**EFFECTS OF *ALOE VERA* ON CHANGES OF GASTRIC MICROCIRCULATION, TNF - ALPHA, AND IL - 10 LEVELS IN *HELICOBACTER PYLORI* INFECTED RATS**

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รัศมี ปราบโจร : ผลของว่านหางจระเข้ต่อการเปลี่ยนแปลงของระบบไหลเวียนเลือดขนาดเล็กในกระเพาะอาหาร และระดับของทีเอ็นเอฟ–แอลฟา และไอแอล–10 ในหนูขาวที่มีการติดเชื้อเฮลิโคแบคเตอร์ไพโลไร (EFFECTS OF *ALOE VERA* ON CHANGES OF GASTRIC MICROCIRCULATION, TNF-ALPHA, AND IL-10 LEVELS IN *HELICOBACTER PYLORI* INFECTED RATS) อ. ที่ปรึกษา : รศ.พญ. ดวงพร ทองงาม, อ.ที่ปรึกษาร่วม : รศ.ดร. สุทธิลักษณ์ ปทุมราช จำนวนหน้า 118 หน้า. ISBN 974-53-1140-5.

การติดเชื้อเฮลิโคแบคเตอร์ไพโลไร เป็นสาเหตุของกระเพาะอาหารอักเสบ และการหลั่งของสารไซโตไคน์ ว่านหางจระเข้เป็นพืชสมุนไพรที่มีผลต่อต้านการอักเสบ จุดประสงค์ของการศึกษานี้เพื่อศึกษาผลของการติดเชื้อเฮลิโคแบคเตอร์ไพโลไรต่อการเกาะติดของเม็ดเลือดขาว ระดับของซีรั่มทีเอ็นเอฟ-แอลฟา และไอแอล-10 และศึกษาผลในการต่อต้านการอักเสบของว่านหางจระเข้ต่อการเปลี่ยนแปลงดังกล่าว การทดลองใช้หนูพันธุ์ Spraque-Dawley เพศผู้ จำนวน 36 ตัว แบ่งหนูเป็น 3 กลุ่ม คือ กลุ่มควบคุม กลุ่มติดเชื้อเฮลิโคแบคเตอร์ไพโลไร และกลุ่มติดเชื้อเฮลิโคแบคเตอร์ไพโลไรที่ได้รับการรักษาด้วยว่านหางจระเข้ หลังติดเชื้อนาน 2 สัปดาห์ หนูแต่ละกลุ่มถูกแบ่งออกเป็น 2 กลุ่มย่อยสำหรับใช้ศึกษาผลในวันที่ 3 และ 8 หลังการรักษาด้วยว่านหางจระเข้ (200 มิลลิกรัมต่อน้ำหนักตัว 1 กิโลกรัม ป้อนทางปาก วันละ 2 ครั้ง) โดยนำเทคนิคทางอินทราไวทัล ฟลูออเรสเซนต์ไมโครสโคปี้ ดูการเกาะติดของเม็ดเลือดขาวบนผนังเอ็นโดทีเลียมของหลอดเลือดดำฝอย และเทคนิคทางอีไลซ่าดูระดับของทีเอ็นเอฟ-แอลฟา และไอแอล-10

ผลการทดลองแสดงว่า กลุ่มติดเชื้อเฮลิโคแบคเตอร์ไพโลไร การเกาะติดของเม็ดเลือดขาว (วันที่ 3 เท่ากับ 10.10 ± 0.67; วันที่ 8 เท่ากับ 13.40 ± 1.00 เซลล์ต่อภาพ; $p<0.01$) และระดับของทีเอ็นเอฟ-แอลฟา (วันที่ 3 เท่ากับ 61.98 ± 18.74; วันที่ 8 เท่ากับ 76.76 ± 23.18 พิโคกรัมต่อมิลลิลิตร; $p<0.05$) เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับกลุ่มควบคุม (การเกาะติดของเม็ดเลือดขาว วันที่ 3 เท่ากับ 2.23 ± 0.48; วันที่ 8 เท่ากับ 2.47 ± 0.25 เซลล์ต่อภาพ) (ทีเอ็นเอฟ-แอลฟา วันที่ 3 เท่ากับ 8.65 ± 1.79; วันที่ 8 เท่ากับ 9.92 ± 2.62 พิโคกรัมต่อมิลลิลิตร) การรักษาด้วยว่านหางจระเข้ช่วยลดการเกาะติดของเม็ดเลือดขาว (วันที่ 3 เท่ากับ 5.81 ± 0.63; วันที่ 8 เท่ากับ 5.45 ± 0.51 เซลล์ต่อภาพ; $p<0.01$) และระดับของทีเอ็นเอฟ-แอลฟา (วันที่ 3 เท่ากับ 14.52 ± 5.53; วันที่ 8 เท่ากับ 26.31 ± 6.38 พิโคกรัมต่อมิลลิลิตร; $p<0.05$) ได้อย่างมีนัยสำคัญทางสถิติ ขณะที่ระดับของไอแอล-10 ในทุกกลุ่มไม่แตกต่างกัน การศึกษานี้แสดงให้เห็นว่าการติดเชื้อเฮลิโคแบคเตอร์ไพโลไรทำให้การเกาะติดของเม็ดเลือดขาว และระดับทีเอ็นเอฟ-แอลฟาเพิ่มขึ้น ว่านหางจระเข้ช่วยลดการเปลี่ยนแปลงดังกล่าวได้ โดยการลดระดับของสารที่ก่อให้เกิดการอักเสบ

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KEY WORD: *HELICOBACTER PYLORI* / GASTRIC MICROCIRCULATION / TNF-α / IL-10 / *ALOE VERA*



RATSAMEE PRABJONE: EFFECTS OF *ALOE VERA* ON CHANGES OF GASTRIC MICROCIRCULATION, TNF-ALPHA, AND IL-10 LEVELS IN *HELICOBACTER PYLORI* INFECTED RATS. THESIS ADVISOR: ASSOC. PROF. DUANGPORN THONG-NGAM, MD. THESIS COADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D. 118 pp. ISBN 974-53-1140-5.



*Helicobacter pylori (H.pylori)* infection causes gastric inflammation and the release of cytokines. *Aloe vera* is the plant medicine that has strong anti-inflammatory effect. The aims of this study were to investigate the effects of *H.pylori* infection on leukocyte adhesion, serum TNF-alpha, and IL-10 levels, and to assess the anti-inflammatory effects of *Aloe vera* on those changes. Thirty-six male Spraque-Dawley rats were divided into 3 groups include control group, *H.pylori* infection group, and *H.pylori* infection with *Aloe vera*-treated group. Two weeks after inoculation animals from each group were divided into 2 subgroups of 3 or 8 days treatment with *Aloe vera* (200 mg/kg BW by gavage twice daily). Intravital fluorescence microscopic technique was performed to examine leukocyte adhesion on endothelium of postcapillary venules. ELISA technique was used to examine serum TNF-alpha and IL-10 levels.

The result showed that in *H.pylori* infection groups leukocyte adhesion (Day 3 = 10.10 ± 0.67; Day 8 = 13.40 ± 1.00 cells/field; $p<0.01$) and TNF-alpha (Day 3 = 61.98 ± 18.74; Day 8 = 76.76 ± 23.18 pg/ml; $p<0.05$) were significantly increased when compared with the control groups (leukocyte adhesion; Day 3 = 2.23 ± 0.48; Day 8 = 2.47 ± 0.25 cells/field) (TNF-alpha; Day 3 = 8.65 ± 1.79; Day 8 = 9.92 ± 2.62 pg/ml). Treatment with *Aloe vera* could significantly reduce amount of leukocyte adhesion (Day 3 = 5.81 ± 0.63; Day 8 = 5.45 ± 0.51 cells/field; $p<0.01$), and significantly reduce TNF-alpha level (Day 3 = 14.52 ± 5.53; Day 8 = 26.31 ± 6.38 pg/ml; $p<0.05$). While IL-10 level has no significant difference in all groups. The results of this study could be concluded that *H.pylori* infection induced increase of leukocyte adhesion, as well as TNF-alpha. *Aloe vera* could reduce those changes through the reduction of pro-inflammatory cytokine.


Field of study…Physiology…. Student's signature…………………………………….

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Co-advisor's signature…………………………………

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# LIST OF ABBREVIATIONS

| Abbreviation | | Meaning |
|---|---|---|
| BW | = | Body weight |
| $^{o}C$ | = | Degree celsius |
| CagA | = | Cytotoxin-associated gene product A |
| CFU/ml | = | Colony forming unit per milliliter |
| cm | = | Centimeter |
| $CO_2$ | = | Carbon dioxide |
| CV | = | Collecting venue |
| DBP | = | Diastolic blood pressure |
| ECs | = | Endothelial cells |
| ELISA | = | Enzyme-linked immunosorbent assay |
| g | = | Gram |
| GBF | = | Gastric blood flow |
| HCl | = | Hydrochloric acid |
| H&E | = | Hematoxylin-eosin |
| hrs | = | Hours |
| HR | = | Heart rate |
| Hsp | = | Heat shock protein |
| HUVECs | = | Human umbilical vein endothelial cells |
| ICAM-1 | = | Intercellular adhesion molecules-1 |
| IL | = | Interleukin |
| iNOS | = | Inducible nitrix oxide synthase |
| ip | = | Intraperitoneal injection |
| kDa | = | Kilo dalton |
| kg | = | Kilogram |
| LPS | = | Lipopolysaccharide |
| m | = | Meter |

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| | | |
|---|---|---|
| M | = | Molar |
| MALT | = | Mucosa-associated lymphoid tissue |
| MAP | = | Mean arterial blood pressure |
| MC | = | Muscle capillary |
| mg | = | Milligram |
| mmHg | = | Millimeter of mercury |
| ml | = | Milliliter |
| mm | = | Millimeter |
| MV | = | Muscle venule |
| MML | = | Macromolecular leakage |
| ηm | = | Nanometer |
| NSS | = | Normal saline solution |
| OD | = | Optical density |
| OipA | = | Outer membrane inflammatory protein |
| PAF | = | Platelet activating factor |
| PBS | = | Phosphate buffer saline |
| PG | = | Prostaglandin |
| PMN | = | Polymorphonuclear leukocyte |
| ROI | = | Reactive oxygen intermediate |
| ROS | = | Reactive oxygen species |
| SA | = | Small artery |
| SBP | = | Systolic blood pressure |
| SMA | = | Submucosal arteriole |
| SMV | = | Submucosal venule |
| SV | = | Submucosal small vein |
| TNF | = | Tumor necrosis factor |
| TX | = | Thromboxane |
| μl | = | Microliter |
| μm | = | Micrometer |

VacA = Vacuolating Cytotoxin

VCAM-1 = Vascular cell adhesion molecules-1

wt = Weight

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# CHAPTER I

# INTRODUCTION

*H.pylori* infection is the most common gastrointestinal bacterial disease worldwide. It is the principle cause of chronic gastritis, and many of the disease associated with gastritis are also associated with *H.pylori*. From epidemiological data, it has shown that in developing countries there is a high incidence of *H.pylori* infection in people ranging from 13% to 70% in the under 20 year-old age group and from 70% to 94% in the over 30 years old (Malfertheiner et al., 1996; Pounder and Ng, 1995). Infection by this bacterium, patients were suffering from gastric ulcer, duodenal ulcer, or chronic gastritis. The symptoms are include; indigestion, heartburn, decrease appetite, nausea, vomiting, abdominal pain under the ribs, hunger pains between meals and in the morning, gas or bloating, acid reflux, and bleeding which may lead to anemia.

Local responses from gastric mucosa after *H.pylori* infection include the inflammatory processes, antigen challenge with overproduction of chemical mediators, activation of leukocyte and endothelial cell (EC) interaction, and alteration in circulating cytokines. These could develop to chronic inflammation and many diseases associated with *H.pylori* gastritis such as peptic ulcer, atrophic gastritis, gastric cancer, and lymphoma (Harford et al., 2000).

Acute gastritis associated with *H.pylori* is characterized by infiltration of lamina propria with inflammatory cells, enhanced release of proinflammatory cytokines such as interleukins (IL), for instance IL-1β

and IL-8, and tumor necrosis factor-α (TNF-α) (Fan et al., 1994; Crabtree, 1996) as well as the generation of reactive oxygen species (Davis et al., 1994; Drake et al., 1994). TNF-α and IL-1β are potent inducers of IL-8 production and up regulation of neutrophil expression of CD11b/CD18 (Crowe et al., 1995). The latter enhances ICAM-1-dependent neutrophil adherence. These are responsible for a positive feedback loop through the stimulatory actions of chemical mediators on neutrophil chemotaxis and activation (Graham et al., 1999). Later the pathology could develop to chronic inflammation, which characterized by infiltration of chronic inflammatory cells such as monocyte, B lymphocyte, and T lymphocyte. Interestingly, activated B and T lymphocytes could produce IL-10. *In vitro*, IL-10 showns potent anti-inflammatory properties include inhibition of synthesis and gene expression of TNF-α, IL-1, IL-6, and IL-8 (Fiorentino et al., 1991; de Waal Malefyt et al., 1991). Hence, IL-10 seems to be an important role to down regulating inflammatory response (Bodger et al., 1997).

The cell envelope of gram negative bacteria (*H.pylori*), lipopolysaccharide (LPS), extracted into water by centrifugation and vortexing methods called *H.pylori* extracts, has been used to study its affect to inflammatory cell activities and found that they are chemotactic for neutrophils and monocytes (Craig et al., 1992; Neilson and Anderson, 1992; Mai et al., 1991). Early studies *in vivo* on the rat mesentery have demonstrated that *H.pylori* extracts cause leukocyte adherence in and emigration from the microcirculation (Yoshida et al., 1993). Current studies, have demonstrated that short-term exposure to water extracts of *H.pylori* induces significant platelet aggregation and macromolecular leakage of labeled albumin (Kalia et al., 1997). Chronic oral administration of water extracts of *H.pylori* induces leukocyte rolling and

adhesion in the rat gastric mucosal microcirculation (Kalia et al., 2000). It was obvious that those previous studies have been done by topical application of *H.pylori* extracts on mesenteric or gastric mucosa, however those performance does not relevant the natural history of *H.pylori* infection in gastric mucosa. Because *H.pylori* infection induced gastric inflammation and one of the major components of gastrointestinal inflammation are changes in vascular structure and function. Therefore, the present study bring a model of *H.pylori* infection which was previous studied by Thong-Ngam and coworker (2005) to study it role on changes in gastric microcirculation thus it may be useful for further investigation to understanding the pathogenic mechanism of inflammation and for searching a new therapeutic strategies such as application plant medicine for efficient therapy against *H.pylori* infection.

Because *H.pylori* is considered as a major risk factor of gastric inflammation, which associated with immune response in host cell and cytokine is one of several proteins that play an important role in the inflammatory process. However, cytokines levels (TNF-α and IL-10) in *H.pylori* infection in rat model have not been reported.

*Aloe vera* is a kind of plant medicines that has been commonly used in basic health care in many countries throughout the world. This plant can be separated into two portions they are *Aloe vera* gel and latex portion. *Aloe vera* gel is containing many biologically active substances (Klein and Penneys, 1988). It has been reported that glycoprotein extracted by *Aloe vera* has a strong anti-inflammatory response (Davis, 1988; Davis et al., 1991,1992; Shelton, 1991) and could exhibit the action of anti-inflammation by reducing changes in cutaneous microcirculation such as reduce leukocyte adherence, wound healing promotion, and

reduce TNF-α and IL-6 levels (Somboonwong et al., 2000; Duansak et al., 2003).

Moreover, *Aloe vera* has been used to treat a variety of gastrointestinal conditions. In Thai Pharmacopeia suggested that in latex portion of *Aloe vera* has anthraquinone glycosides that could improve indigestive disease. In aloe gel portion it has been reported that oral administration of the mucilagous gel from *Aloe vera* has prophylactic and curative effect against gastric lesion induced by hydrochloric acid (0.6 N HCl) and acetic acid (30% and 100%) (Mahattanadul, 1995). Mucopolysaccharide and glycoproteins content that found in aloe gel have been shown that there was a synergistic action that is responsible for the antigastric ulcer action (Grindlay and Reynolds, 1986; Blitz, 1963).

From previous studies, they demonstrated that *Aloe vera* could exhibit anti-inflammatory response, maintain balance of cutaneous microcirculation and affect to cytokines levels in burn model and also exhibit prophylactic and curative effects against gastric lesion induce by chemical irritation. Then *Aloe vera* may be appropriate for application to treat gastric inflammation in *H.pylori* infected rat.

Therefore, we design the experiment in order to study the effect of *H.pylori* infection on changes in gastric microcirculation by using intravital fluorescent microscopic technique and on TNF-α and IL-10 production. Especially, the main objective of the present study is to study the ability of *Aloe vera* to reduce changes in rat gastric microcirculation and its affect to cytokines levels in *H.pylori* infected rat.

# CHAPTER II

# LITERATURE REVIEWS

## *HELICOBACTER PYLORI (H.PYLORI)*

*H.pylori* was first reported in 1983 by two Australian investigators, Barry Marshall and Robin Warren, who reported isolation of spiral organisms from mucosal biopsies of patients with chronic active gastritis. First named including *Campylobacter pyloridis*, *Campylobacter pylori*, and *Campylobacter-like organism*, their name were changed to *Helicobacter pylori* when biochemical and genetic characterization of the organism shown that it was not a member of the Campylobacter genus (Peterson and Graham, 2002).

## CHARACTERISTIC OF *H.PYLORI*

*H.pylori*, is a small gram-negative bacteria (3 x 0.5 µm), wavy or comma-shaped rod, has four to six sheathed flagella at one pole, host protein binding for adhesion to gastric epithelium (Figure 1), and slow-growing in microaerophilic condition; 5% oxygen ($O_2$); 50% carbon dioxide ($CO_2$) at 37 $^oC$ (Yamada et al., 1991). This bacterium is an unusual organism in that it is able to survive in the human stomach and also to multiply in the acid environment of the stomach. *H.pylori* is a non-invasive bacterium, it lives in the mucus layer overlying the epithelium, not invade tissue although a fraction of the bacterial cells directly adhere to the epithelium (Figure 2). *H.pylori* grows

predominantly in the antral region of the stomach which produced acid less than corpus and body region (Blaser, 1992).

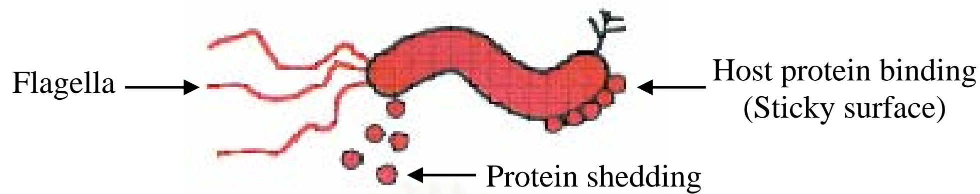


**Figure 1** Characteristic of *H.pylori* (Malfertheiner et al., 1996).

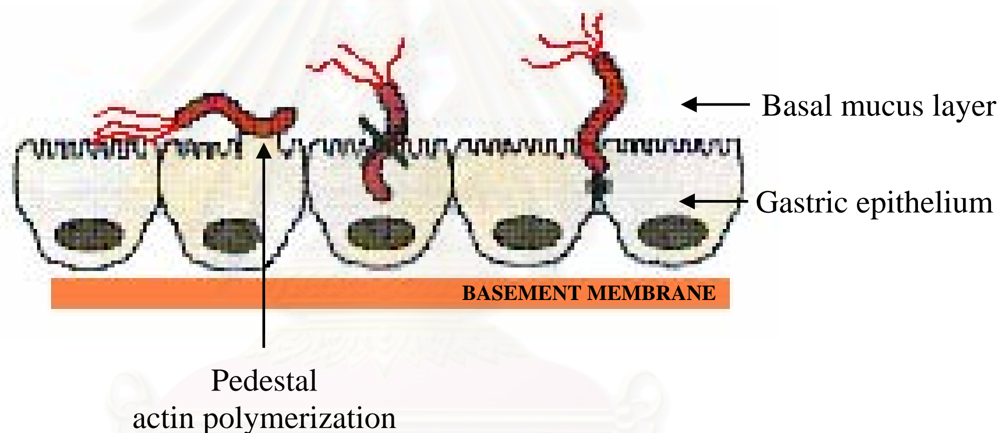


**Figure 2** Non-invasive bacterium, *H.pylori*, adhere to surface epithelium (Malfertheiner et al., 1996).

## VIRULENCE FACTORS OF *H.PYLORI*

Virulence factors of *H.pylori* may be divided into colonization factors and factors that are responsible for tissue injury (Peterson and Graham, 2002) as shown in Table 1.

| Promote Colonization |
|---|
| Flagella (for motility)<br>Urease<br>Adherence factors |
| **Factors Mediating Tissue Injury** |
| Lipopolysaccharide (LPS)<br>Leukocyte recruitment and activating factors<br>Vacuolating cytotoxin (VacA)<br>Cytotoxin-associated gene product A (CagA)<br>Heat shock proteins (HspA, HspB) |

**Table 1** Virulence factors of *H.pylori* that promote colonization and induce tissue injury (Peterson and Graham, 2002).

## COLONIZATION FACTORS

Colonization factors are these attributes of *H.pylori* that allow it to establish its presence in the stomach and to persist despite the body attempts to rid itself of infection. These factors permit *H.pylori* to thrive in a niche that is in hospitable to virtually every other enteric organisms. Colonization factors can be divided into:

### Motility via flagella

The structure of *H.pylori* is an important factor in allowing these bacteria to colonize the mucus-secreting gastric mucosa. Its spiral shape and the environment of the stomach and into the neutral mucosal layer where pH is near neutral to permit optimal growth.

