [7]. The chemical structure of curcumin was determined by Roughley and Whiting (figure 9) [138].



Curcumin

Figure 9 Chemical structure of curcumin (diferuloylmethane)

Pharmacokinetic study and safety

Curcumin is dissolved in organic solvents such as Dimethylsulfoxide (DMSO), oil, alcohol, and petroleum agents. Interestingly, curcumin has been demonstrated the safety in human and animals. Human appeared to be able to tolerate high doses of curcumin without significant

side-effects. A phase 1 study by Cheng *et al.* [139], found no adverse effects of curcumin ingestion for 3 months of doses up to 8000 mg/day. Other human studies of curcumin included the following: a double-blinded, crossover trial in 18 patients with rheumatoid arthritis [140], a randomized, placebo-controlled trial with 45 postsurgical patients [141]. The doses of curcumin in these studies ranged from 1125 mg/day to 2500 mg/day. Only one postsurgical patient reported mild transient giddiness. No other adverse reactions were reported, including any changes in blood chemistry reports. Thus curcumin appears to be safe in human even using at high doses.

In animals, the previous study demonstrated that curcumin is rapidly metabolized and poorly absorbed in Sprague-Dawley rats. Administrating curcumin orally were made by Wahlström and Blennow [142]. They demonstrated that this compound in a dose of 1 to 5 g/kg BW given to rats apparently did not cause any adverse effects and it was excreted about 75% in the feces, while traces appeared in the urine. In addition, measurements of blood plasma levels and biliary excretion showed that curcumin was poorly absorbed by the gastrointestinal tract. Curcumin was disappeared within 30 min after adding to microsomes suspensions or hepatocyte suspensions. Furthermore, it was capable of disappearing from the blood after intravenous or after addition to the liver perfusion system. Moreover, oral LD_{50} was found to be 12.2 g/kg BW in rats [143]. In addition, a study in which rats were fed with curcumin 1.8 g/kg BW per day for 90 days and monkeys were fed with curcumin 0.8 mg/kg BW per day for 90 days showed no adverse effects [144].

Activities of curcumin

Hundreds of *in vitro* and animal studies have been published describing antioxidant, anti-inflammation, anti-protozoa, anti-bacteria, nematocidal activity, anti-venom, anti-HIV, and anti-tumor properties of curcumin [10, 13, 145-147].

Antibacterial activity

Bhavani Shankar and Murthy [148] investigated the activity of turmeric fractions against some intestinal bacteria *in vitro*. In this work, curcumin at the dose of 2.5-50 mg/ml inhibited *Staphylococcus aureus*. Mahady *et al.* [9] found that both of methanol extract of dried powder tumeric rhizome and curcumin inhibited *H. pylori* growth, with significantly activity against the CagA positive strains.

Anti-inflammatory activity

In previous studies, there are two models of inflammation to be studied. First, chronic models (cotton pellet and granuloma pouch), where the inflammation and granuloma

development during a period of time (several days), indicating the proliferative phase of inflammation. Second, acute models, where acute effects of anti-inflammatory agents can be studied, testing their inhibitory action on the development of rat paw edema.

Mukophadhyay *et al.* [149] demonstrated the activity of curcumin and other semi-synthetic analogues in carrageenin-induced rat paw edema and cotton pellet granuloma models of inflammation in rats. In these experiments the authors used ferulic acid and phenylbutazone (reference drug) as a treatment. Curcumin and its analogues showed similar action to the reference drug, potent anti-inflammatory in the chronic model of inflammation and in carrageenin-induced paw edema in rats. Among the curcumin analogues, triethyl-curcumin was the most, when compared with the others and with the drug reference, whereas tetrahydro-curcumin showed no activity in chronic model. In the acute inflammation condition, all the substances were more effective. The authors concluded that the activity of the compounds used in these experiments, would depend on the model of inflammation.

Moreover, Arora *et al.* [143] investigated the anti-inflammatory activity in different fractions of the petroleum ether extract of the rhizomes of turmeric (two constituents) in animals. They found that the extracts reduced the granuloma growth and no toxic effects were observed. Srimal and Dhawan [150] found that curcumin inhibited the carrageenan-induced edema in rats in dose range of 20-80 mg/kg BW and has a lower ulcerogenic index. Besides, Blood pressure and respiration of anaesthetized rats were not affected by curcumin.

Curcumin down-regulates the NF-KB.

In many studies showed that curcumin is an anti-inflammatory substance because it can inhibit the activation of the major transcription factor NF-KB. This transcription factors required for the expression of many genes linked with cell proliferation and host immune response [118]. Duvoix *et al.* [151] described this effect in K562 leukemia cells in which curcumin strongly inhibits TNF-α-induced NF-KB and binding to the corresponding target sequences on glutathione S-transferase P1-1 (GSTP1-1) gene promoter or consensus binding sites. Bharti *et al.* [152] discovered that inhibition of IKK complex blocks both IKB-α phosphorylation as well as NF-KB p65 translocation and thus leads to NF-KB inhibition. Many reports confirmed these results and published that curcumin inhibits IL-1α-, TNF-α-, 12-O-tetradecanoylphorbol-13-acetate (TPA)-, lipopolysaccharide (LPS)- and thrombin-induced NF-KB activation. [153-159].

NF-KB inhibition by curcumin is certainly an interesting strategy against diseases such as the pathogenesis of alcoholic liver disease, in which NF-KB is activated [160]. In addition, NF-KB is an important transcription factor implicated in proangiogenic genes. Curcumin showed cancer chemoprevention by down regulation of proangiogenic genes, such as VEGF gene [161, 162]. In ovarian cancer study, NF-KB signaling blockade significantly inhibited *in vitro* and *in vivo* expression of two major proangiogenic molecules, VEGF and IL-8. The- decreased expression of VEGF and IL-8 directly correlated with decreased tumorigenicity, decreased vascularization of lesions, decreased formation of malignant ascites, and prolonged survival of mice. These findings suggest that inhibition of NF-KB activity can suppress angiogenesis and progressive growth [163].

Interestingly, Foryst-Ludwig and co-workers [164] studied on *H. pylori*-infected gastric epithelial cells and suggested that curcumin, not a toxic agent on cell culture, can inhibit NF-KB activation and IL-8 production. In addition, curcumin inhibited *H. pylori*-induced scatter cells.

Curcumin down-regulates the enzymes involved in inflammation.

Interestingly, it was published that curcumin inhibits cyclooxygenase 2 (COX-2) as well as lipoxygenase (LOX), two enzymes involved in inflammation [165]. Indeed, cytokine-induced COX-2 transforms arachidonic acid in prostaglandins during acute inflammatory episodes. COX-2 is also the prevalent isoform during chronic inflammations. Curcumin at the doses of 100 and 200 mg/kg of body weight (BW) inhibited the granuloma formation. Moreover, treatment of the animals with 200 mg/kg BW of curcumin for 4 days reduced the prostaglandin-E_2 (PGE-2) content [166]. In addition, LOX transforms arachidonic acid in leukotrienes, which take part in leukocytes recruiting and play a role in inflammation [167]. Besides, curcumin down-regulates leukocyte adhesion. Curcumin blocked the attachment of monocytes to endothelial cells by inhibiting the expression of endothelial cell adhesion molecules ICAM-1, VCAM-1, and ELAM-1 [168]. Furthermore, this yellow substance can suppress inflammatory cytokines TNF-α and IL-1 [169, 170] that disturb endothelial cell and induced expression of adhesion molecules [115, 171].