**Urease**

Urease is a protein enzyme, which was produce by *H.pylori* and present on the surface of bacteria. This enzyme is essential for convert urea into ammonia and bicarbonate, two strong bases which in turn neutralize the acid and provide a safe environment. Therefore, the acid is not able to destroy the *H.pylori*. Then these organisms can survive in the acidic condition in the stomach.

**Adherence factors**

The ability of *H.pylori* to bind specifically to gastric-type epithelium is termed tissue tropism, a property that prevents the organism from being shed during gastric epithelial cells and mucus turnover. Adherence may also be important in targeting toxins and leukocyte recruitment factors in host epithelium. An N-acetylneuraminyllactose-binding fibrillar hemagglutination has been described for *H.pylori*, as far a specific gastric glycerolipid receptor on gastric mucosal cells. Tight attachment of the fibrillar adhesion on the bacterium to the carbohydrate receptor on the mucosal cell results in the formation of an attaching-effacing lesion (adherence pedestal), which in turn leads to actin polymerization and possibly epithelial cell disruption.

## FACTORS MEDIATING TISSUE INJURY

*H.pylori* does not invade tissue but lives in the mucus layer overlying the epithelium. Infection can result in inflammation due to transport of *H.pylori* products into the gastric mucosal tissue. The bacterial products that mediating tissue injury can be divided into:

**Lipopolysaccharide**

Lipopolysaccharide (LPS) or called endotoxin is the cell envelope of gram-negative bacteria, *H.pylori*. The LPS of individual *H.pylori* strains are structurally heterogeneous and may have variable degree of endotoxicity (Blaser et al., 1992). It primarily through the lipid A component, stimulate the release of cytokines and possess endotoxic properties. Other reaction of LPS includes interference with the gastric epithelial cell-laminin interaction, which may lead to loss of mucosal integrity, inhibit of mucin synthesis, and stimulation of pepsinogen secretion.

**Leukocyte Recruitment and Activating Factors**

*H.pylori* elaborates a number of LPS-independent soluble surface proteins with chemotactic properties to recruit monocytes and neutrophils to the lamina propria and to activate these inflammatory cells. These including:

***H.pylori* Neutrophil-Activating Protein**

*H.pylori* neutrophil-activating protein (HP-NAP) is a 17-kDa protein expressed by *napA* gene. It is a chemotactic for human neutrophils and capable of promoting their adhesion to, in addition, it stimulates the production of reactive oxygen intermediate (ROI) of human neutrophils and monocytes. HP-NAP is localized in the bacterial cytosol and it is released upon autolysis, it can mediate *H.pylori* binding to host cell and to the stomach mucus via interaction with carbohydrates. Recently Montemurro et al. (2002) reported that HP-NAP is a potent against of mast cells capable of inducing degranulation of performed chemical mediators and the production of IL-6.

**Platelet-Activating Factor (PAF-acether)**

*H.pylori* produces a PAF-acether, an ether phospholipids with a variety of proinflammatory activities which play a role in the pathogenesis of gastric mucosa (Blaser, 1992).

## Vacuolating Cytotoxin (VacA)

VacA gene encodes a 140,000 molecular weight protein that is processed to a mature toxin of 90 kDa. Study *in vitro* demonstrated that this gene could induce cytoplasmic vacuolation in eukaryotic cells (Cover, 1996). VacA gene is found in all *H.pylori* strains but only about 40% of strains exhibit vacuolating cytotoxin activity. This is because vacA nucleotide sequence varies between strains (Kalia et al., 2002). The vacA gene has two families of alleles of the middle region (m1, m2) and at least two families of alleles of signal sequence (s1, s2). Type s2m2 vacA encodes a non-vacuolating form of vacA. The vacA genotype s1 is strongly, type s1m1 vacA encodes the most toxic form of vacA while type s1m2 vacA encodes a form of the toxin that induces vacuolation in a narrower range of epithelial cell lines than type s1m1 vacA (Peterson and Graham, 2002). Despite the vacA genotype s1 is strongly, but not exclusively, associated with the presence of cagA (Kalia et al., 2002). Investigating the role of vacA in the pathogenic action of *H.pylori* revealed that vacA caused acute inflammation with mast cell accumulation and also shown to bind to mast cells *in vitro* and induce cytokine release from mast cells, which may promote tissue damage (Supajatura et al., 2002).

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## Cytotoxin-Associated Gene Product A (CagA)

CagA, a 120 to 140 kDa molecular weight highly antigenic protein. This gene is present in 60% to 70% of *H.pylori* strains and is a marker for

the presence of the cag pathogenicity island (cag PAI), which comprises about 30 genes (Kalia et al., 2002). The presence of the cag PAI is associated with a more prominent inflammatory tissue response than is seen with strains lacking this virulence factor. Infection with *H.pylori* cagA positive strains could induce the production of chemokine (IL-8) from gastric epithelial cells which is a potent inflammatory cytokine to enhance inflammatory response in gastric mucosa (Peterson and Graham, 2002).

**Heat Shock Proteins (Hsp)**

Heat shock proteins (HspA, HspB) are immunodominant antigens in various diseases including *H.pylori* infection. It has been shown that 60 kDa Hsp (Hsp60) is expressed in the follicular dendritic cells of the gastric mucosa in patients with gastric mucosa-associated lymphoid tissue (MALT) lymphoma and that antibodies to human Hsp60 can be detected in MALT lymphoma patients. Involves to the immune response, it has been shows the elevated levels of immunoglobulin G1 antibody which response to *H.pylori* Hsp60 in patients gastroduodenal disease and MALT lymphoma (Ishii et al., 2001).

**STOMACH**

The stomach (Figure 3) is a J-shaped tube connecting the esophagus and the duodenum. Its primary functions are the mechanical and chemical disruption of food and its even distribution into the absorptive portion of the gastrointestinal tract. It is situated in the upper abdomen, below the diaphram, and is fixed in two places, at the esophagogastric junction above and gastroduodenal junction below. The lesser and greater curvature constitute the medial and lateral margins,

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respectively, of the stomach, and correspond to the regions where the lesser and greater omenta attach.

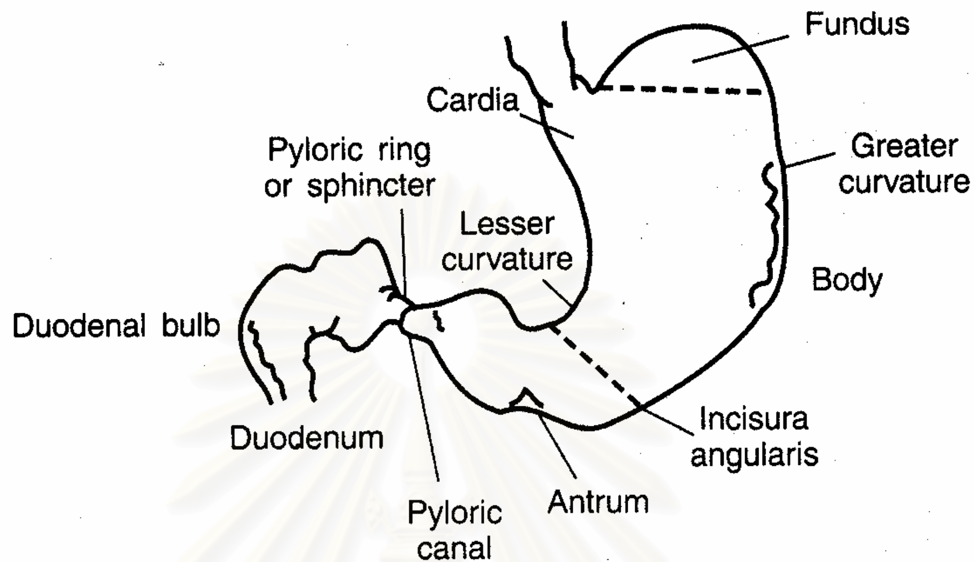



**Figure 3** Anatomy of stomach (Thomson and Shaffer, 1992).

The stomach begins at the gastroesophageal junction, where the tubular esophagus ends and the rugal folds of the stomach begin. The cardia is comprised approximately the proximal 0.5 to 2 cm. of the stomach. The fundus and body (synonyme: corpus) are the large area of the stomach, and they extends between the cardia and antrum. The fundus consists of the dome of the stomach that lies above an imaginary horizontal line through the diaphragmatic pinch. The antrum occupies the distal fourth of the stomach and extends from an indentation on the lesser curvature, to the pyloric sphincter (synonyme: pyloric antral).

The wall of the stomach has four layers (Figure 4); mucosa, submucosa, muscularis propria, and serosa.

**The mucosa**

The mucosa of the stomach is made up of epithelium and surrounding connective tissue called the lamina propria. The epithelium can be divide into the surface faveolae (synonyme: gastric pits) and the deeper gastric glands. Both of them produce the surface mucus layer that is part of the stomach's mucosal protection system. The surface epithelium and faveolae are comprised of columnar cell. The apical portion of the cell contains mucin. It expressed the antigens M1, a mucin antigen, cathepsin E, and aspartic proteinase. The lamina propria comprises the supporting framework for the epithelium. The epithelium rests as a basement membrane. The framework is comprised of a fine meshwork of reticulin with occasional collagen and elastin fibers; the supporting capillary network and nerve fibers occupy this space as well.

**The submucosa**

The submucosa consists of loose connective tissue in which are embedded lymphatics, blood vessels, and scattered mononuclear cell, including mast cells.

**The muscularis propria**

The muscularis propria is comprised of three layers of smooth muscle: the outer longitudinal, middle circular, and inner oblique. The outer longitudinal layer is most concentrated along both curvatures. The middle circular layer encircles the body of the stomach and forms the pyloric sphincter distally. The inner oblique fibers pass down from the fundus over both anterior and posterior walls. The nerves and ganglion cells are located between the outer longitudinal and middle circular muscle layers.

**The serosa**

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

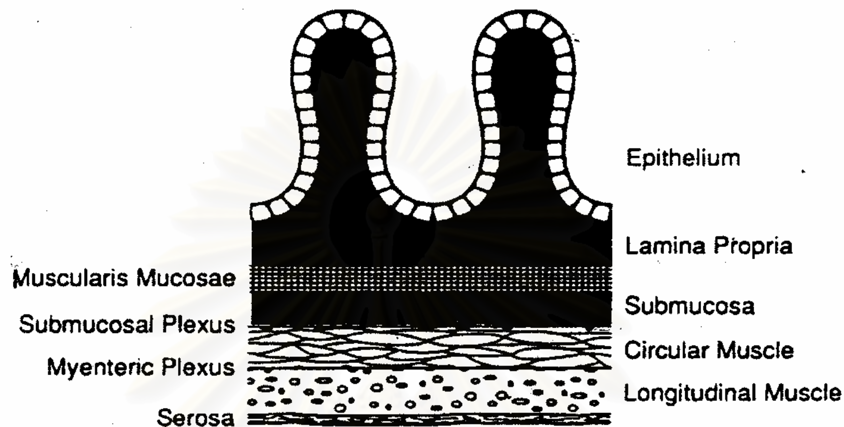



**Figure 4** Structure of gastric wall (Johnson, 1997).

## VASCULAR ANATOMY OF STOMACH

The stomach has rich network of anastomosing vessels derived from the various branches of the celiac trunk. The latter emanates from the anterior surface of the thoracic aorta just below the aortic hiatus. The celiac trunk is a shot vessel that quickly divides into three arteries (Figure 5): the left gastric artery, the splenic artery, and the common hepatic artery. Each of these divisions supplies a portion of the stomach. The left gastric artery supplies the fundus and left superior portion of the lesser curvature. The common hepatic artery gives rise to the gastroduodenal artery and the right gastric artery, which then anastomoses with the left gastric arterial supplies the inferior lesser curvature of the stomach. The splenic artery gives rise to the short gastric arteries, which also supply the fundus and body along the greater curvature of the stomach. The right

and left gastroepiploic arteries also form an anastomosis along the greater curvature (Graham et al., 1999; Thomson and Shaffer, 1992).

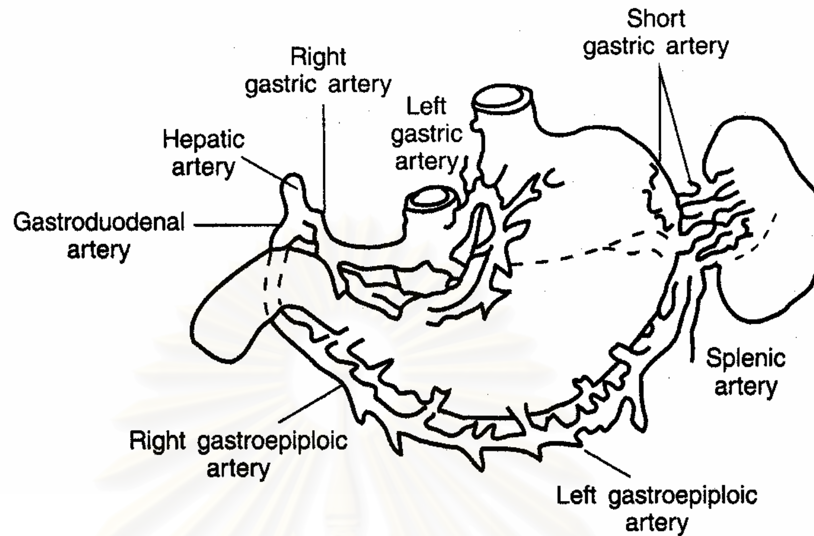



**Figure 5** Blood supply the stomach (Thomson and Shaffer, 1992).

## GASTRIC CIRCULATION

The circulation of the gastric wall has arrangement, which has branching hierarchy and relative dimensions. The posterior branch of the left gastric artery, gives rises to a 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. The arteries that supply the muscle layer are branch to small arteries (SA) that give rise to ascending muscle arterioles, which supply muscle arterioles in the circular and longitudinal muscle layers. These arterioles run perpendicular to the muscle fibers and divide into the longitudinal and circular muscle capillaries (MC), which run parallel to the muscle fiber. Capillaries end in muscle venules (MV), which form descending venules

through both muscle layers and return blood to the submucosal venules (SMV) or submucosal small vein (SV) before leaving the submucosa (Peti-Peterdi et al., 1998). The gastric vasculature and the various branches of the gastric circulation are shows in Figure 6.

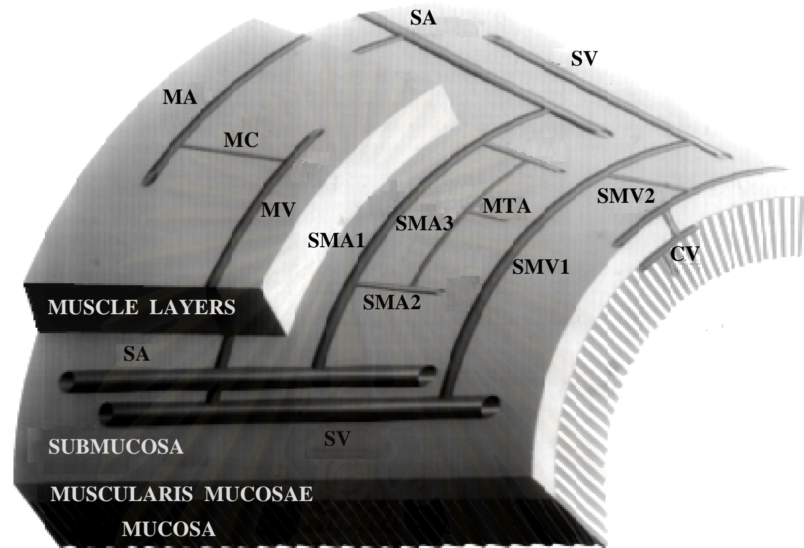



**Figure 6** Schematic representation of microvasculature in gastric wall. Blood vessels were identified numerically according to their branching order and vascular and vascular hierarchy: small artery (SA), submucosal primary (SMA1), secondary (SMA2), and tertiary (SMA3) arterioles, mucosal terminal arterile (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 (Peti-Peterdi et al., 1998).

## PATHOGENESIS OF *H.PYLORI* INFECTION

Infection is acquired by the oral route and, after ingested, the organism penetrates through the viscid mucus layer and multiplies in

close proximity to the apical membranes of the surface epithelial cells. A proportion of the organisms become adherent and attach to the plasma membrane, wherease other penetrate into the potential gap between epithelial cells. The inflammatory response developed from day 8 to 39 after ingestion these organisms. The earliest effect of *H.pylori* infection is induced transient hypochlorhydria, that is increased in basal acid secretion and followed by reduction in acid secretion (Peterson and Graham, 2002; Graham et al., 1988) and, therefore, the acid is not able to destroy the *H.pylori* organisms.

The epithelium responses to infection by marked degenerative changes, including mucin depletion, cellular exfoliation, and syncytial regenerative changes (Graham et al., 1999).

Acute *H.pylori* gastritis, endoscopic appearances are erythema, edema, hemorrhage, erosions, and raised white plaques. Histological features of acute gastritis represent the host's mucosal immune response to the organism, including numerous of neutrophils infiltration with little or no chronic inflammatory cell infiltrate into both lamina propria and epithelial lining (Wyatt, 1995).

Chronic *H.pylori* gastritis, the histological features represent the host's mucosal immune response to the organism, including polymorphonuclear leukocytes (PMNs), eosinophils, and mononuclear cells infiltrate. The latter include B and T lymphocytes and typically induced development of lymphoid follicles monocytes and plasma cells. The lymphocytic component of the inflammatory response is referred to a MALT. Biopsies of gastric antral also usually demonstrate focal epithelial cell damage as well as glandular atrophy and intestinal metaplasia may be

present in antral and/or corpus mucosa in patients with *H.pylori* infection (Wyatt, 1995). To postulate steps of *H.pylori* infection were shown in Table 2.

| **Postulated steps in *H.pylori* infection** |
| --- |
| Ingestion of *H.pylori* |
| Movement into and through mucus layer |
| Attachment mucosa |
| Bacterial multiplication |
| Invasion, tissue damage |
| Internalization |
| Acute eradication attempt by host |
| Neutrophilic response |
| Chronic inflammatory cell response |
| Humoral immune response |
| Control of infection and down regulation of immune response to the pattern of chronic disease |

**Table 2** Postulated steps in *H.pylori* infection (Goodgame et al., 1995).

## IMMUNE RESPONSE FOLLOWING *H.PYLORI* INFECTION

Host defense against microbial infection is a complex system of both specific and non-specific reactions. Among the non-specific defence mechanisms acting on bacteria before they reach the mucus layer in the stomach are digestive enzymes, lysozyme, lactoferin and other components with antimicrobial activity that are produced in the oral cavity and the stomach. The mucus layer is the final non-specific barrier against the bacteria reaching the gastric mucosal cell. The glycoproteins

of the mucus layer build a net that contains lectin-like adhesions that prevent the penetration by bacteria. However, the spiral shape of the bacterium and its flagella enable *H.pylori* to penetrate through this glycoprotein gel. These characteristics of *H.pylori* make it one of the very few bacteria able to reach the gastric epithelial cells.

On reaching the gastric mucosa, *H.pylori* adheres to the epithelial cells and may penetrate the tight junctions between them. Bacterial antigens, chemotaxins and other components are liberated and are taken up in the lamina propria. These liberated antigens, chemotaxins and other compounds attract and activate leukocytes, initially PMNs and macrophages. The latter secrete interleukins (e.g. IL-1β, IL-6, and IL-8), and tumor necrosis factor (TNF-α), which are potentially able to phagocytes and kill the bacteria. The phagocytes also release toxic oxidative radicals and proteolytic enzyme. *H.pylori* antigens are presented to immature B lymphocytes, which interact with T-helper lymphocytes to become mature immunoglobulin (include IgA-, IgD-, IgE-, and IgM-) producing plasma cells (Malfertheiner et al., 1996).

The acute phase of *H.pylori* gastritis is short lived. In a minority of people, the organism may be spontaneously cleared, the polymorph infiltrate resolves, and appearances return to normal. In most, however, the initial natural immune response fails to eliminate the bacterium and the infection appears to be typically long lived in adults. Over the next 3 or 4 weeks there is a gradual accumulation of chronic inflammatory cell engaged in a gastric mucosa. Most individuals have chronic active gastritis, nonatrophic gastritis, and superficial gastritis. This histologic form of *H.pylori* gastritis is usually asymptomatic but may be associated with duodenal ulcer disease, chronic atrophic gastritis, gastric

adenocarcinoma or gastric lymphoma (Peterson and Graham, 2002; Graham, 1999).

## PATHOGENESIS OF *H.PYLORI* INDUCE INFLAMMATION

*H.pylori* stimulates the release of a variety of inflammatory mediators both directly by bacterial products such as LPS, vacuolating cytotoxin, and neutrophil-activating factor and indirectly as a result of interaction with gastric epithelial cells (Crowe et al., 1995; Crabtree, 1996). *H.pylori* LPS and released surface proteins stimulate lamina propria mononuclear cells and macrophage to produce proinflammatory cytokine such as IL-1β, TNF-α, and generation of reactive oxygen species (ROS) (Davis et al., 1994; Drake et al., 1994). The cytokines are potent inducers of IL-8 expression in many cell types. Moreover *H.pylori* organisms are able to interacting with epithelial cell surface to produce IL-8. The release of inflammatory cytokines and the recruitment of inflammatory cells can induce generation of reactive oxygen metabolites and up-regulation of neutrophil expression of CD11b/CD18 (Crowe et al., 1995). The latter enhance intercellular adhesion molecules-1 (ICAM-1)-dependent neutrophil adherence (Figure 7). With neutrophil adhesion its produce large amounts of reactive oxygen intermediate (ROI) that may further affect intracellular signal transduction pathway, which induce the cell loss of certain function and come changes in microvascular permeability (Shimada and Terano, 1998). These are responsible for a positive feedback loop through the stimulatory actions of chemical mediators on neutrophil chemotaxis and activation (Graham et al., 1999). Later the pathological could develop to chronic inflammation, which characterized by infiltration of chronic inflammatory cells such as monocyte, T lymphocyte, and B lymphocyte. The pathology could

develop to diseases associated with *H.pylori* gastritis such as peptic ulcer, atrophic gastritis, gastric cancer, and lymphoma (Harford et al., 2000).

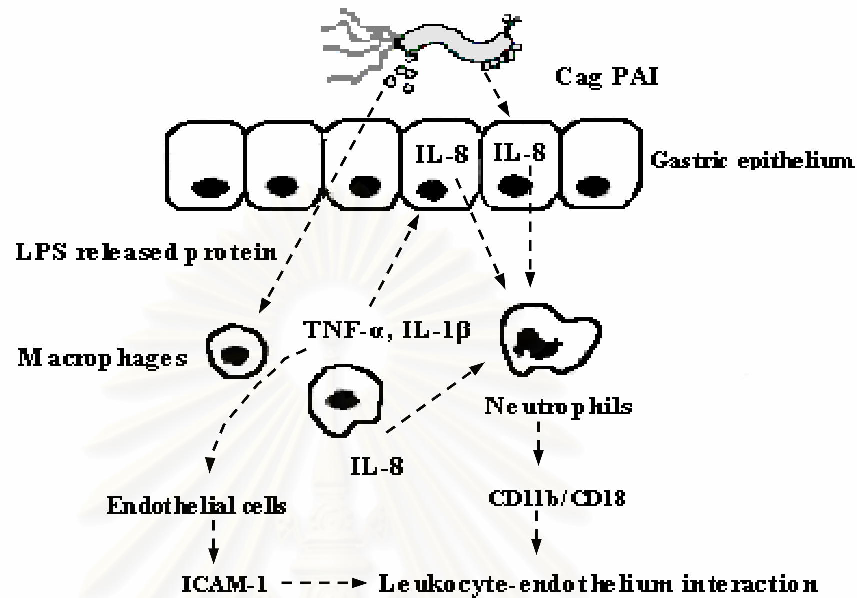



**Figure 7** Pathogenesis of *H.pylori* induced gastric inflammation (Modified from Shimada and Terano, 1998).

## LEUKOCYTE - ENDOTHELIAL ADHERENCE FOLLOWING *H.PYLORI* INDUCING INFLAMMATION

Leukocytes are central mediators of microvascular endothelial injury in many acute pathologic processes (Boykin et al., 1980; Harlan, 1987a; Movat and Cybulsky, 1987; Weiss, 1989). Adherence leukocytes have been identified as possibly contributing to the microvascular occlusion seen following exposed to extract of *H.pylori*, which could induce resistance to blood flow and releasing cytotoxic products into the peripheral circulation. Hence leukocyte adhesion is often a prerequisite for pathological tissue injury both systemically and locally. Many studies have demonstrated an increase in various inflammatory cells such as

monocytes, macrophage and PMNs activation when they are exposed to *H.pylori* extracts *in vitro* (Mai et al., 1991; Neilson and Anderson, 1992; Mai et al., 1992; Craig et al., 1992). Yoshida et al. (1993) have demonstrated an increase in the leukocyte adhesion and emigration in mesenteric venules following exposed to *H.pylori* extracts. An increased in leukocytes adhesion to endothelial cells (ECs) were occurred via the expressed of CD11a/CD18 and CD11b/CD18 on leukocytes and ICAM-1 on ECs. Leukocyte-mediated injury is dependent in part on leukocyte adherence to the vascular endothelial cell surface and leukocytes aggregation in the microvasculature. Leukocyte-EC adherence results in the formation of a microenvironment between the leukocyte and the EC (Harlan, 1987b). In this microenvironment, leukocyte-derived protease and toxic oxygen products produced by both the EC and leukocyte exist in high locally concentrations. These highly reactive substances partially protected from inactivation by circulating plasma anti-proteases and free radical scavengers, then produce endothelial injury resulting in intercellular gap formation, increase in microvascular permeability, edema, and thrombosis. The aggregation of leukocytes further compromises the microvascular circulation by plugging of capillaries and postcapillary venules, extending the tissue ischemia, and subsequent tissue loss.

The sequence of even that allows the traveling of leukocytes to site host defense is designed the multistep paradigms of leukocyte recruitment as the following:

Circulating leukocytes are recruited to sites of inflammation and tissue injury by a highly coordinated process that occurs primarily in postcapillary venules. As leukocyte exit capillaries, hemodynamic forces

give rise to an outward radial movement of leukocytes toward the venular endothelium. The initial adhesive interaction between the leukocytes and venular endothelium is rolling. This low-affinity (weak) interaction is subsequently strengthened, such that the leukocytes attach to the EC and remain stationary as shown in Figure 8. The leukocytes are then able to migrate into the interstitium through spaces between adjacent EC. These interactions are regulated by sequential activation of different families of adhesion molecules expressed on the surface of neutrophils and EC. Selectin-like adhesion glycoproteins, called the selectins, mediate leukocyte rolling. Both P- and E-selectin expression are increased on EC when the appropriate stimuli are present in inflamed tissue. P-selectin, which is stored in EC as a pool, can be rapidly mobilized to the cell surface by stimuli such as inflammatory cytokine (TNF-α). Endothelial cell adhesion molecules that mediated firm adhesion of leukocytes include ICAM-1 and vascular cell adhesion molecules (VCAM-1) both of these are expressed on venular EC.

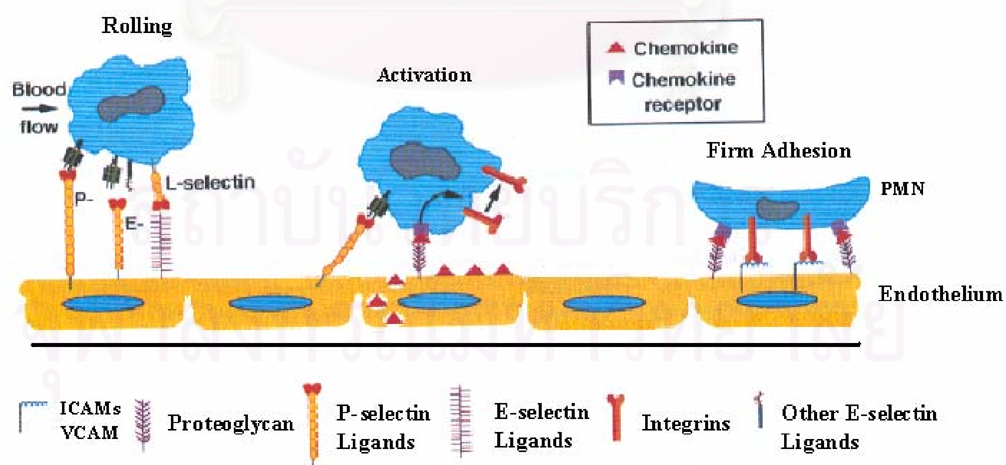



**Figure 8** Cascade of leukocyte-endothelium interaction (Freedberg et al., 2003).

## ROLE OF CYTOKINES ON INFLAMMATION

Cytokines are small polypeptide molecules of between 5 and 50 kDa that play pivotal roles in communication between cells of the hematopoietic system and other cells in the body. It can be expressed by a number of different cell lineages but which are most commonly associated with cells of the immune system. Cytokine can work in an autocrine, paracrine, or endocrine fashion and influence not only immune cells but also epithelium, endothelium, mesenchyme, and the extracellular matrix. Over production of cytokine can lead to clinical sequelae such as inflammatory disease which can contribute to the systemic effects such as anorexia, fever, anemia, and thrombocytosis, but some of cytokine also have important roles to play in the suppression of both physiological and pathological inflammation, and in healing and repair. Moreover cytokine influence many aspects of leukocyte function including differentiation, growth, activation, and emigration.

Cytokines are fundamentally involved in the control of the immune and inflammatory response. They enable the cellular components of these responses to communicate with each other and can also determine the nature response. Certain molecules such as IL-1, IL-6, TNF-α, and the chemokines, are predominantly synthesized and released by activated macrophages. IL-1 and IL-6 have the capacity to stimulate both arms of the immune response by activating T cells to produce IL-2 and express the IL-2 receptor, and also by inducing B-cell proliferation, maturation and increased immunoglobulin synthesis. TNF-α similarly has an important and wide-ranging role in the inflammatory response and in host resistance, with its ability to induce or suppression of a number of genes. These include genes for growth factors and cytokines, transcription

factors, receptors, inflammatory mediators and acute-phase response (Allan et al., 1997).

The other major cytokines, which include IL-2, IL-4, IL-5, IL-10, IL-13 and γ-interferon (IFN), are predominantly synthesized by activated T lymphocytes. These molecules are now subdivided into the Th1 (IL-2 and IFN) and Th2 (IL-4, IL-5, IL-10 and IL-13) subgroups because of their different actions and effects on the immune response. The Th1 subgroup directs a cell-mediated immune response, whereas the Th2 cytokines generate a humoral response (Allan et al., 1997; Mosmann and Coffman, 1989; Maggi et al., 1992).

**PROINFLAMMATORY CYTOKINE**

Proinflammatory cytokines are comprised of TNF (α and β forms) and IL-1 (α and β forms). These cytokines functions as primary cytokines, it appears to play an important role in gastrointestinal immunoregulation and physiology. Both TNF and IL are potent inductors of inflammation and are expressed in increased amount by mononuclear cells from inflamed as well as infectious disease.

TNF-α is comprised of 235 amino acids, it produced mainly by activated macrophages in response to tissue injury or infection. TNF-α has been shown to activate neutrophils, promote T and B cell proliferation, induce cell adhesion molecule expression on EC, induce a variety of cells to produce a host of additional cytokines (Thomas et al., 1999), and to induce expression of chemokine and IL-1 and IL-6 (Colletti et al., 1996) that provide a chemotactic gradient allowing the directed migration of leukocyte into a site of inflammation.

In *H.pylori* infected patients, TNF-α is the most powerful effect on acute inflammatory reaction which could affect to the leukocytes and ECs activation, the acute inflammatory reactions are also related to the increase production of other cytokines such as IL-1β, IL-6, and IL-8, (Beales et al., 1997). The production of these cytokines are not limited at the site of infection but are further produced in a numerously which contribute to the systemic circulation. Interestingly, TNF-α are endogenous pyrogen, if they was produce in numerously, can cause a number of metabolic changes in a variety of cell types and contribute to the hepatic acute-phase response, and other systemic effects such as anorexia, fever, anemia, and thrombocytosis (Allan et al., 1997).

**ANTIINFLAMMATORY CYTOKINE**

IL-10 is a 18 kDa peptide comprise 178 amino acids. This cytokine was first identified as “cytokine synthesis inhibitory factor" because of its ability to suppress cytokine synthesis in certain T cells (Bodger et al., 1997). IL-10 was produced by chronic inflammatory cells such as T lymphocytes, B lymphocytes, and monocytes. The function of IL-10 is inhibition of synthesis and gene expression of TNF-α, IL-1α, IL-1β, IL-6, IL-8, and colony stimulating factor in monocytes (Fiorentino et al., 1991, de Waal Malefyt et al., 1991). In addition, IL-10 shows potent anti-inflammatory properties, including suppression of IL-2 and interferon-gamma (IFN-γ) production by T lymphocytes (Fiorentino et al., 1989, Del Prete et al., 1993), inhibition of mitogen induced T cell proliferation and of the effector functions of activated monocytes/macrophages (de Wall Malefyt et al., 1991). It has been shown that IL-10 acts on the macrophage to inhibit the production of cytokines such as IL-1, IL-6 and TNF-α by LPS-activate macrophages (Fiorentino et al., 1991).

In patients with inflammatory bowel disease, IL-10 could down regulates IL-1β and TNF-α secretion as well as messenger RNA expression (Schreiber et al., 1995). In patients with chronic *H.pylori* gastritis has been found the increase production of IL-10 and TNF-α. The production of IL-10 level was related to chronic inflammation score and *H.pylori* status. They proposed that mononuclear cells and gastric epithelium are possible sources of IL-10, which are capable of secreting other cytokine and the increasing of IL-10 may contributing to limit tissue damage cause by inflammation (Bodger et al., 1997)

## *IN VITRO* STUDIES ON *H.PYLORI* AND INFLAMMATION

Since *H.pylori* organism has become accepted as an important human pathogen for the development of gastritis and many of the diseases associated with *H.pylori* gastritis. This discovered induce to sparked interest to studies about the mechanisms of *H.pylori* induced inflammation. The latter is characterized by marked infiltration of potent inflammatory cells into the gastric lamina propria (Wyatt, 1995; Crabtree, 1996). This observation may be cause from *H.pylori* producing and releasing some form of chemotactic substance that could attract and activate leukocytes.

From the studies in 1991 and 1992 many investigators suggested that *H.pylori* can cause gastrointestinal inflammation by its produced and releases chemotactic substances that attract and activate neutrophils and other inflammatory cells, with the activated leukocytes causing tissue injury (Wallace, 1991, Graham, 1992). In support of the latter hypothesis is the observation that the degree of *H.pylori* infection and severity of the mucosal injury are directly correlated with the extent of neutrophil

infiltration. (Kozol et al., 1991; Graham et al., 1987; Bayerdorffer et al., 1992). From *in vitro* studies have demonstrated that the surface proteins of *H.pylori*, extracted into water by centrifugation and vortexing methods, called *H.pylori* extracts, are chemotactic substances for PMNs, monocytes, and macrophages (Craig et al., 1992; Neilson and Anderson, 1992; Mai et al., 1991). In 1996 Takemura et al. studied the effects of *H.pylori* extracts on activate neutrophils to disrupt EC monolayers and to identify the mechanisms involved in this process. Human neutrophils were incubated with monolayers of human umbilical vein endothelial cells (HUVECs) in the presence or absent of *H.pylori* extracts. From this studies found that *H.pylori* extracts could activate human neutrophils to produced EC detachment from HUVEC monolayers. EC detachment was prevented by a monoclonal antibody directed against CD11/CD18 on neutrophils or a monoclonal antibody against ICAM-1 on ECs. HUVEC monolayer disruption was also prevented by superoxide dismutase, catalase, and a monoclonal antibody against elastase. These studies indicated that *H.pylori* extracts can activate human neutrophils to injure HUVEC monolayers only when human neutrophil are allowed to adhere to HUVECs and may provide an explanation for the *H.pylori* extracts to induce microvascular dysfunction *in vivo* model.

## *IN VIVO* STUDIES ON *H.PYLORI* AND MICROCIRCULATION

For the quantitatives studies of hemodynamics and morphologic parameters in the microcirculation intravital microscopy were used to detect microcirculatory changes affected from *H.pylori* was first described by Yoshida et al. in 1993 who used intravital microscopic technique to detect the effects of topical administration of *H.pylori* extracts on changes in mesenteric microcirculation. Superfusion of the

exposed mesentery with *H.pylori* extracts resulted in increase leukocyte adhesion and emigration in venules. This result was confirmed by *in vitro* study which detected the PMN-EC adhesive interactions by using human PMNs and monolayers of HUVECs. The results revealed that *H.pylori* extracts induced expression of CD11b and CD18 on surface of PMN and then affect to HUVEC to express ICAM-1. From these studies suggested that products of *H.pylori* elicit gastrointestinal inflammation by promoting PMN adhesion to ECs via CD11a/CD18 and CD11b/CD18 dependent interactions with ICAM-1. The damaging effects of neutrophil adhesion to the vasculature was also shown by Takemura et al. who demonstrated *in vitro* that neutrophils activated with an extract of *H.pylori* could disrupt EC monolayers, which could provide an explanation for the increased protein leakage observed *in vivo* (Takemura et al., 1996).

Despite, *H.pylori* extracts exhibited chemoattractant for inflammatory cell and can cause disturbance in rat mesenteric microcirculation. However, *H.pylori* infection is commonly localized to the stomach. Therefore, in 1997 Kalia et al. was adapted to studied in gastric mucosal microcirculation by topical application *H.pylori* extracts on rat gastric mucosa and used intravital microscopy to assessed macromolecular leakage of labeled albumin (MML), leukocyte adherence/rolling and platelet activity in mucosal vessels. The results revealed that *H.pylori* extracts induced increases in adherent platelet thrombi and MML after five minutes only but the leukocyte adherence was not observed following *H.pylori* extracts administration. These results suggested that the early events associated with *H.pylori* infection are platelet aggregation. However, the leukocyte activation was also observed after chronic administration of *H.pylori* extracts by gavage three

times daily at three-day intervals. Acridine red was used to quantitate gastric mucosal leukocyte and platelet activity by using fluorescent *in vivo* microscopy. Another rat was received additional acute *H.pylori* extracts after 1 hr and recording were made for a further 1 hr. From these studied they found that chronic administration of *H.pylori* extracts induced increase the number of rolling and adherent leukocytes throughout the study. And acute topical administration of *H.pylori* extracts induced a further significant increase in adherent leukocytes (Kalia et al., 2000). Therefore, the initial response to *H.pylori* may be activate the platelet and may be involved in recruitment of leukocyte in later stage. These activated cells may contribute to the development of gastric mucosal damage. And repeated infection may induce inflammatory cells become sensitive to the stimuli and make to the pathological more virulence. *H.pylori* strains are highly diverse with different strains which associated with the present or absence of gastric mucosal inflammation and peptic ulcers in patients. From the clinical studies found that the different clinical outcomes in *H.pylori* infected patients could be dependent upon pathogenic strains, toxigenic or non-toxigenic strain, exerting greater disturbances within the gastric microcirculation. Then in 2002 the acute effects of genotypically different *H.pylori* strain on the rat gastric mucosal microcirculation *in vivo* by used fluorescent *in vivo* microscopy to determine MML, platelet and leukocyte activation, and mucosal vessel diameter. Three different strains of *H.pylori* were studied: $cagA^+$ strongly toxigenic vacA s1/m1 strain, $cagA^+$ $vacA^-$ non toxigenic isogenic mutant, $cagA^-$ only, $cagA^-$ vacA s2/m2 strain producing a non-toxigenic form of the vacA protein. Each strain was extracted into water and brought to topical application on gastric mucosa of anaesthetized rat. The microcirculatory changes were observed after administration *H.pylori* extracts. The results found that

$cagA^+$ vacA toxigenic strains induced significant and sustained MML by five minutes. Transient and less leakage was observed with its isogenic $vacA^-$ mutant and other nontoxigenic strains regardless of cagA status. Significant increases in leukocyte adhesion, platelet aggregation, and postcapillary venule vasoconstriction were observed with the cagA and toxigenic strain. In conclusion, extracts of *H.pylori* are capable of inducing marked disturbances within the rat gastric mucosal microcirculation. The disturbances seem to be dependent on the production of an active vacuolating cytotoxin and varying effects on the gastric mucosal microcirculation may explain the clinically diverse outcomes associated with genotypically different strains (Kalia et al., 2002).

Because *H.pylori* is considered as a major risk factor of gastric inflammation which associated with immune response in host cell, cytokine is one of several proteins that associated with inflammatory process, in 1999 Brzozowski et al. had studied effect of *H.pylori* extracts on rate of gastric ulcer healing in Wistar rat. Gastric ulcer were produced by serosal application of acetic acid in rat with or without gastric fistula treated with saline or *H.pylori* extracts was administered intragastrically on days 1, 3, 5 and 7 upon ulcer induction. On day 3, 9, and 15, animals were lightly anesthetized with ether, the abdomen was opened and the gastric blood flow (GBF) in ulcer area and plasma cytokine such as IL-1$\beta$ and TNF-$\alpha$ levels were measured. From these studies they found that rat treated with *H.pylori* extracts, ulcer healing was significantly delayed, GBF at ulcer margin marked fall. On day 15 of ulcer healing, the plasma concentration of IL-1$\beta$ and TNF-$\alpha$ was significantly elevated while in control rat both of cytokines was negligible. This study can conclude that *H.pylori* extracts delayed ulcer healing due to the reduction in the gastric

microcirculation at the ulcer margin and the over expression of inflammatory cytokine.

From the literature reviews as mentioned above, we can conclude that *H.pylori* extracts could induce gastric inflammation, which may involve in the disturbances in gastric microcirculation and changes of cytokines levels. However, it was obvious that using *H.pylori* extracts may not be mirror the pathophysiological changes in gastric microcirculation and cytokines levels of *H.pylori* infected rat. And previously have not been study the effect of *H.pylori* infection on changes of rat gastric microcirculation and cytokines levels.

## MODEL OF *H.PYLORI* INFECTION IN THIS STUDY

The experimental model of *H.pylori* gastritis which was used in this study was induced by pretreatment with oral dose of streptomycin 5 mg/ml suspend in tap water and give to animal drink freely for three consecutive days before the first *H.pylori* inoculation. On the day of inoculation rat were fasted overnight and then was induced by given the *H.pylori* organisms (about $10^8$ to $10^{10}$ CFU/ml of normal saline) to the rat by gavage (1ml/rat) twice daily for three consecutive days. After the last inoculation these animals were housed with free access to tap water and standard laboratory chow for two weeks. With inoculation of *H.pylori*, the mild to moderate gastric inflammation were developed within two weeks as confirmed by hematoxylin-eosin (H&E) staining in the previous studied (Thong-Ngam et al., 2005), thus we use this model in the present study. Since it has been found that at two weeks after *H.pylori* infection, the pathophysiology did not become progressively more severe during the following months (Li et al., 1999). This infection in rat model is

considered to be an experimental model that closed resemblance to gastric inflammation in human. The damaging action of *H.pylori* was infiltrated by inflammatory cells into lamina propria, enhanced release of proinflammatory cytokines, especially TNF-α, and generated reactive oxygen species (ROS) (Brzozowski et al., 1999).