Curcumin and gastrointestinal tract

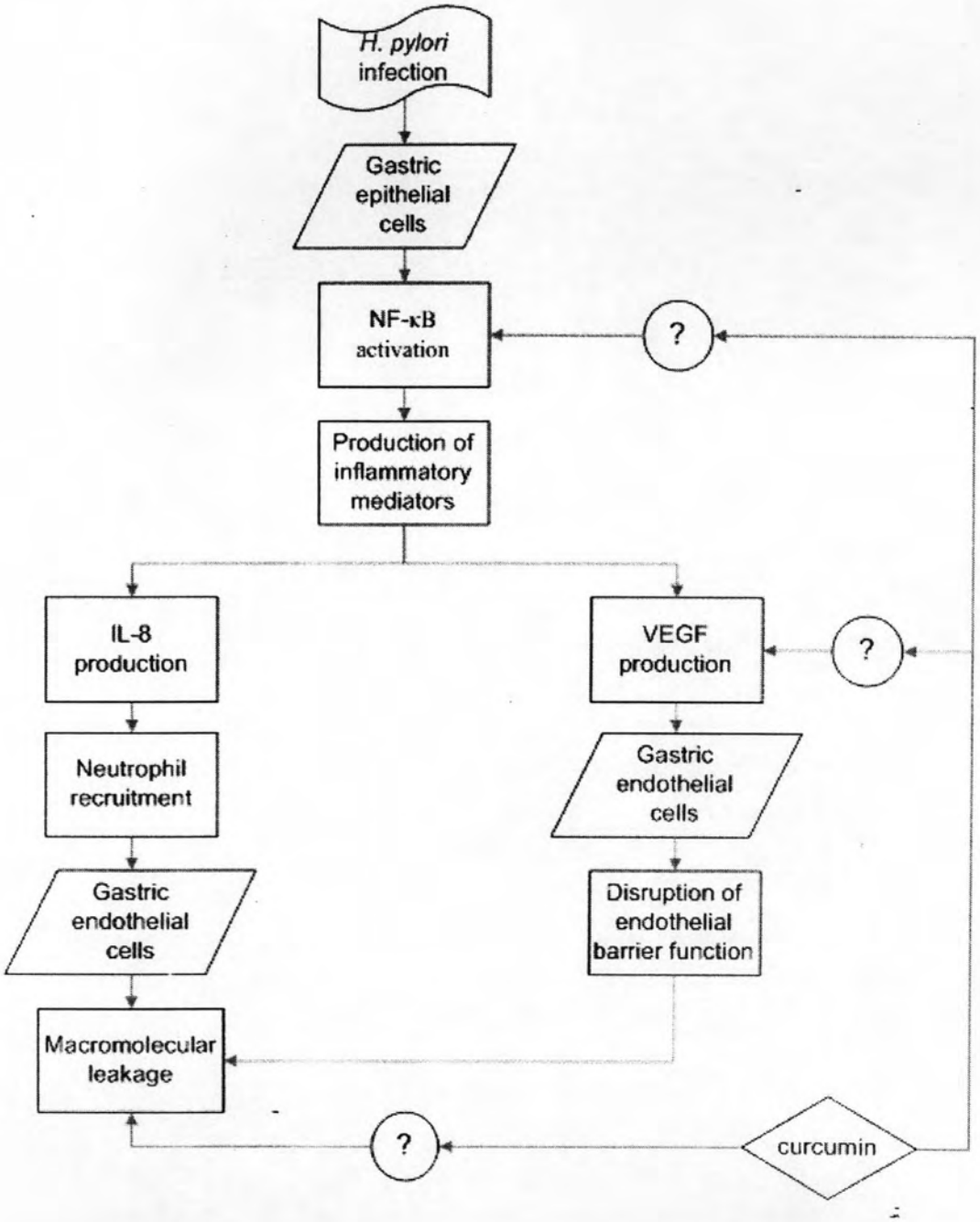
Curcumin protects the gastrointestinal tract against irritants. Pancreatitis improves after curcumin treatment, which blocks key inflammatory signals [172]. Indomethacin-induced ulcer in rats were orally treated with tumeric suspended in 10% propylene glycol (0.25, 0.5, or 0.75 g/kg

BW) for 3 days. Tumeric at dose 0.5 g/kg BW showed protecting and enhancing healing of gastric ulcer [173]. In 2000, Chuang *et al.* [174] showed that curcumin at concentrations of 200 mg/kg BW or 600 mg/kg BW could effectively inhibit diethylnitrosamine-induced liver inflammation in rats. Other interesting action of curcumin was demonstrated by Park *et al.* [175]. Curcumin inhibited liver injury in carbon tetrachloride (CCl_4) induced hepatotoxicity in rats.

From various *in vitro* and animal studies, they point to the possible mechanisms of the anti-inflammatory activity of curcumin. In previous studies, there are acute and chronic inflammation models that were treated with curcumin at various doses, solvents, and treatment days. However, there is rarely *in vivo* study of anti-inflammation property of curcumin on gastric inflammation. This study used curcumin doses from the *in vivo* experiment of anti-inflammation property of curcumin on diethylnitrosamine-induced liver inflammation [174]. Therefore, curcumin at dose 200 mg/kg BW or 600 mg/kg BW were used for identification of anti-inflammation property. Furthermore, the concentration of DMSO maximally, 0.1% v/v did not interfere with cell viability [166]. Therefore our study used **0.1%** v/v DMSO as the curcumin solvent.

In consider to the literature view from above, it might be said that *H. pylori*, a gastrointestinal pathogen, induces gastric inflammation by activating transcription factor NF-KB in gastric epithelial cells. Then, the production of inflammatory mediators including IL-8 and VEGF has been occurred. The macromolecular leakage is evoked by IL-8-induced neutrophil infiltration. Moreover, VEGF may involve in the macromolecular leakage by stimulating disruption of endothelial barrier function. Interestingly, curcumin, an anti-inflammatory substance, may improve *H. pylori*-induced gastric inflammation by preventing the macromolecular leakage. With this idea, curcumin could reduce NF-KB activation as well as VEGF production. Therefore, this present study is desired to evaluate effects of curcumin on *H. pylori*-induced gastric inflammation by monitoring the macromolecular leakage from gastric PCVs, serum VEGF level, and NF-KB subunit p65 expression in gastric epithelial cells as shown in the following conceptual framework and research objectives.

Conceptual framework



The objectives of this study are:

To examine the effects of curcumin on *H. pylori*-induced the following changes:

1. Macromolecular leakage of PCVs at 0 and 30 minute after fluorescence injection
2. Serum VEGF level
3. NF-KB p65 expression in infected area of gastric epithelial cells