Actually, *H.pylori* induced gastritis in rat model has been done in several studies but there used the difficulty technique, expensive, and longer period (Li et al., 1999, Zeng et al., 1998). Besides, those previous studies have been designed with the aim of establishing histologic gastritis that would observe in human, but have not been study on gastric microcirculatory changes.

***ALOE VERA***

**BOTANY OF *ALOE VERA***

*Aloe vera* or scientific name is *Aloe vera* (Linn.) Burm.f. It is the plant belongs to the lily family, family *Liliaceae* and subfamily *Aloineae*, which comprises more than 600 species (Grindlay and Reynold, 1986; Winter and Yang, 1996). Only a few species of more than 600 species were currently used by the pharmaceutical or cosmetic industries. These three main species are *Aloe felox Miller* (Cape Aloes), *Aloe perryi Baker* (Socotrine Aloes), and *Aloe vera* (Linn.) Burm.f. (Grindlay and Reynolds, 1986)

*Aloe vera* grows best in a dry chalky soil or in a sandy loam, good drainage is essential to prevent "root rot". This plant needs warm semi-tropical conditions, it dislikes over exposure to the sun, which causes stunted plants with a low gel content. *Aloe vera* is a perennial plant with

freshy leaves arising in a rosette from a short stem. The fully mature plants are up to 0.5-1 m long, comprise 15 to 30 leaves per plant. The leaves are crowed on the top of their stem, spreading, grayish and glaucous, 20 to 50 cm long, 8 to 10 cm across at the base, 1 to 2.5 cm thick, tapering to a point, with saw-like teeth along their margin. Flowers bone on the upper of a slender stalk, 50 to 100 cm high. Forms of the species vary in sizes of leaves and colors of flowers (Grindlay and Reynolds, 1986). The epidermis of the leaves has a thick cuticle, and beneath is a zone of parenchyma which obtains pericylic cells. The latex portion of the plant or yellow juice obtained from the pericyclic cells. The central bulb of the leaf contains the colorless mucilaginous pulp, made up of large thin-walled mucilaginous cell containing the aloe gel itself. Along the junction between the pulp and the parenchyma are arranged the numerous vascular bundles, with accompanying inner bundle sheath cells. The bundle sheath cells at the phloem poles are thin-walled and axially elongated, and contain the bitter yellow sap which exudes from the leaves when they are cut (Grindlay and Reynold, 1986; Klein and Penneys, 1988).

**CHEMICAL CONSTITUENTS**

The components of *Aloe vera* have been identified into two portion include latex portion (aloe leaf exudates) and aloe gel portion. Several studies of chemical constituent analysis in the latex portion have found anthraquinone glycosides derivatives, which are responsible for the strong laxative effects of aloes. The fresh gel had been found to consist of pH 4.7 (Rowe et al., 1941), 99.5% water and 0.5% solid component (Gjerstad, 1971; Mckeown, 1983). Analysis of the solid components

revealed that *Aloe vera* has a largest number of active substances as shown in Table 3.

| **Latex portion** | **Constituents** |
|---|---|
| **Anthraquinones** | Aloin, Emodin, Barbaloin, Chrysophanic Isobarbaloin, Resistannol, Anthranol, Aloe-emodin, Aloetic acid |
| **Aloe gel portion** | **Constituents** |
| **Saccarides** | Cellulose, Glucose, Mannose, L-rhamnose, Aldopento |
| **Enzymes** | Cyclooxygenase, Oxidase, Catalase, Lipase, Amylase, Alkaline phosphatase, Carboxypeptidase |
| **Vitamins** | $B_1$, $B_2$, B6, Choline, Folic acid, α-tocopherol, β-carotene, C |
| **Essential amino acids** | Lysine, Threonine, Valine, Leucine, Isoleucine, Phenylalanine, Methionine |
| **Nonessential amino acids** | Histidine, Arginine, Hydroxyproline, Aspatic acid, Glutamic acid, Proline, Salicylic acid, Glycine, Alanine, Tyrosine, Arachidonic acid |
| **Miscellaneous** | Cholesterol, Triglycerides, Steroids, Lignin, β-sitosterol, Uric acid, Lectin-like substance, Gibberellin |
| **Inorganic compounds** | Calcium, Chromium, Sodium, Chlorine, Copper, Manganese, Magnesium, Zinc, Iron |

**Table 3** Summarizes the active constituents of *Aloe vera* (Modified from Vogler and Ernst, 1999).

## PHARMACOLOGICAL EFFECTS OF *ALOE VERA*

Several studies have shown that many chemical substances that contains in aloe gel portion has vary pharmacological effects. Among of them is included a strong anti-inflammatory action by active substances in aloe gel such as polysaccharide, enzyme, certain amino acid, and vitamins. The prophylactic and curative effects of *Aloe vera* on gastric ulcer is one of the majestic effect of this plant, because it has mucopolysaccharide and glycoproteins content that are a synergistic action to exhibited antigastric ulcer (Grindlay and Reynolds, 1986; Blitz, 1963; Barry, 1983). According to the anti-inflammation and anti peptic ulcer action, it has been proposed that *Aloe vera* appears to exert cytoprotective via endogenous prostaglandins, which prevent the mucosa of the stomach becoming inflamed (Robert et al., 1979). The pharmacological action of *Aloe vera* on anti-inflammation and anti-gastric ulceration were summarized in the following

Moreover, the bactericidal activity of *Aloe vera* is one of several effects of this plant. It has been reported from the *in vitro* study that *Aloe vera* gel could inhibited growth on plates inoculated with *Staphylococcus aureus*, *Streptococcus pyrogens*, *Corynebacterium xerose* and *Salmonella paratyphi* (Lorenzetti et al., 1964). Robson and coworker (1982) has been reported the bactericidal of *Aloe vera* against a gram negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli* or gram positive bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*. However, from the *in vitro* study there was found that *Aloe vera* has no antibiotic properties for inhibited growth or bactericidal against *H.pylori*. *In vivo* bactericidal activity of *Aloe vera* against *H.pylori* has not been reported.

**Antiinflammatory activities**

*Aloe vera* has an important role in the traditional medicine of many contemporary cultures. The use of *Aloe vera* is being promoted for a range variety of conditions including stomachic, skin irritation, burn, dermatitis, arthritis, reduce swelling, peptic ulcer, antifungal, antimicrobial, and prevent infections (Grindlay and Reynold, 1986). Regarding the therapeutic effects of *Aloe vera* on inflammation, it was investigated in many studies as the following:

The anti-inflammatory effects of *Aloe vera* were reported. Shelton (1991) reported that *Aloe vera* gel has many solid components such as gibberellin which act like growth hormone could reduce inflammation, lectin-like substance that act as hemagglutination proteins to reduce inflammation, many vehicles such as petrolatum, mineral oil, and aqauphor all of which could inhibit arachidonic oxidation and to be reduce prostaglandin production, then it could reduce irritation and inflammation at ulcer area. Other active substances in *Aloe vera* such as amino acids, histidine, glutamic acid, leucine, and vitamins C and B, both amino acids and vitamins showed strong anti-inflammatory activities (Hanley et al., 1982). In year 1982 Saito et al. found that lectin aloctin A in *Aloe vera* was active against edema and adjuvant arthritis in rats. They had also found that lectin aloctin A had promoted mitosis in lymphocytes (Suzuki et al., 1979). Lymphocyte is known as a chronic inflammatory cell, activated lymphocytes could produce anti-inflammatory cytokine (IL-10), the latter may aid in anti-inflammation and promoted mitogenic activity which aid in tissue regeneration after wounding.

Several pharmacological studies have been performed in an attempt to identify active substance for anti-inflammatory action of *Aloe vera* in varying model. Robson et al. (1982) tested the effects of *Aloe vera* gel on thermal burn in a standard guinea pig experiment. The depth of dermal ischemia was measured by perfusion with indian ink and an immunohistochemical analysis was used to analyse levels of prostaglandins (PG) and thromboxanes (TX). The results were that *Aloe vera* had similar effects to the methylprednisolone and methimazole giving improved perfusion of capillaries and a reduction in thromboxane $B_2$ ($TXB_2$) and prostaglandin $F_2$ ($PGF_2$) which showed complete dermal ischemia by 24 hrs. Moreover, salicylic acid and inorganic compounds such as emodin, aloe emodin, and aloin, all of which can be broken down by the Kolbe reaction to form salicylates. Salicylates are both analgesic and anti-inflammatory, inhibiting the production of PG from arachidonic acid by inhibiting cycloxygenase (Moore and Hoult, 1982). Another chemical substances in *Aloe vera* gel were found such as anthraguinone, barbaloin, and aloe-emodin both of which are related compound of anthraquinone, found in sufficient quantities to act as false substrate inhibitors blocking prostanoid synthesis, since they have a similar chemical structure to PG substrates (Hegger and Robson, 1983). Penney (1982) found a number of vehicles compounds in lyophilize aloe gel and fresh gel, which was inhibited arachidonic oxidation *in vitro*, reflecting inhibition of PG synthesis, Because PG and TX are compounds involved in the long-term inflammatory response in damage tissue and have a number of different physiological effects including vasoconstriction and promotion of fever and pain. Then in clinical application to frostbite or inflammatory disease aloe could cause vasoconstriction, preserve the vasculature, and suppression fever and pain.

Magnesium lactate is one of many chemical substances that found in *Aloe vera*. In 1977 Hirata and Suga found that magnesium lactate could inhibit the *in vivo* conversion of histidine to histamine in mast cell by inhibiting histidine decarboxylase (Lehninger, 1981). Histamine, a known vasodilator, produces form mast cells, it could induce platelet aggregation and increase vascular permeability in inflamed tissue. Then inhibition of histidine decarboxylase by magnesium lactate in aloe may aid in inhibit inflammation. Similarly, Rubel et al. (1983) proposed that magnesium lactate in *Aloe vera* is an inhibitor of histidine decarboxylase thereby it might prevent histamine production and result in decreasing vasodilation and irritation in gastric mucosa. Moreover, glycoprotein alprogen which found in *Aloe vera* gel had similar effect as magnesium lactate because it could inhibit histamine and leukotriene production from activated mast cell that isolated from lung guinea pig (Ro et al., 2000).

Moreover, in *Aloe vera* gel, many enzymes were also found such as catalase, bradykinase, and carboxypeptidase. It has been reported that carboxypeptidase in *Aloe vera* gel could inhibit bradykinin production by hydrolyzing bradykinin and angiotensin I *in vitro*. Bradykinin is both a vasodilator and potent pain producing agent at site of acute inflammation. Then treated with *Aloe vera* may decrease pain at the site of acute inflammation (Fujita et al., 1979). The bradykinase was reported to be enzyme in *Aloe vera* gel that could hydrolyze bradykinin and angiotensin I to convert into angiotensin II, resulting in suppressing vasodilation and pain at site of inflammation (Rubel et al., 1983).

The anti-inflammatory activities of chemical substances in *Aloe vera* were also studied. *Aloe vera* and gibberellin was measured by measuring the inhibition of PMN infiltration into a site of gelatin-induced

inflammation. Both *Aloe vera* and gibberellin similarly inhibited inflammation. This result revealed that gibbrellin or a gibbrellin-like substance is an active anti-inflammatory compound in *Aloe vera* (Davis and Moro, 1989). The sprectrum of anti-inflammatory activity of *Aloe vera* was evaluated in a number of models of inflammation in the hind paw of the experimental rat induced by kaolin, carragenan, albumin, dextran, gelatin, and mustard. They found that *Aloe vera* provided a broad sprectrum of anti-inflammatory activity in the various irritant-induced edema rat model (Davis et al., 1989). Furthermore, Davis et al. (1994) showed that the *Aloe vera* sterols, lupeol, campesterol, and B-sitosterol had significantly anti-inflammatory effects.

Therapeutic effects of *Aloe vera* on cutaneous microcirculation in second degree burn rats were studied by Somboonwong et al. in 2000. Burn wound rats were treated with once daily application of normal saline or lyophilized *Aloe vera* gel for 7 and 14 days. The effects of *Aloe vera* on cutaneous microcirculation including leukocyte adhesion, vascular permeability, and arteriolar diameter were measured by intravital fluorescent microscopy. On day 7 *Aloe vera* could significant reduce vascular permeability and arteriolar diameter but leukocyte adhesion was not different among the normal saline solution (NSS) and *Aloe vera* treated groups. On day 14, vasoconstriction occurred after the wound had been left untreated. Only in the *Aloe vera* treated groups, arteriolar diameter was increased up to normal condition and postcapillary venular permeability was not difference from the control group. The amount of leukocyte adhesions were less than control and NSS treated groups. Besides, the healing area of *Aloe vera* treated wound was more than those of the control and NSS treated ones during 7 and 14 days after burn. Then it can conclude that *Aloe vera* could exhibit the action of both anti-

inflammation and wound healing promotion when applied on burn wound. According to the anti-inflammatory activity of *Aloe vera* in second burn rats Duansak et al. (2003) demonstrated that application of *Aloe vera* gel in burn wound rats once daily at three days interval 3, 7, and 14 days, *Aloe vera* could significant reduce leukocyte adhesion on day 14. It also found that *Aloe vera* could significant reduce TNF-α and IL-6 levels at a three monitored time points. Then *Aloe vera* could inhibit the inflammatory process following burn injury.

**Therapeutic effects of *Aloe vera* on peptic ulcer disease**

*Aloe vera* gel has been used to treat a variety of gastrointestinal conditions. In Thai Pharmacopeia suggested that anthraquinone glycosides that found in latex portion of *Aloe vera* could improve indigestive disease.

In Aloe gel portion, *Aloe vera* has been used taken internally to treat peptic ulcers. Twelve patients diagnosed as suffering from peptic ulcer, confirmed by x-ray evidence of duodenal lesions, were given an emulsion of *Aloe vera* in petrolatum. After complete cures, the x-ray examination showed complete healing. The author proposed that *Aloe vera* gel was attributed to inhibition of hydrochloric acid (HCl) secretion and a general detoxifying effect (Blitz et al., 1963). Later in 1977 Hirata and Suga demonstrated that several biologically active substances such as aloenin, magnesium lactate, aloe-emodin, barbaloin, and succinic acid were found in *Aloe vera* could exhibit an inhibitory action on the gastric juice secretion of rats. Aloctin A is a glycoprotein isolated from *Aloe vera* was used to examine it effects on gastric secretion and on acute gastric lesion in rats. Aloctin A was given intravenously significantly inhibited

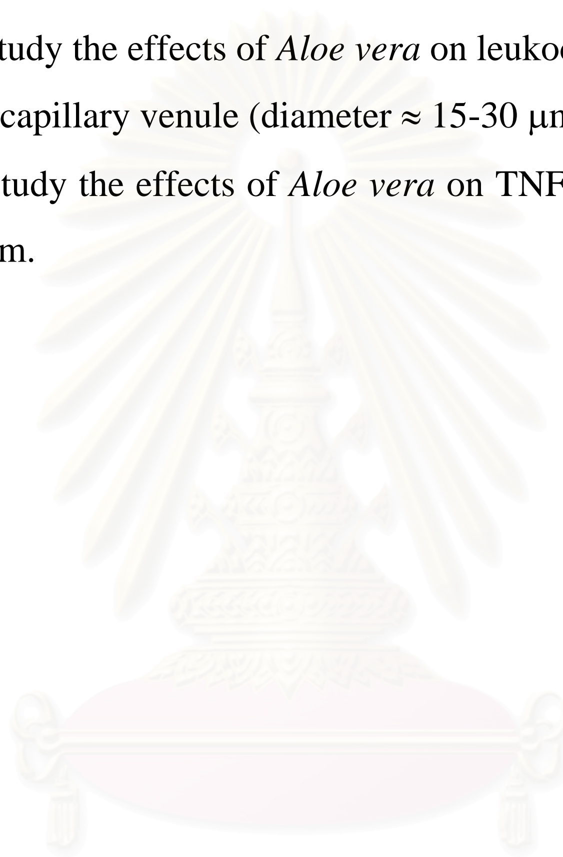
the development of ulcers and indomethacin-induced gastric lesions in rats. It also inhibited water-immersion stress lesions induced in pylorus-ligated rats. Moreover, it has been reported that mucilaginous gel from the pulp extract given by oral administration has prophylactic and curative effect against gastric lesion induced by aspirin irritation and on nervous stress in rats. This effect is due to its protection of gastric mucosa and its support of mucosal resistance against the sequalae of chemical irritation and nervous stress without interfering with the gastric pH (Galal et al., 1975; Kandil and Gobran, 1982). According to Robert et al. 1979 they proposed that *Aloe vera* appears to exert cytoprotective effect via endogenous PG, which prevent the mucosa of the stomach becoming inflamed and necrotic or maintaining the cellular integrity of the gastric mucosa when exposed to noxious agents. Mucopolysaccharide and glycoproteins content that found in aloe gel are a synergistic action that is responsible for the antigastric ulcer action (Grindlay and Reynolds, 1986; Blitz, 1963; Barry, 1983) which act like the gastric mucin to protect the gastric mucosa from damage, the antithromboxane B2 effect of the gel which cause reduction of vasoconstriction and give improved perfusion of gastric mucosal capillaries and the glycoproteins, namely Aloctin-A or Lectin P-2, which have a healing effect on the produced ulcer (Mahattanadul, 1995). Recent study, Mahattanadul (1995) evaluated the prophylactic and curative effects of *Aloe vera* on gastric ulcer induced by cytodestructing agents, 0.6 N HCl and acetic acid (30% and 100%) in rats. *Aloe ver* were prepared in different forms; freshly gel; fresh freezed-dried powder (changed from gel by freeze dry method using lyophillizer) and two months prestored freeze-dried powder. All of three forms had similar antigastric ulcer effects. The results shown that *Aloe vera* dose of 400 mg/kg/day given per oral had significant efficacy both in protecting gastric mucosa against the injuries caused by necrotizing agent, 0.6 N

HCl, and in healing ulcers already induced by acetic acid (30% and 100%). In comparison of the curative effect of *Aloe vera* with that of the standard cytoprotecting agent, sucralfate, they used the same treatment and the same dose, all three preparations of *Aloe vera* gel showed the same curative efficacy as sucralfate on gastric ulcer induced by acetic acid. These findings suggested that *Aloe vera* gel exerts antigastric ulcer action by directly protecting the gastric mucosa and exerts cytoprotective activity associated with an enhancement of local healing process. According to the prophylactic effect of *Aloe vera*, it could protect gastric mucosa against the injuries caused by necrotizing agent. Recently, it has been reported the effect of ethanol extract of *Aloe vera (Liliaceae)* on acute mucosal lesions induced by 0.6 M HCl and on acid output in pylorus ligated and lumen perfused rats. They found that *Aloe vera* could protect the gastric mucosa and is endowed with gastric acid anti-secretory activity by dose dependently (Yusuf et al., 2004).

From the literature reviews mentioned above, it was obvious that *Aloe vera* has been found to contain various kinds of active components. It could be reduced inflammation, preserved balance of microcirculation, affect to cytokines levels, and have prophylactic and curative effects on gastric ulcer. However, the anti-inflammatory activity of *Aloe vera* on gastric microcirculation and cytokines levels in *H.pylori* infected rats have not been study. Therefore, it is the hypothesis of the present study that the active components in *Aoe vera* gel may reduce changes in gastric microcirculation, reduce TNF-$\alpha$, and increase IL-10 levels in serum of *H.pylori* infected rats.

**Therefore, the objectives of this study are:**

1) To study the effects of *H.pylori* infection on leukocyte adhesion in postcapillary venule (diameter ≈ 15-30 μm).
2) To study the effects of *H.pylori* infection on TNF-α and IL-10 levels in serum.
3) To study the effects of *Aloe vera* on leukocyte adhesion in postcapillary venule (diameter ≈ 15-30 μm).
4) To study the effects of *Aloe vera* on TNF-α and IL-10 levels in serum.



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# CHAPTER III

# MATERIALS AND METHODS

## CHEMICAL SUBSTANCES

Aloe gel powder [Lipo, chemical (USA.)] 99.5%
Streptomycin (M&H Manufacturing Co., Ltd)
Fluorescein marker acridine orange (Sigma Chemical Co.)
Heparin
Sodium pentobarbital
Formalin solution
Phosphate buffer solution
Sterile normal saline solution
Distilled water

## ANIMAL PREPARATIONS

A total of 36 male Spraque-Dawley rats weighing 200 to 250 grams were used in this study. The animals were divided into three groups of twelve animals each as the following:

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### Control group

The animals were received NSS, which is a transporter media of *H.pylori* at a volume of 1 ml/rat by gavage twice daily at an interval of four hrs for three consecutive days. After that animals were gavaged with NSS the animals were housed with free access to water and standard

chow two weeks. Then the animals were treated with distilled water, a vehicle of *Aloe vera* (1ml/rat) by gavage twice daily until the day of performing intravital fluorescent microscopy.

***H.pylori* infection with distilled water treatment group (*H.pylori* infection group)**

The animals were inoculated with *H.pylori* by using the model of Thong-Ngam et al. (2005). Briefly, the *H.pylori* suspension (about $10^8$ to $10^{10}$ CFU/ml; 1 ml/rat) was given to the rats by gavage twice daily, with an interval of four hrs, for three consecutive days. Two weeks after *H.pylori* inoculation, the animals were treated with distilled water at a volume of 1 ml/rat by gavage twice daily at an interval of four to six hrs until the day of performing intravital fluorescent microscopy.

***H.pylori* infection with *Aloe vera* treatment group (*Aloe vera* treated group)**

In order to make the *H.pylori* infection, the same protocol was performed as the previous model. After two weeks of *H.pylori* inoculation, the animals were treated with *Aloe vera* gel at a dose of 200 mg/kg BW suspended in distilled water by gavaged twice daily with an interval of four to six hrs, until the day of performing intravital fluorescent microscopy (Modified from Mahattanadul, 1995).

*Aloe vera* used in this study was "Lyophilized *Aloe vera* gel", prepared by the Lipo Chemical Co. USA. Lyophilization was a process for the preparation of dried *Aloe vera* gel with unchanged properties (Meadow, 1983).

The animals in each group were equally subdivided into two subgroups for the experimental periods of third and eight days after distilled water or *Aloe vera* treatment. For further evaluation of gastric microcirculatory changes, intravital microscopic technique was performed (Menger and Lehr, 1993). The procedures of animal preparation were concluded in diagram as shown in Figure 9.

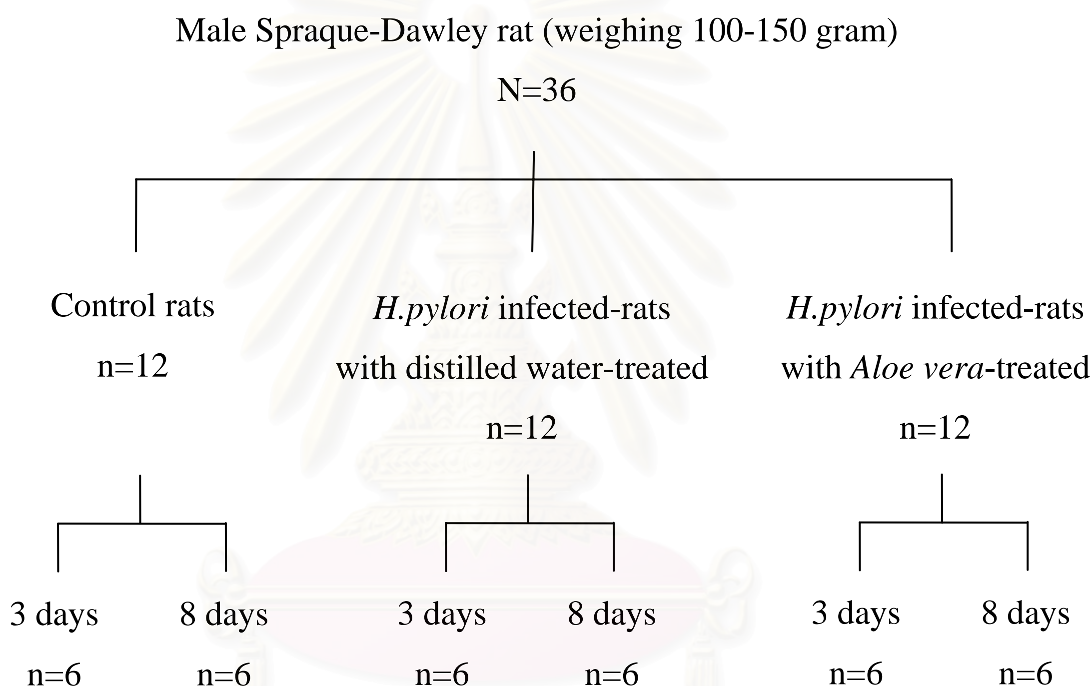


**Figure 9** Diagram of experimental animal groups.

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## METHODS

### *H.PYLORI* INOCULATION IN THE RAT STOMACH

The *H.pylori* suspension (about $10^8$ to $10^{10}$ CFU/ml) in NSS was given to the rats (1ml/rat) by gavage twice daily (Figure 10), with an interval of four hrs, for three consecutive days. Three days before the first *H.pylori* inoculation, the rats were free access to 5 mg/ml streptomycin suspended in tap water. During *H.pylori* inoculation, the animals were fasted overnight but free access to tap water (Thong-Ngam et al., 2005). After the last inoculation these animals were housed with free access to tap water and standard laboratory chow for two weeks. It had reported that, two weeks after inoculation, *H.pylori* could induce mild to moderate gastritis, dominated by infiltration of macrophages and lymphocytes into lamina propria (Li et al., 1999, Thong-Ngam et al., 2005).

### EXPERIMENTAL PROTOCOL FOR INTRAVITAL FLUORESCENCE MICROSCOPIC STUDY OF GASTRIC MICROCIRCULATORY CHANGES

On the day of experiment, the animal was anesthetized with intraperitoneal injection (ip) of 45 mg/kg BW of sodium pentobarbital. A constant level of anesthesia was maintained throughout the experiment by intraperitoneal injection of supplement dose (20% of original dose) of the anesthetic agent every 30-45 minutes (Koller and Kaley, 1990).

Under spontaneous respiration, the trachea was canulated to facilitate respiration. Then a fine polyethylene catheter (PE 10, inner diameter 0.28 mm) was inserted into the right common carotid artery

until it reached the aortic arch. The common carotid arterial pressure (CAP) was recorded via this catheter by using pressure transducer (Nikon model RM 6000). The other catheter was placed into jugular vein for injection of fluorescent (fluorescein marker acridine orange, 0.5 mg/kg BW) into the blood stream. Both catheters were filled with heparinized saline.

After laparotomy, a 1.0 cm incision was made by scissors in the anterior wall of the exteriorized stomach parallel to the “limiting ridge”. Observations were made from the glandular portion of stomach. Care was taken to avoid injurious through blood vessels, the stomach was gently extended and held in place by a stay suture as shown in Figure 11. During the surgery, the area was kept by allowing drops of the warmed normal saline to fall on the incision. The body temperature of the animal was kept constant at 36-37 $^{o}$C by mean of heating pad. After preparing the stomach for fluorescent *in vivo* microscopy the animal was places on microscopic stage of the fluorescent microscope equipped with transillumination and epiillumination optics (Nikon Optiphol-2). After intravenous application of acridine orange, epiillumination was achieved with a 50 W, mercury lamp with a 488 ηm attached to excitation filter and 515 emission barrier filters. An intravital microscope with a 40x long working distance objective (CF Achromat) were to observe microvessels in the stomach. A video camera mounted on the microscope projected the image onto a black-white monitor. The images of microvessels were stored on videotape (Sony, SLV-X311) for playback analysis using a video cassette recorder. A videotape connected to a video timer (UTG-33) for time later recorder. During the experiment, microvessels images could be printed by using video graphic printer (Sony, UP-890 CE). All

instruments used for quantitative studies of hemodynamic and the morphologic parameters in microcirculation were shown in Figure 12.

**Studies of leukocyte-endothelium interaction in postcapillary venule**

To observe the adherent leukocyte in postcapillary venule, the intravital microscope with a 40x long working distance objective (CF Achromat) were used to observe the microvessels in muscle layer at the body region of the stomach (Figure 11). Anatomical of blood vessel that supply the body region of stomach were observed for a landmark in every animals. After 15 minutes of equilibration period, the fluorescent marker, acridine orange was infused intravenously (Sigma chemical Co., USA, 0.5 mg/kg BW) for 5 minutes (Lehr et al., 1991) for visualized the adherent leukocyte. During experiment, leukocytes were recorded on videotape for further observation of leukocyte adhesion.

Videotape of each experiment was played back and then adherent leukocytes on postcapillary venules (diameter ≈ 15 to 30 μm) were counted by visual observation. Adhesive leukocytes were defined as cells which did not move or detach from the endothelial lining within the entire observation for a period equal to or greater than 30 seconds whereas rolling leukocytes were defined as nonadherent leukocytes passing through the observed vessel segment within the observation period (Gaboury and kubes, 1994). The image analysis that used to count number of adherent leukocyte was showed in Figure 13.

## Limitation of this experiment

### *H.pylori* induce gastric inflammation but not ulceration in rat gastric mucosa

To induce *H.pylori* infection in this study we used the model of Thong-Ngam and coworker (2005). There reported that two weeks after *H.pylori* infection the mild to moderate gastric inflammation were developed as detected under light microscopy by pathophysiologist. However, the gastric ulceration was not observed in *H.pylori* infected rat. In our experiment, we also did not found gastric ulcer in experimental animal model. According to many studies which had reported that inoculation with *H.pylori* organisms to normal rat stomach resulted in mild to moderate gastric inflammation but no ulceration in gastric mucosa. They thought that rats can presumably adapt to the infection but the mechanisms remain unknown (Zeng et al., 1998; Li et al., 1999).

### Macromolecular leakage was not observed in this study

For observe MML or leukocyte adhesion in postcapillary venule at body region of stomach. The availability of different fluorescent markers for *in vivo* study are fluorescein isothiocyanate dextran (MW 150,000; 15 mg/kg BW) (FITC-dextran)-labeled plasma and acridine orange [acridine orange hemi (zinc chloride) salt; 0.5 mg/kg BW] to stain mitochondria especially in leukocyte. As we try in our experiment, we did not observed MML because after intravenous application with FITC-dextran, it made brightness in plasma and the protein leakage were occurred rapidly within 15 minutes. These change is the cause that we could not observe leukocyte activity. Moreover, in inflamed tissue the reaction from

microcirculation is changes of leukocyte activity, which is the cause of MML. Then we only observed the adherent leukocyte.

**Figure 10** Inoculation of *H.pylori* organisms to rat's stomach by gavage

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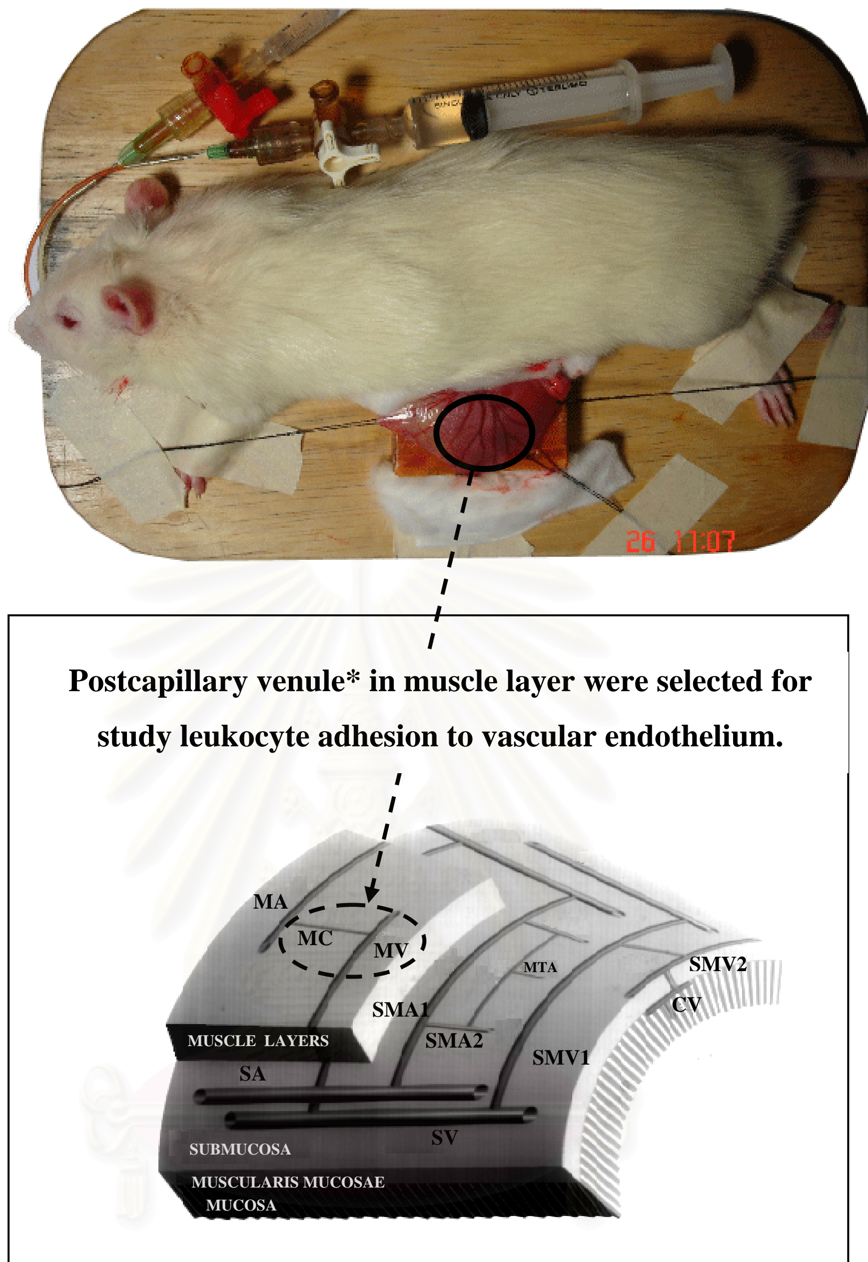


**Figure 11** Preparation model for study leukocyte activity on gastric postcapillary venules in muscle layer at body region of the stomach.

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*The PVCs were selected to study including:

1) Inner diameters are 15-30 μm.

2) Blood flow in PCV must collect from branch of capillary end and flow directed to the collecting venule.

3) PCVs are located at the body region of stomach.

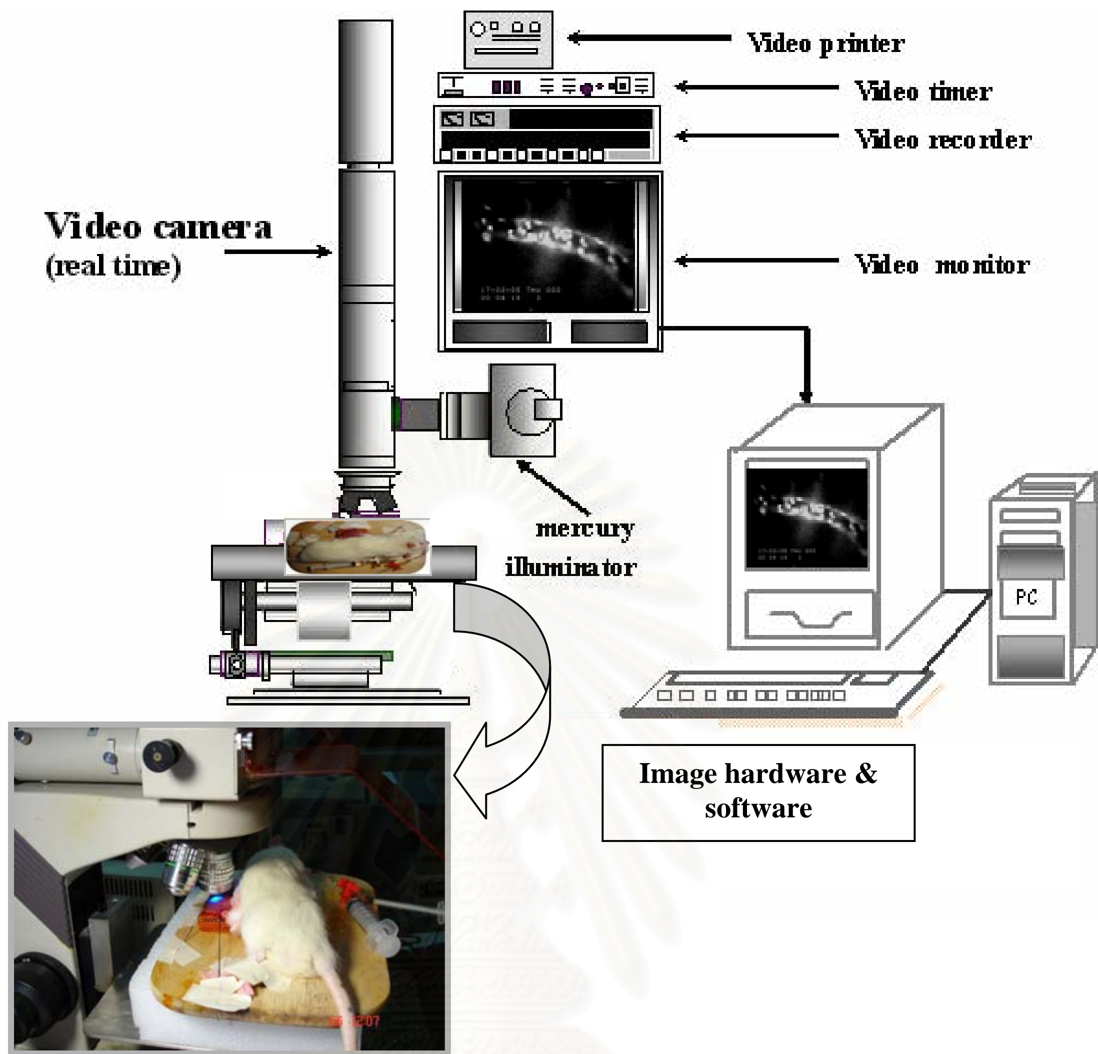
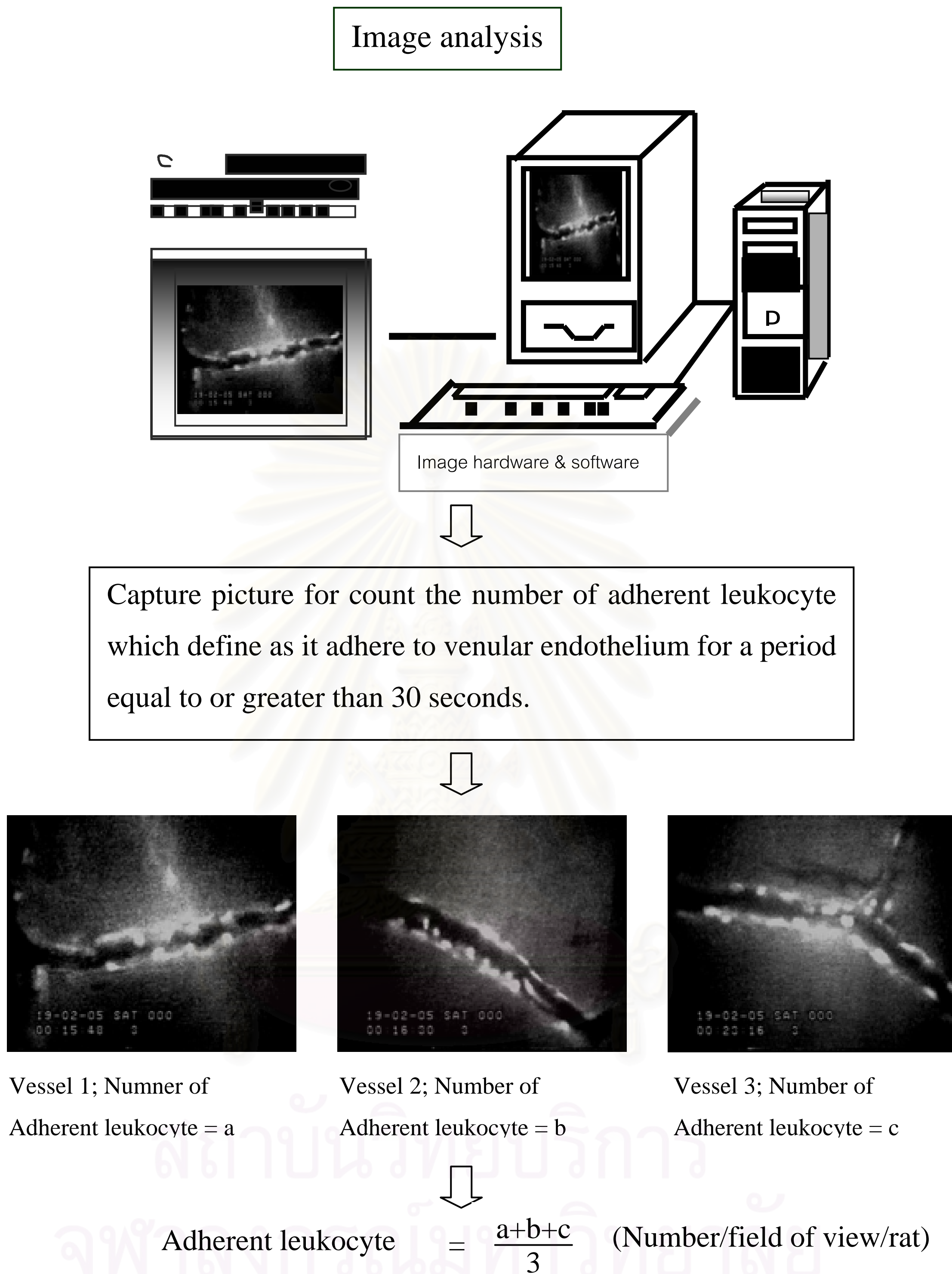



**Figure 12** Intravital fluorescence microscopy and instrument used for quantitative studies of leukocyte adhesion in microvasculature.




The result from each group was expressed as mean of 6 tests on 6 rats.

**Figure 13** Image analysis used for determined number of adherent leukocyte.

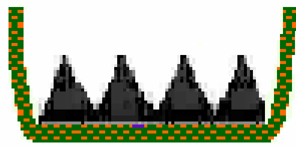
**ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)**

Enzyme-linked immunosorbent assay (ELISA) is a useful method in estimating the concentration of antibody or some other protein in a sample such as culture supernatant, urine, plasma, and serum. ELISA is performed in 96 well plates which permits high throughput results. The bottom of each well is pre-coated with a protein (called inactivated antigens) to which will bind the antibody that we want to measure.

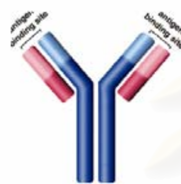
To measure TNF-α and IL-10 levels in serum, blood was collected by cardiac puncture after finishing the intravital fluorescent procedure. Then blood sample was allowed to clot for 2 hrs at room temperature. Clotted blood was brought to centrifugation for 20 minutes at approximately 2000g. Then serum was removed and stored at -70 $^{o}$C until the day of analysis.

At analyze, the serum with antibodies (called primary antibodies) is incubated in a well. After some time, the serum is removed and weakly adherent antibodies are washed off with a series of buffer rinses. To detect the bound antibodies, a secondary antibody is added to each well. The secondary antibody would bind to all antibodies. Attached to the secondary antibody is an enzyme conjugate, which can metabolize colorless substrates into colored products. After an incubation period, the secondary antibody solution is removed and loosely adherent ones are washed off as before. The final step is the addition of the enzyme substrate and the production of colored product in wells with secondary antibodies bound. The steps of reactions were shows in Figure 14. When the enzyme reaction is completed, the entire plate is placed into a plate reader and the optical density (O.D.) is determined for each well. The

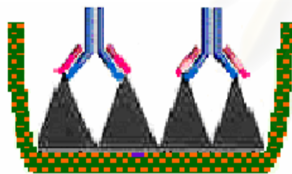
amount of color produced is proportional to the amount of primary antibody bound to the proteins on the bottom of the well.



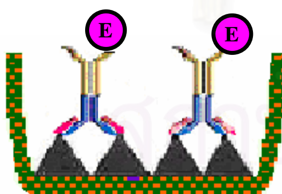
Antigen pre-coated at bottom of each well



Serum contains antibody



Serum which contains antibodies bind to the antigen on the plate




Secondary antibodies which couple to an enzyme bind to the antibodies

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**Figure 14** Show ELISA activities.

**TNF-α assay**

The ELISA kit of R&D systems, Inc. (Minneapolis, MN, USA) was used to determine level of TNF-α in serum. The assay procedures were performed as following:

**Assay procedures**

All samples, standards and controls were assayed in duplicate.

Step 1. The reagent, standard, and samples were prepared as introduce in the reagents preparation part.

Step 2. Add 50 µl of assay diluent RD1-41 to each well.

Step 3. Add 50 µl of standard, control or sample to each well. Mix by gently tapping the plated frame for 1 minute. Cover with the adhesive strip and incubate for 2 hrs at room temperature.

Step 4. Aspirate each well and wash, this process were made repeatly fourtimes for a total of five washes. Wash by filling each well with wash buffer (400 µl) using an autopipette. After the last wash, the remaining wash buffer was removed by aspirating or by inverting the plate and blotting it against clean paper towels.

Step 5. Add 100 µl of rat TNF-α conjugate to each well. Then cover with a new adhesive strip. Incubate for 2 hrs at room temperature.

Step 6. The aspirate/wash as in step 5 was made repeated.

Step 7. Add 100 µl of substrate solution to each well. Incubate for 30 minutes at room temperature and must protect from light.

Step 8. Stop reaction by add 100 µl of stop solution to each well. The plate was tapped gently to ensure through mixing.

Step 9. The O.D. of each well was determined within 30 minutes, by used a microplate reader set at 450 ηm of spectophotometer.

**IL-10 assay**

Serum level of IL-10 was determined by using the ELISA kit of R&D systems, Inc. (Minneapolis, MN, USA). The experimental protocol used for IL-10 assay is similar to that of TNF-α assay. However, some reagent and reagent concentration were different.

For calculation of results, O.D. of each standard, control, and sample were read in duplicate. Each O.D. and standard concentration were convert to logistic parameter. Then bring to construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw fit curve through the point on the graph for obtained the equation. Bring the equation from graph to calculation TNF-α or IL-10 value.

The standard curves and calculation method for TNF-α and IL-10 were shown in Figure 15 and 16, respectively. The measured O.D. (450 ηm) of each unknown was then converted to its corresponding concentration by using these standard curves.

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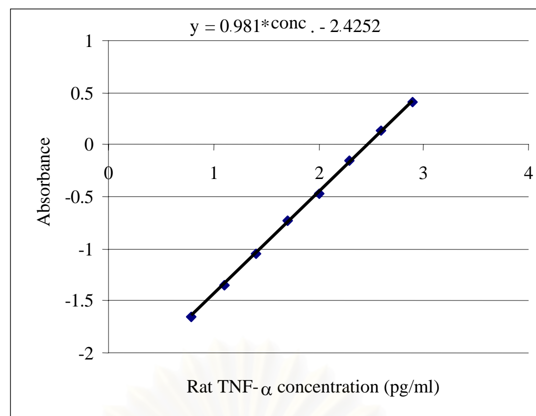
The standard curve of TNF-α




**Calculation method for TNF-α value**

Average O.D. blank = 0.037 ………………..….data from our experiment

O.D.1 of sample = 0.24 …………………..data from *H.pylori* infected rat

Corrected O.D. = O.D.1 of sample - average O.D. blank

= 0.24 - 0.037 → 0.203 → log 0.203 = -0.693

Bring -0.693 to instead in the equation from the standard curve of TNF-α

-0.693 = 0.981*conc. - 2.4252

conc. = 1.766 ………………………………………logistic data

Antilog = 58.345 pg/ml

O.D.2 of sample = 0.293 …………………data from *H.pylori* infected rat

Corrected O.D. = 0.293 - 0.037 →0.256 → log 0.256 = -0.592

Bring -0.592 to instead in the equation from standard curve of TNF-α

-0.592 = 0.981*conc. - 2.4252

conc. = 1.869 ……………………………………...logistic data

Antilog = 73.961 pg/ml

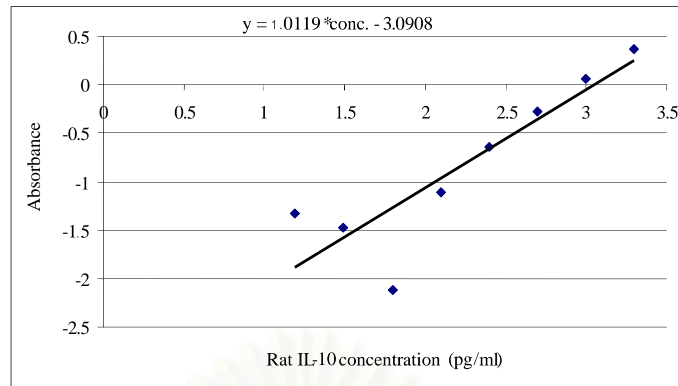
Mean of TNF-α $= \frac{58.345 + 73.961}{2}$ pg/ml/rat

TNF-α = 66.153 pg/ml/rat

The result from each group was expressed as mean of 6 tests on 6 rats.

**Figure 15** The standard curve of TNF-α and calculation method.

The standard curve of IL-10




**Calculation method for IL-10 value**

Average O.D. blank = 0.061...…………………data from our experiment

O.D.1 of sample = 0.649 …………………data from *H.pylori* infected rat

Corrected O.D. = O.D.1 of sample - average O.D. blank

= 0.649 - 0.061 → 0.588 → log 0.588 = -0.231

Bring -0.231 to instead in the equation from standard curve of IL-10

-0.231 = 1.0119*conc. - 3.0908

conc. = 2.827 …………………………..…………logistic data

Antilog = 671.429 pg/ml

O.D.2 of sample = 0.663 …………………data from *H.pylori* infected rat

Corrected O.D. = 0.663 - 0.061 → 0.602 → log 0.602 = -0.220

Bring -0.220 to instead in the equation from standard curve of IL-10

-0.220 = 1.0119*conc. - 3.0908

conc. = 2.837 ……………………………………..logistic data

Antilog = 687.068 pg/ml

Mean of IL-10 $= \frac{671.429 + 687.068}{2}$ pg/ml

IL-10 = 679.249 pg/ml

The result from each group was expressed as mean of 6 tests on 6 rats.

**Figure 16** The standard curve of IL-10 and calculation method.

## DETECTION OF *H.PYLORI* ORGANISMS IN GASTRIC TISSUES

After all parameters were collected, gastric tissues from *H.pylori* infection groups were taken for examined the organism. The present of *H.pylori* was determined by positive rapid urease test and positive histopathology.

### Enzymatic test by using rapid urease test

After blood were sampled for detection cytokines levels. The animal was sacrificed with intravenous infusion of 45 mg/kg BW by used overdose of sodium pentobarbital. At death, the stomach was removed and opened along the greater curvature. The mucosal side was excised and sterile normal saline was used to remove the exudates in gastric mucosa. The gastric mucosa was cut by scissors from the antrum because *H.pylori* most colonized in this area (Li et al., 1999). Gastric specimens were placed in contact with a gel that contains urea and phenol red (a pH indicator). If animal were infected with *H.pylori*, this organism will produces large amounts of urease. The enzyme converts urea into ammonia and $CO_2$ with a resultant increase in the pH of the medium and gives a pink color as shown in Figure 17. Rapid urease tests generally are read periodically over 2 to 3 hrs after the biopsy and then again at 24 hrs. The urease test has sensitivity and specificity in the 86% to 97% range and 86% to 98% range respectively (Nakamura, 2001).

### Histopathology

After gastric tissues were taken for urease test, the remaining gastric tissues were fixed in 10% formaldehyde in 0.2 M sodium

phosphate buffer, pH 7.4 at room temperature. Stomach was processed by standard methods. Briefly, gastric tissues were embedded in paraffin, sectioned at 5 μm thickness. Then tissues specimens were stained with H&E and picked up on glass slides for light microscopy for detection *H.pylori* in gastric mucosa. In the cases that unclear, the presence of *H.pylori* were detected with Warthin-Starry staining.

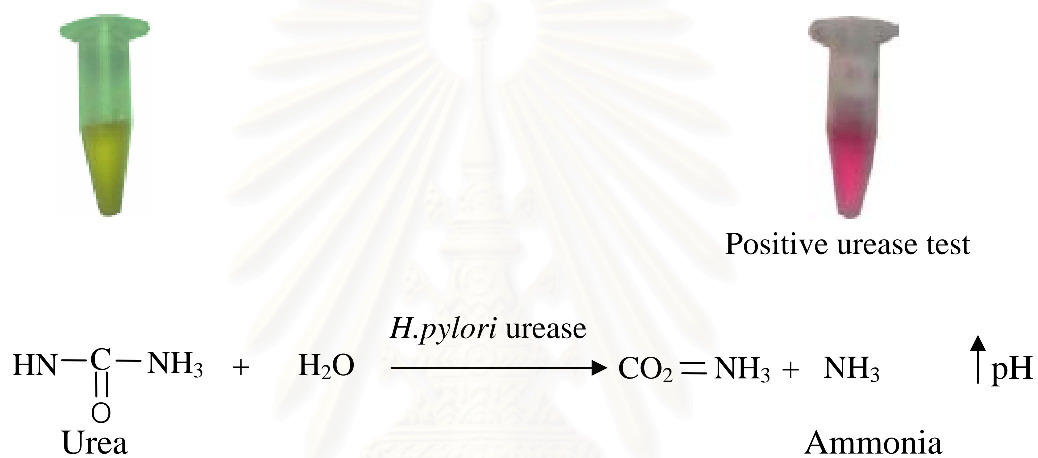



**Figure 17** Rapid urease test: presence of *H.pylori* urease is indicated by ammonia production.

## STATISTICAL ANALYSIS

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Results were expressed as means ± standard error. One-way analysis of varience (ANOVA) was performed to examine the different of each parameter between control group, *H.pylori* infection group, and *Aloe vera* treated group.

# CHAPTER IV

# RESULTS

## *H.PYLORI* INFECTION IN RAT STOMACH

Induce *H.pylori* infection to rat's stomach in the present study using the previous model (Thong-Ngam et al., 2005) was found that, *H.pylori* was detected by positive urease test 19 of 23 rats (82.61%) in *H.pylori* infection groups and were positive 17 of 20 rats (85%) in *Aloe vera* treated groups. There were all negative results in control groups (Table 4).

By microscopic examination, the results were concordance to the rapid urease test. *H.pylori* were observed mainly in gastric pits of the antrum from 36 *H.pylori* infected rats (19 in *H.pylori* infection groups; 82.61% and 17 in *Aloe vera* treated groups; 85%) (Figure 18 and 19). There were both in the lumen and on the surface of epithelial cells. No *H.pylori* were found in gastric pits of the control groups.

## EFFECTS OF *H.PYLORI* INFECTION ON PHYSIOLOGICAL CHARACTERISTIC AND ROLE OF *ALOE VERA*

Two weeks after *H.pylori* inoculation into the stomach of 100-150 grams Spraque-Dawley rat. The physiological characteristics of animal were expressed as the changes of hemodynamic and body weight (BW). At the day of experiment the animals were detected the hemodynamic parameters including systolic blood pressure (SBP), diastolic blood

pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) by using pressure transducer. The means and the standard error of means of SBP, DBP, MAP, and HR were shown in Table 5, 6, 7, 8 and Figure 20, 21, 22, 23, respectively. The results indicated that on day 3 there were not significant difference between the SBP in control group, *H.pylori* infection group, and *Aloe vera* treated group (111.78 ± 2.36, 121.34 ± 2.63, 113.11 ± 1.74 mmHg, respectively). On day 8 the SBP were significantly increased in *H.pylori* infection group and *Aloe vera* treated group (123.78 ± 4.37, 118.89 ± 3.71 mmHg, respectively) when compared to control group (108.17 ± 4.22 mmHg, $p<0.05$), however in *Aloe vera* treated group the SBP was slightly decreased but not significant difference to *H.pylori* infection group. On day 3 the DBP was not significant difference among the control group, *H.pylori* infection group, and *Aloe vera* treated group (94.11 ± 2.76 mmHg, 103.00 ± 5.33, 100.56 ± 4.64, respectively). On day 8 the DBP in control group, *H.pylori* infection group, and *Aloe vera* treated group (93.17 ± 2.48, 100.72 ± 1.55, 96.67 ± 1.41 mmHg, respectively) was concordance to day 3. The MAP were significantly increased in *H.pylori* infection groups on day 3 and day 8 (109.11 ± 4.28, 108.41 ± 2.11 mmHg, respectively) compared to day 3 and day 8 control groups (99.99 ± 2.18, 98.17 ± 2.93 mmHg, respectively, $p<0.05$), in *Aloe vera* treated groups MAP were slightly decreased both on day 3 and day 8 after treatment (104.74 ± 3.57, 104.07 ± 2.11 mmHg, respectively). The HR on day 3, there were not significant difference between the HR in control group, *H.pylori* infection group, and *Aloe vera* treated group (406.67 ± 9.89, 373.33 ± 6.67, and 390.00 ± 13.42 beats/min, respectively). On day 8 the HR in *H.pylori* infection group were significantly decreased (339.00 ± 19.07 beats/min) when compared to control group (403.33 ± 6.15 beats/min, $p<0.05$), while *Aloe vera* treated group (370.00 ± 22.95 beats/min) were not significant

difference from *H.pylori* infection group and control group. The body weight on day 3 was not significant difference among the control group, *H.pylori* infection group, and *Aloe vera* treated group (230.83 ± 5.61, 227.67 ± 8.28, 223.33 ± 11.49 kg, respectively). According to day 8 there were not significant difference between BW in control group, *H.pylori* infection group, and *Aloe vera* treated group (242.00 ± 4.78, 239.17 ± 9.25, 240.67 ± 10.29 kg, respectively). The data were shown in Table 9 and Figure 24.

**EFFECTS OF *H.PYLORI* INFECTION ON LEUKOCYTE ADHESION AND ROLE OF *ALOE VERA***

The intravital fluorescence microscopic technique was used to determine amount of leukocyte adhesion in control groups, *H.pylori* infection groups, and *Aloe vera* treated groups. The adherent leukocyte was defined when the cell adhere to endothelium of postcapillary venule remained stationary for at least 30 seconds and expressed as the number of cells per field of view. The means and the standard error of means were shown in Table 10 and Figure 25. The results were found that, two weeks after *H.pylori* infection followed by gavage with vehicle or *Aloe vera* for 3 and 8 days. The leukocyte adhesion on day 3 and day 8 were significantly increased in *H.pylori* infection group (10.10 ± 0.67, 13.40 ± 1.00 cells/field, respectively) when compared to control group (2.23 ± 0.48, 2.47 ± 0.25 cells/field, respectively, $p<0.01$). In *Aloe vera* treated group, leukocyte adhesion on day 3 and day 8 (5.81 ± 0.63, 5.45 ± 0.51 cells/field, respectively) were significantly reduced when compared to *H.pylori* infection group ($p<0.01$). Then *H.pylori* infection could induce increased the leukocyte adhesion to endothelium of postcapillary venule. Treatment with *Aloe vera* could reduce amount of leukocyte adhesion.

The intravital microscopic demonstration of leukocyte adhesion in control groups, *H.pylori* infection groups, and *Aloe vera* treated group on day 3 and day 8 after treatment were shown in Figure 26 and 27.

## EFFECTS OF *H.PYLORI* INFECTION ON TNF-α AND IL-10 LEVELS IN *H.PYLORI* INFECTED RAT AND ROLE OF *ALOE VERA*

TNF-α and IL-10 levels were detected by ELISA technique, the means and the standard error of means in control groups, *H.pylori* infection groups, and *Aloe vera* treated groups were shown in Table 11, 12, and Figure 28, 29.

For TNF-α level, the results indicated that in *H.pylori* infection group TNF-α levels on day 3 and day 8 (61.98 ± 18.74 pg/ml, 76.76 ± 23.18 pg/ml, respectively) were significant higher than those of day 3 and day 8 control groups (8.65 ± 1.79, 9.92 ± 2.62 pg/ml, respectively, $p<0.05$). In *Aloe vera* treated group, TNF-α levels were significantly reduced on day 3 and day 8 (14.52 ± 5.53, 26.31 ± 6.38 pg/ml, respectively, $p<0.05$) when compared to the *H.pylori* infection group. There were not significant difference between TNF-α level in *Aloe vera* treated group and control group on day 3 and day 8.

For IL-10 level, the results were shown that there were not significant difference between IL-10 levels on day 3 and day 8 in control group (256.80 ± 47.66, 383.99 ± 62.58 pg/ml, respectively), *H.pylori* infection group (266.66 ± 128.01, 663.60 ± 105.33 pg/ml, respectively) and *Aloe vera* treated group (275.05 ± 115.84, 681.17 ± 197.49 pg/ml, respectively).

Thus *H.pylori* infection could induce the increasing of TNF-α level. *Aloe vera* could reduce TNF-α level, but have no affect on IL-10 level at two monitored time points.

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**Table 4** *H.pylori* infection detected by urease test and histopathology.

| Group | Number | Urease test | | Histopathology | | Succesful rate |
|---|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative | |
| Control groups | 12 | 0 | 12 | 0 | 12 | 0% |
| *H.pylori* infection groups | 23 | 19 | 4 | 19 | 4 | 82.61% |
| *Aloe vera* treated groups | 20 | 17 | 3 | 17 | 3 | 85% |

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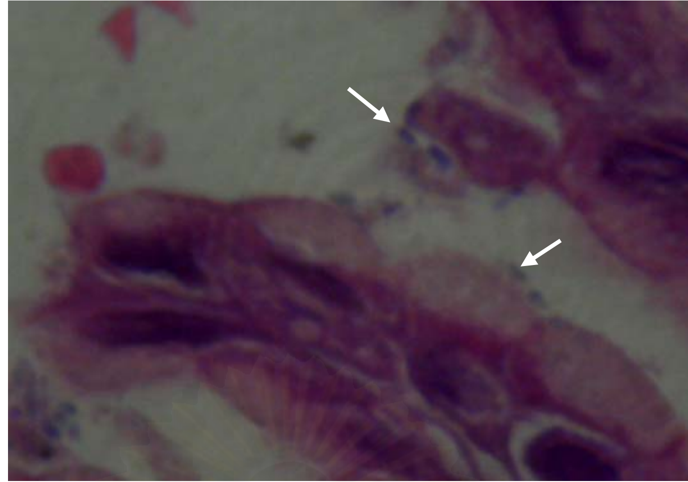

**Figure 18** Antral mucosa from *H.pylori* infected rat, demonstrating the *H.pylori* organisms in the gastric mucosa. H&E staining (x 600).

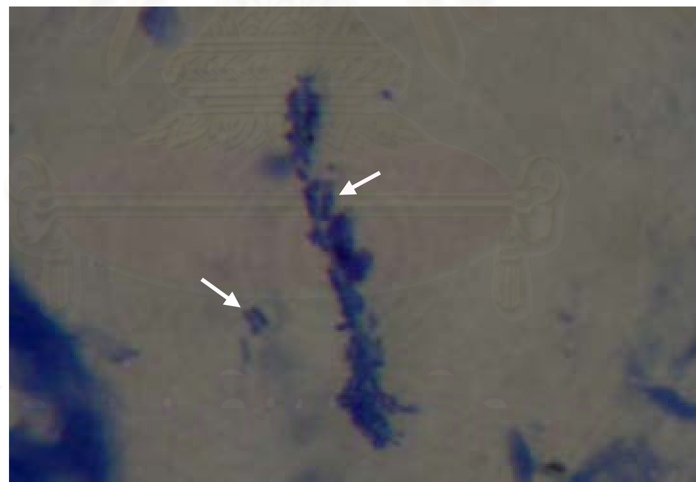

**Figure 19** Antral mucosa from *H.pylori* infected rat, demonstrating *H.pylori* organisms clumping in gastric mucosa. Warthin-starring stain (x 600).

**Table 5** Means ± SE of systolic blood pressure (SBP; mmHg) of control groups, *H.pylori* infection groups, and *Aloe vera* treated groups.

| Day after treatment | Systolic Blood Pressure (mmHg) | | |
|---|---|---|---|
| | Control group (n=6) | *H.pylori* infection group (n=6) | *Aloe vera* treated group (n=6) |
| Day 3 | 111.78 ± 2.36 | 121.34 ± 2.63 $^{ns}$ | 113.11 ± 1.74 $^{\S\ ns}$ |
| Day 8 | 108.17 ± 4.22 | 123.78 ± 4.37 $^{*}$ | 118.89 ± 3.71 $^{\S\ *}$ |

* significant difference as compared to control group ($p<0.05$)

ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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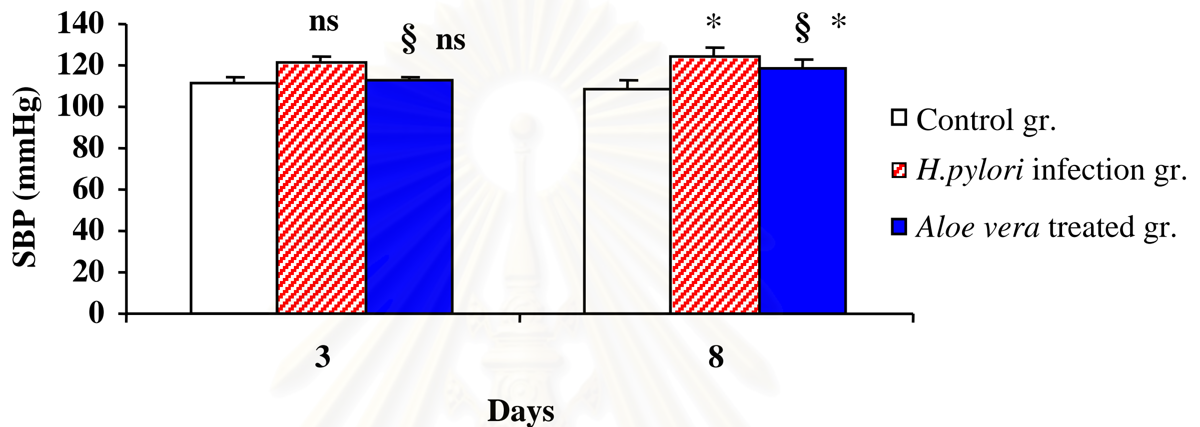
**Figure 20** Bar graph showing the means ± SE of systolic blood pressure (SBP; mmHg) of control group, *H.pylori* infection group, and *Aloe vera* treated group on day 3 and day 8 after treatment. Each group represented the mean of 6 tests on 6 rats.




* significant difference as compared to control group ($p<0.05$)

ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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**Table 6** Means ± SE of diastolic blood pressure (DBP; mmHg) of control groups, *H.pylori* infection groups, and *Aloe vera* treated groups.

| Day after treatment | Diastolic Blood Pressure (mmHg) | | |
|---|---|---|---|
| | Control group (n=6) | *H.pylori* infection group (n=6) | *Aloe vera* treated group (n=6) |
| Day 3 | 94.11 ± 2.76 | 103.00 ± 5.33 ns | 100.56 ± 4.64 § ns |
| Day 8 | 93.17 ± 2.48 | 100.72 ± 1.55 ns | 96.67 ± 1.41 § ns |

ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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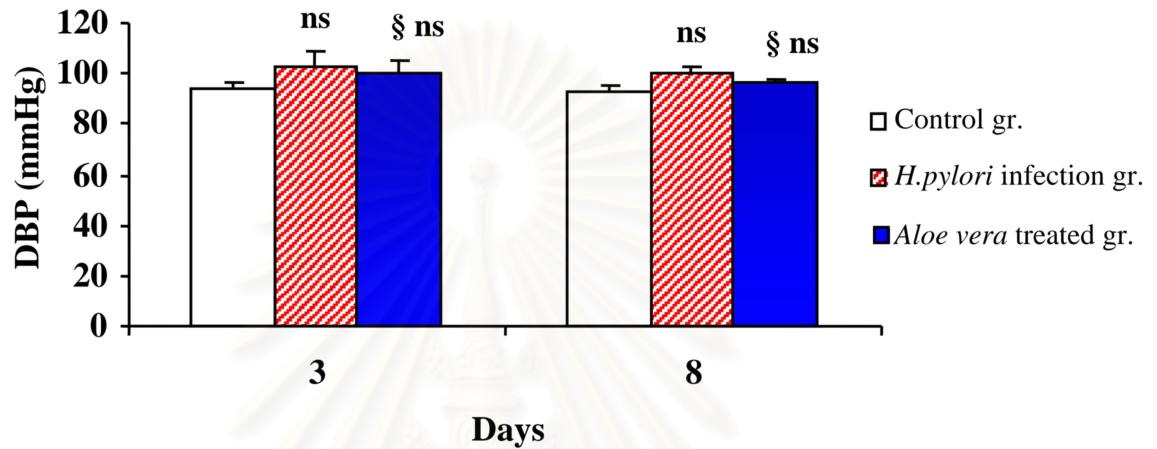
**Figure 21** Bar graph showing the means ± SE of diastolic blood pressure (DBP; mmHg) of control group, *H.pylori* infection group, and *Aloe vera* treated group on day 3 and day 8 after treatment. Each group represented the mean of 6 tests on 6 rats.




ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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**Table 7** Means ± SE of mean arterial pressure (MAP; mmHg) of control groups, *H.pylori* infection groups, and *Aloe vera* treated groups.

| Day after treatment | Mean Arterial Pressure (mmHg) | | |
|---|---|---|---|
| | Control group (n=6) | *H.pylori* infection group (n=6) | *Aloe vera* treated group (n=6) |
| Day 3 | 99.99 ± 2.18 | 109.11 ± 4.28 [*] | 104.74 ± 3.57 [§ ns] |
| Day 8 | 98.17 ± 2.93 | 108.41 ± 2.11 [*] | 104.07 ± 2.11 [§ ns] |

* significant difference as compared to control group ($p<0.05$)

§ No significant difference as compared to *H.pylori* infection group

ns No significant difference as compared to control group

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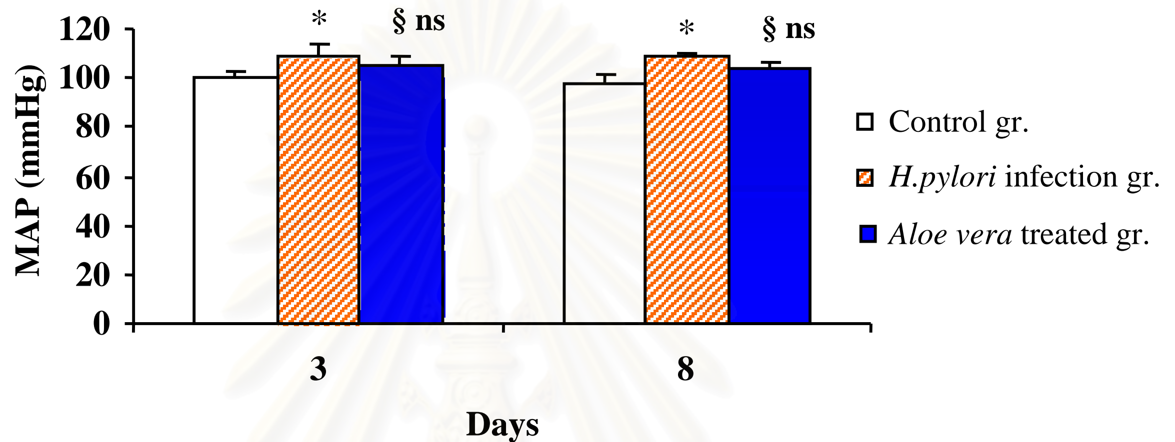
**Figure 22** Bar graph showing the mean ± SE of mean arterial pressure (MAP; mmHg) of control group, *H.pylori* infection group, and *Aloe vera* treated group on day 3 and day 8 after treatment. Each group represented the mean of 6 tests on 6 rats.




* Significant difference as compared to control group ($p<0.05$)

ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

**Table 8** Means ± SE of heart rate (HR; beats/min) of control groups, *H.pylori* infection groups, and *Aloe vera* treated groups.

| Day after treatment | Heart rate (beats/min) | | |
|---|---|---|---|
| | Control group (n=6) | *H.pylori* infection group (n=6) | *Aloe vera* treated group (n=6) |
| Day 3 | 406.67 ± 9.89 | 373.33 ± 6.67 [ns] | 390.00 ± 13.42 [§ ns] |
| Day 8 | 403.33 ± 6.15 | 339.00 ± 19.07 [*] | 370.00 ± 22.95 [§ ns] |

* Significant difference as compared to control group ($p<0.05$)

ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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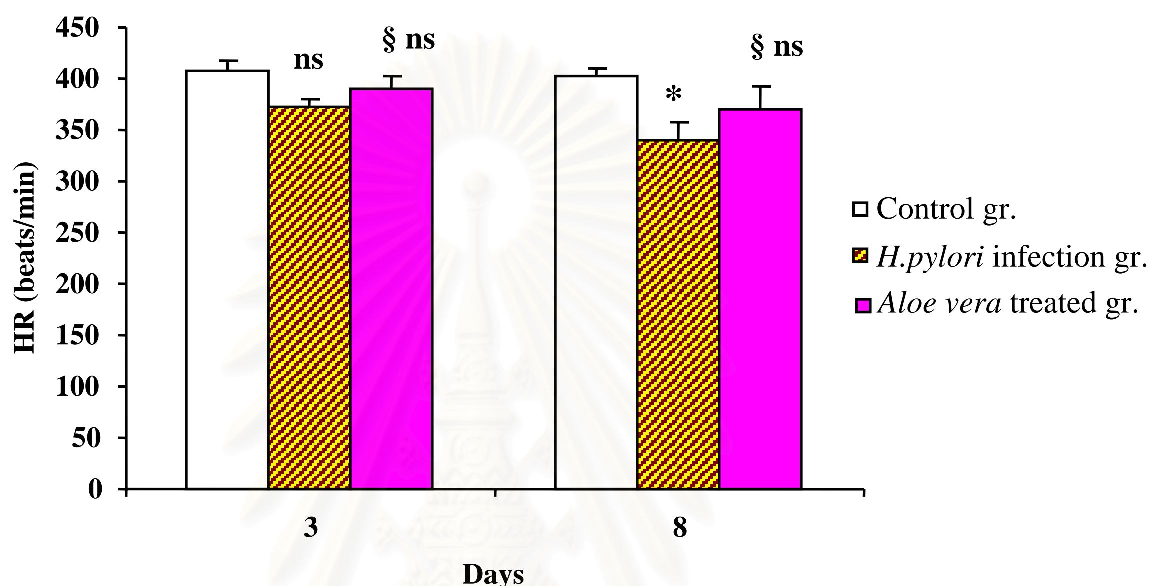
**Figure 23** Bar graph showing the means ± SE of heart rate (HR; beats/min) of control group, *H.pylori* infection group, and *Aloe vera* treated group on day 3 and day 8 after treatment. Each group represented the mean of 6 tests on 6 rats.




* Significant difference as compared to control group ($p<0.05$)

ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

**Table 9** Means ± SE of body weight (BW; kg) of control groups, *H.pylori* infection groups, and *Aloe vera* treated groups.

| Day after treatment | Body weight (kg) | | |
|---|---|---|---|
| | Control group (n=6) | *H.pylori* infection group (n=6) | *Aloe vera* treated group (n=6) |
| Day 3 | 230.83 ± 5.61 | 227.67 ± 8.28 [ns] | 223.33 ± 11.49 [§ ns] |
| Day 8 | 242.00 ± 4.78 | 239.17 ± 9.25 [ns] | 240.67 ± 10.29 [§ ns] |

ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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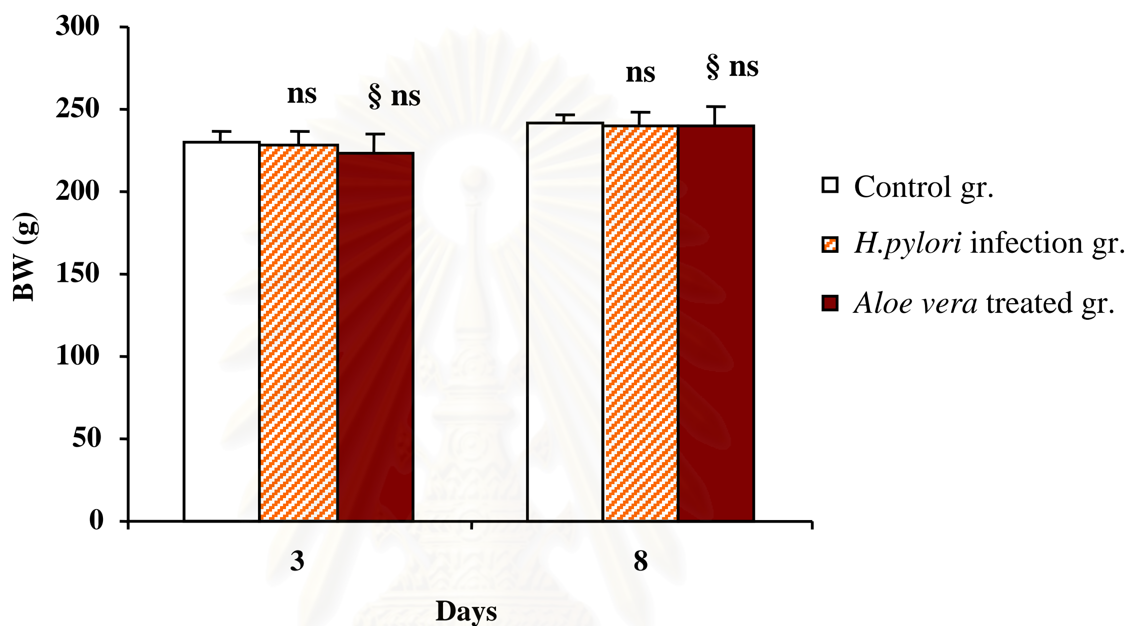
**Figure 24** Bar graph showing the means ± SE of body weight (BW; g) of control group, *H.pylori* infection group, and *Aloe vera* treated group on day 3 and day 8 after treatment. Each group represented the mean of 6 tests on 6 rats.




ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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**Table 10** Means ± SE of adherent leukocyte (per field of view) of control groups, *H.pylori* infection groups, and *Aloe vera* treated groups.

| Day after treatment | Adherent leukocyte (per field of view) | | |
|---|---|---|---|
| | Control group (n=6) | *H.pylori* infection group (n=6) | *Aloe vera* treated group (n=6) |
| Day 3 | 2.23 ± 0.48 | 10.10 ± 0.67 [#] | 5.81± 0.63 [† #] |
| Day 8 | 2.47 ± 0.25 | 13.40 ± 1.00 [#] | 5.45 ± 0.51 [† #] |

# Significant difference as compared to control group (p<0.01)

† Significant difference as compared to *H.pylori* infection group (p<0.01)

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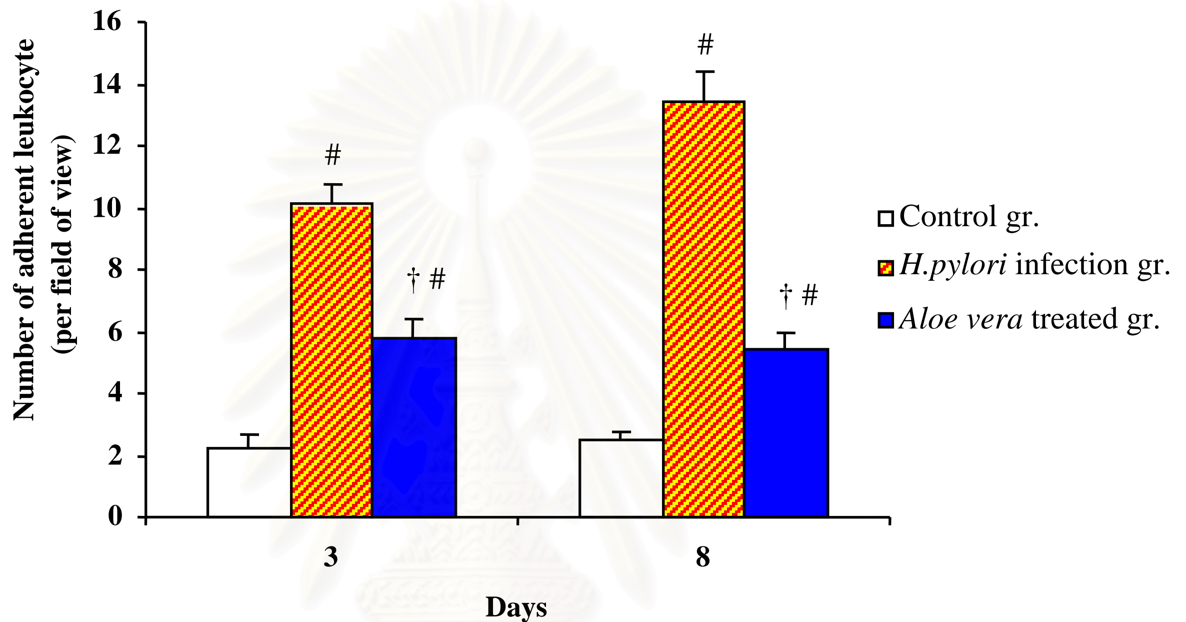
**Figure 25** Bar graph showing the means ± SE of adherent leukocytes (per field of view) of control group, *H.pylori* infection group, and *Aloe vera* treated group on day 3 and day 8 after treatment. Each group represented the mean of 6 tests on 6 rats.




# Significant difference as compared to control group (p<0.01)

† Significant difference as compared to *H.pylori* infection group (p<0.01)

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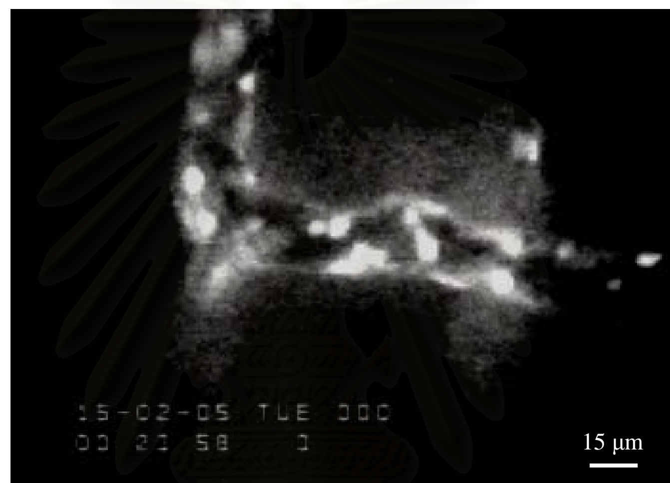
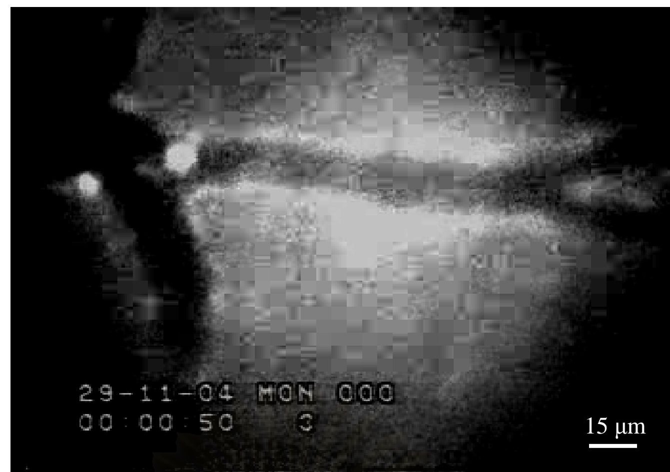
(a)



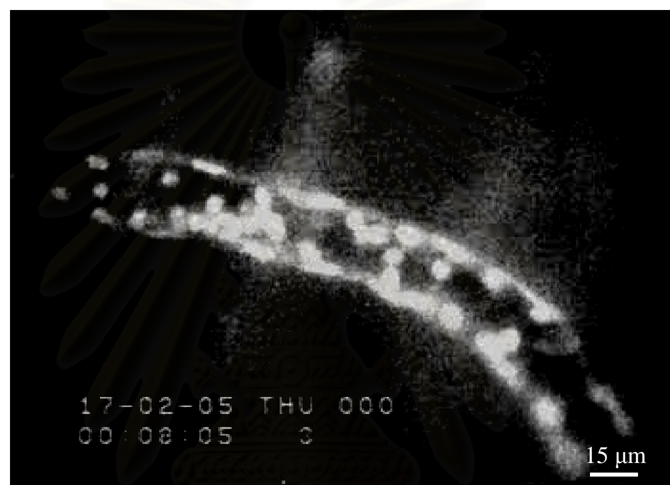

(b)

(c)

**Figure 26** Intravital fluorescent microscopy demonstrated leukocyte adhesion in control group (a), *H.pylori* infection group (b), and *Aloe vera* treated group (c) on day 3 after treatment (x 40).




(a)




(b)

(c)

**Figure** 27 Intravital fluorescent microscopy demonstrated leukocyte adhesion in control group (a), *H.pylori* infection group (b), and *Aloe vera* treated group (c) on day 8 after treatment (x 40).

**Table 11** Means ± SE of TNF-α (pg/ml) of control groups, *H.pylori* infection groups, and *Aloe vera* treated groups.

| Day after treatment | TNF-α (pg/ml) | | |
|---|---|---|---|
| | Control group (n=6) | *H.pylori* infection group (n=6) | *Aloe vera* treated group (n=6) |
| Day 3 | 8.65 ± 1.79 | 61.98 ± 18.74 $^{*}$ | 14.52 ± 5.53 $^{\dagger\ ns}$ |
| Day 8 | 9.92 ± 2.62 | 76.76 ± 23.18 $^{*}$ | 26.31 ± 6.38 $^{\dagger\ ns}$ |

* Significant difference as compared to control group (p<0.05)

† Significant difference as compared to *H.pylori* infection group (p<0.05)

ns No significant different as compared to control group

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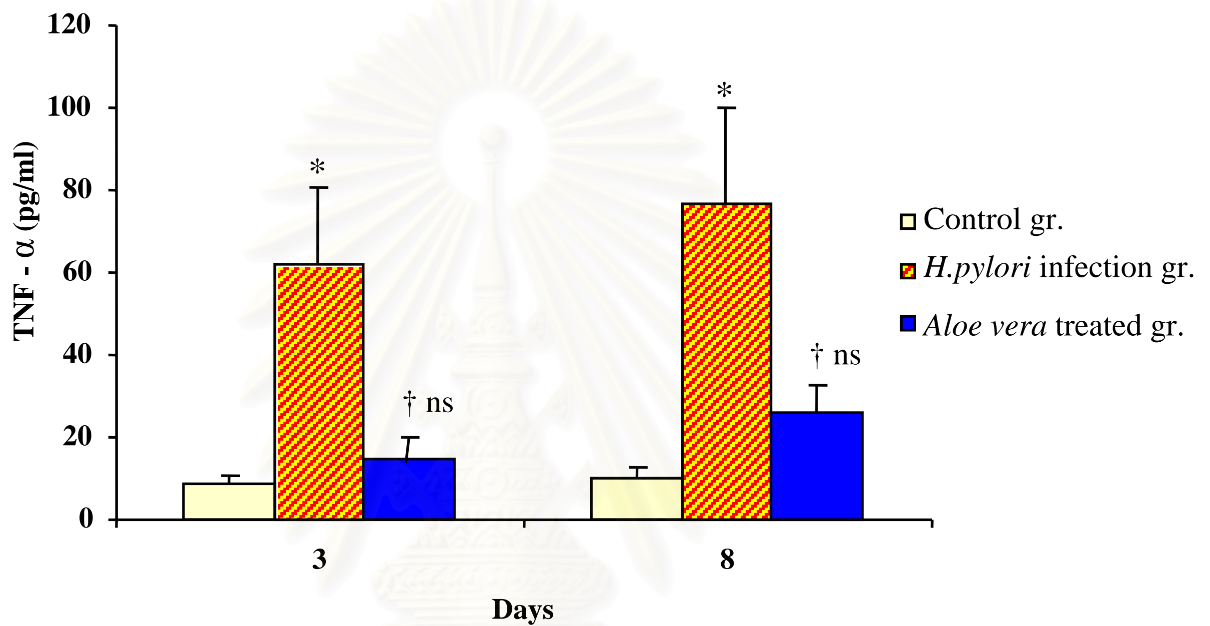
**Figure 28** Bar graph showing the means ± SE of TNF-α (pg/ml) of control group, *H.pylori* infection group, and *Aloe vera* treated group on day 3 and day 8 after treatment. Each group represented the mean of 6 tests on 6 rats.




* Significant difference as compared to control group (p<0.05)
† Significant difference as compared to *H.pylori* infection group (p<0.05)
ns No significant difference as compared to control group

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**Table 12** Means ± SE of IL-10 (pg/ml) of control groups, *H.pylori* infection groups, and *Aloe vera* treated groups.

| Day after treatment | IL-10 (pg/ml) | | |
|---|---|---|---|
| | Control group (n=6) | *H.pylori* infection group (n=6) | *Aloe vera* treated group (n=6) |
| Day 3 | 256.80 ± 47.66 | 266.66 ± 128.01 [ns] | 275.05 ± 115.84 [§ ns] |
| Day 8 | 383.99 ± 62.58 | 663.60 ± 105.33 [ns] | 681.17 ± 197.49 [§ ns] |

ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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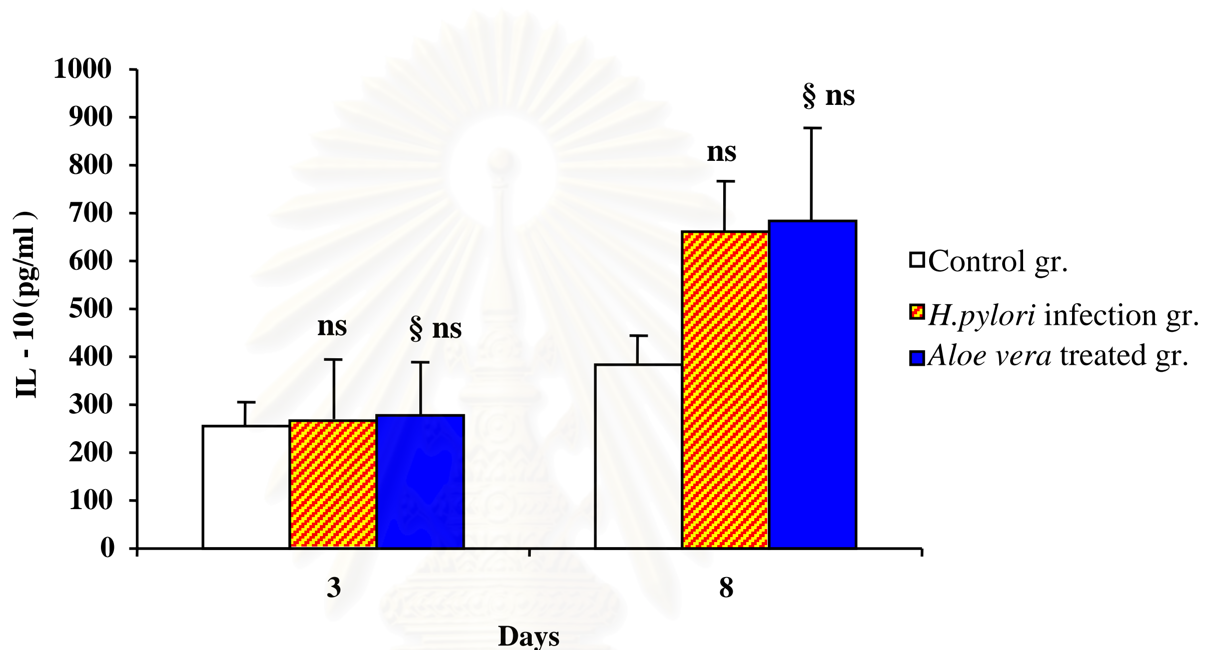
**Figure 29** Bar graph showing the means ± SE of IL-10 (pg/ml) of control group, *H.pylori* infection group, and *Aloe vera* treated group on day 3 and day 8 after treatment. Each group represented the mean of 6 tests on 6 rats.




ns No significant difference as compared to control group

§ No significant difference as compared to *H.pylori* infection group

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# CHAPTER V

# DISCUSSION

## RAT MODEL OF *H.PYLORI* INFECTION IN THIS STUDY

The technique of induction *H.pylori* infection to rat's stomach in the present study was concordance to the previous study (Thong-Ngam et al., 2005). There was more successful rate 82.61% in *H.pylori* infection groups and 85% in *Aloe vera* treated groups when compared to the previous study (successful rate 69.84%).

It was obvious that, from the rapid urease test and histopathologic results in *Aloe vera* treated groups, there were positive results in both of tests. It indicated that *Aloe vera* had no effects to rid or inhibit growth of *H.pylori* organisms.

Increasing successful rate of *H.pylori* infection in the present study, it may be involved to the toxigenic strain of *H.pylori*, the host's immune response, or technically. The strain of *H.pylori* is importantly for the pathogenesis of disease. In this study *H.pylori* organisms were obtained from peptic ulcer patient, those pathogenic strains could increase rate of infection as well as could induce the inflammation in animal stomach. Another study had been colonized with *H.pylori* that obtained from peptic ulcer patient and colonize to normal rat stomach, before inoculation, rats were pretreated with oral dose of omeprazole for reduce acidic condition in stomach (Li et al., 1999). That study was closely to our work, but we pretreated with streptomycin for getting rid any organisms those may live

in rat stomach. Sydney strain (SS1 *H.pylori*) has been used to colonize in mouse and rat stomach. They found that at 8, 12, and 24 weeks after inoculation the stomach was developed to chronic gastritis (Zeng et al., 1998). Inoculation with non-toxigenic strain to normal rat stomach caused unsuccessful but colonized with toxigenic strain in ulcer operated rat could induce chronic inflammation (Li et al., 1998). In the present study we did not check the strain of *H.pylori* but there should be pathogenic strains because the organisms were originally obtained from peptic ulcer patients. We confirmed the successful infection by positive rapid urease test and positive histopathology. By histopathologic examination, the mild to moderate gastritis were developed. According to our results, in 1999 Li et al. had reported the histopathologic changes in rats gastric mucosa after long term *H.pylori* infection (2, 6, 12 weeks). After two weeks of inoculation, there were found moderate gastric inflammation with infiltration of PMNs, macrophages, and lymphocytes. So it may be said that *H.pylori* could induce changes in gastric microcirculation that we demonstrated in this study.

## PHYSIOLOGICAL CHARACTERISTIC OF ANIMAL USED IN THE STUDY

In this study, the animals model were infected with *H.pylori* organisms. After two weeks of *H.pylori* inoculation, followed by gavage with *Aloe vera* or vehicle, the MAP on day 3 and day 8 and SBP on day 8 were marked increased in *H.pylori* infection group, but the DBP was slightly increased in this groups. Increasing of SBP and MAP, and decreasing of HR in *H.pylori* infection group indicated that in chronic *H.pylori* infection, it could harmful to the systemic circulation. That caused from *H.pylori* release toxicity to disturbance the homeostasis of

vascular endothelium or induced vasoconstriction. The pathophysiology is not limited only at site of infection but also affects to the systemic circulation after chronic inflammation. Then we found the hemodynamic changes in *H.pylori* infected rat in this study. Kalia and coworker (2002) have demonstrated that topical application with *H.pylori* extracts toxigenic strain could induce transient vasoconstriction of postcapillary venule within five minutes. According to the vasoconstriction of postcapillary venules, the reduction in gastric mucosal blood flow was also demonstrated after topical application with *H.pylori* extracts on gastric mucosa (Atuma et al., 1995) Moreover, Brzozowski and coworkers (1999) has reported the marked fall in the GBF at the ulcer margin following challenged with *H.pylori* extracts. In the present study there were not significant difference in BW between *H.pylori* infection group and control group. It indicated that after *H.pylori* infection, animals were no or slightly suffering from the infectious disease, as well as the life style of animal such as drinking, eating, and activities which revealed the metabolic changes of body were no changes.

Treatment with *Aloe vera*, our experimental data found that SBP, DBP, MAP, HR, and BW in *Aloe vera* treated group were not significant different from *H.pylori* infection group. However, it looked like that SBP, DBP, and MAP were slightly decreased followed by treatment with *Aloe vera*. It indicated that many chemical substances in *Aloe vera* may affect to the vasomotion, which may result to the changes of those hemodynamic parameters and vessel diameter. However in this study we did not directly to examine the changes in vascular diameter.

## EFFECTS OF *H.PYLORI* INFECTION ON LEUKOCYTE ADHESION AND ROLE OF *ALOE VERA*

In our experiment, we observed the leukocyte activity in gastric microcirculation through intravital fluorescence microscope by using acridine orange to label leukocyte.

Two weeks after *H.pylori* infection followed by gavage with vehicle for 3 and 8 days, the present experiment exhibited marked enhancement of leukocyte adhesion. The results of this study revealed that *H.pylori* release some toxicity or toxicity from cell envelope of gram negative bacteria to activate those cells. It has been found that the LPS from cell envelope of *H.pylori*, extracted into water could exhibit chemotactic substance to activate inflammatory cells to interact with venular endothelium (Yoshida et al., 1993). Generally, gastrointestinal inflammation is accompanied with vasodilatation followed by vasoconstriction, changes in vascular endothelium, blood flow, shear rate, platelet activity, and especially PMNs activity (Kalia et al., 2002). According to the PMNs activity, which comprise with sequence of activation, rolling, and adhesion, PMNs adhere to ECs resulted in the aggregation of inflammatory cells at site of inflammation (Freedberg et al., 2003). Thus the found of increase adherent leukocyte in the present study was mirrored the inflammatory response from gastric mucosa caused from *H.pylori* infection. This change agreed with those previous studies that reported in acute *H.pylori* gastritis could found the neutrophils activation detected by histopathology (Kozol et al., 1991; Bayerdorffer et al., 1992). According to histopathologic studied, they have been demonstrated an accumulation of PMNs, mononuclear

leukocytes, macrophage, and lymphocytes in gastric mucosa followed by long term *H.pylori* infection (Li et al., 1999).

Moreover, our data were according to those of previous studies that using topical application of *H.pylori* extracts with $cagA^{+}$, vacA s1m1 toxigenic strain on mesenteric or gastric mucosa could significantly increase adherent leukocyte. They also found the increase of leukocyte emigration, platelet activation, and MML (Kurose et al., 1994; Kalia et al., 2002). These results were explained that extracts of *H.pylori* contain substances that increased surface expression of adhesion glycoprotein (CD11/CD18) on leukocyte and expression of ICAM-1and P-selectin on vascular endothelium (Kurose et al., 1994). The increased leukocyte chemotactic activity elicited an oxidative burst that responsed from leukocyte to damage tissues.

In our experiment, the increased of leukocyte adhesion in *H.pylori* infected rat as we showed in Figure 26 (b), 27 (b) indicated that it occur followed from a chronic *H.pylori* infection in gastric mucosa. *H.pylori* toxigenic strain could release the various of bacterial toxins (as we mention in chapter II) to enhance the synthesis of chemokines in the gastric epithelium (Shimada and Terano, 1998). Those induced infiltration of inflammatory cells, enhanced the release of proinflammatory cytokine such as IL-1β, IL-6, and TNF-α, as well as the generation of ROS (Brzozowski et al., 1999). The release of ROS and proinflammatory cytokine by macrophage, and mast cells are initiated. By means of these mediators, they induced the expression of leukocyte-endothelium interaction.

Interestingly, our results showed in Table 10 and Figure 26 (c), 27 (c) that twice daily treatment of *Aloe vera* (200 mg/kg BW) could reduce the leukocyte-endothelium interaction significantly both on day 3 and day 8. The results of this experiment were according to our hypothesis, we believed that many active substances that contain in *Aloe vera* were absorbed to the circulation. Then many active substances were acted synergist with many pathways such as suppress of inflammatory mediators or anti-oxidative stress for help to preserve the homeostasis of vascular endothelium and resulted in the reduction amount of leukocyte adhesion. The over production of inflammatory mediators such as TNF-α, IL-6, IL-8 or ROS could induce the enhancement of leukocyte-endothelium interaction. According to the pathologic process, Duansak and coworkers (2003) have been proposed that *Aloe vera* might have some active ingredients which are able to inhibit or suppress TNF-α and IL-6 level then leukocyte-endothelium interaction were also reduced after burn. As studied in burn wound rats, *Aloe vera* could inhibit leukocyte recruitment (Davis, 1994). These findings were explained by immunomodulatory activity of high molecular polysaccharide in *Aloe vera* (Hart et al., 1989). Besides, low molecular constituents of aqueos aloe gel has been demonstrated that it could inhibit the release of ROS from activated human PMNs. Sabech et al. (1993, 1996) found antioxidant substance such as glutathione peroxidase and superoxide dismutase in aloe gel. The exclusion of those antioxidant from contact points of PMN with endothelium was the significant factor regulating PMN-leukocyte interaction (Siflinger-Birnboim and Malik, 1996). Therefore, it is possible to say that active substance in *Aloe vera* could reduce amount of leukocyte adhesion in *H.pylori* infected rats through it suppress those mediator that initiated the process of leukocyte-

endothelium interaction. Then we have made for further evaluation the effects of *Aloe vera* on those cytokines.

## EFFECTS OF *H.PYLORI* INFECTION ON TNF-α AND IL-10 LEVELS AND ROLE OF *ALOE VERA*

ELISA technique was performed to detect the level of serum TNF-α and IL-10 in this study.

Results obtained in our study showed that there was marked increase in serum TNF-α in *H.pylori* infection groups, both on day 3 and day 8 after received vehicle. After treatment with *Aloe vera*, serum TNF-α was significantly decreased both on day 3 and day 8 compared to *H.pylori* infection groups. For IL-10 level, there were not significant difference between IL-10 in control groups, *H.pylori* infection groups, and *Aloe vera* treated groups at two monitored time points. However, it looked like that on day 8, IL-10 was slightly increased in *H.pylori* infection group and *Aloe vera* treated groups.

Infection with *H.pylori* in gastric mucosa is known to activate the production of many inflammatory mediators including IL-1, IL-6, IL-8, and TNF-α. The production of these inflammatory mediators are not limited at the local site of infection but are also superimposed by the acute inflammatory reactions. Since the gastric mucosa was further challenged by infection, the overproduction of chemical mediators can cause leukocyte-endothelial interaction and overproduction of cytokines which might be contribute to the systemic effects. Previous studies had shown the elevation of plasma TNF-α, and IL-1β in ulcer operated rat and received *H.pylori* extracts (TNF-α at day 9 = 82 ± 7 pg/ml, day 15 = 102

± 5 pg/ml). There were significant different from the ulcer group without *H.pylori* extracts (TNF-α at day 9 = 3 ± 0.6 pg/ml, day 15 = 5 ± 1.2 pg/ml). It indicated that *H.pylori* extracts provoke the production of cytokine (Brzozowski et al., 1999). According to the study of Perez-perez et al. (1995) found that incubation *H.pylori* LPS with the macrophages derived from rat bone marrow could activate the production of TNF-α. Furthermore, expression of TNF-α in gastric mucosa were also demonstrated in rat with intragastric surface epithelial application of *H.pylori* LPS (50 μg/animal). On day 4, they found the increasing of TNF-α level (24 pg/mg protein), and these were reduced by sucralfate treatment (12 pg/mg protein) (Slomiany et al., 1998). All of these studies were concordance to our results.

In our experiment, it was obvious that in *H.pylori* infection group and received vehicle for 8 days, IL-10 has a trend to increase, but not significant difference when compared with the age matched control group. The production of IL-10 at day 8 after vehicle or *Aloe vera* treatment in *H.pylori* infected rats involved to the longer period than at 3 days. So, IL-10 were super produced by *H.pylori* activated chronic inflammatory cells.

Following chronic *H.pylori* infection, many chronic inflammatory cells such as eosinophils, B and T lymphocytes were activated by *H.pylori* and contribute to the chronic inflammation (Malfertheiner et al., 1996, Fan et al., 1994, Hatz et al., 1996, Eidt and Stolte, 1993). Besides, the histopathologic examination could be found the small aggregation of lymphocyte and lymphoid follicles in antral mucosa in *H.pylori* infected rats. Because activated lymphocytes could produce IL-10, then the elevation of IL-10 in our results were obtained. According to those previous studies in human with chronic *H.pylori* gastritis, supernatants

TNF-α and IL-10 were significantly increased compared to control, and they also found the correlation between IL-10 level and chronic inflammatory score (r = 0.04) (Bodger et al., 1997). The study of Li and coworkers (1999) had reported that inflammations were observed at two weeks after *H.pylori* infection and did not become progressively more severe during the following months. In our experiment, increasing of IL-10 in *H.pylori* infected rat may be protective, limiting tissue damage caused by inflammation. It may be also contributed toward to the failure of the immune response to eliminate the organism. The present study may explain the pathogenesis in rat that why many studies not found the more severity gastritis or ulcer in *H.pylori* infected rats.

Interestingly, our results showed that twice daily treatment of *Aloe vera* (200 mg/kg BW) could reduce or prevent the elevation of TNF-α both on day 3 and day 8. While IL-10 level has no changed. It may be explain through the anti-inflammatory properties of *Aloe vera*, since in aloe gel has been investigated and found a various types of sterols and those sterols have been proposed as an anti-inflammatory agent. Furthermore, *Aloe vera* has been used to investigate the role on TNF-α and IL-6 levels in burn wound rats. They found that levels of TNF-α and IL-6 were decreased significantly after *Aloe vera* treatment compared to control at 3, 7, and 14 days post burn (Duansak et al., 2003). However, in our study there were not significant different between IL-10 level in *Aloe vera* treated and untreated groups. It may say that *Aloe vera* has no effect on IL-10 in *H.pylori* infected rats.

## HYPOTHESIS FOR THE EFFECTS OF *ALOE VERA* ON *H.PYLORI* INDUCED GASTRIC INFLAMMATION

Overall results of this study, we would like to propose the possible mechanisms of *Aloe vera* to reduce gastric inflammation in *H.pylori* infected rat.

As we know in this experiment, infection with *H.pylori* could induce gastric inflammation. The bacterium could activate inflammatory cells or gastric epithelial cells to produce a numerous of TNF-α, it also activate leukocytes to interact with endothelium. Furthermore, in this experiment we also found the slightly increased of IL-10 level after long term *H.pylori* infection (2 weeks plus 8 days vehicle or *Aloe vera* treatment). It according to that in long term period of infection, chronic inflammatory cell such as B and T lymphocytes were activated to synthesis IL-10.

The possible mechanism of *Aloe vera* on leukocyte adhesion and on TNF-α level in *H.pylori* infected rats showed in Figure 30. The idea is that *Aloe vera* might have many active ingredients that act as anti-inflammatory agent. And our results showed that *Aloe vera* could reduce amount of leukocyte adhesion. Regarding the experimental result of TNF-α and IL-10, *Aloe vera* treated groups showed a significant decreased of TNF-α, while IL-10 was not changed. It was obvious that in *H.pylori* infected rats increasing of TNF-α levels were maintained for a long time since gastric mucosal were challenged by infection. Then it may be said that *Aloe vera* has directly effect to reduce TNF-α level, but not affect to IL-10 for reduce gastric inflammation in *H.pylori* infected rats. Taken together, *Aloe vera* could help to inhibit leukocyte-endothelium

interaction through the reduction of TNF-α level or by its direct effect to preserve the balance of those tissues.

However, other pathway such as antioxidant properties of active substances in *Aloe vera* should be proposed because it may help to maintain homeostasis of endothelium, which might be further investigation.

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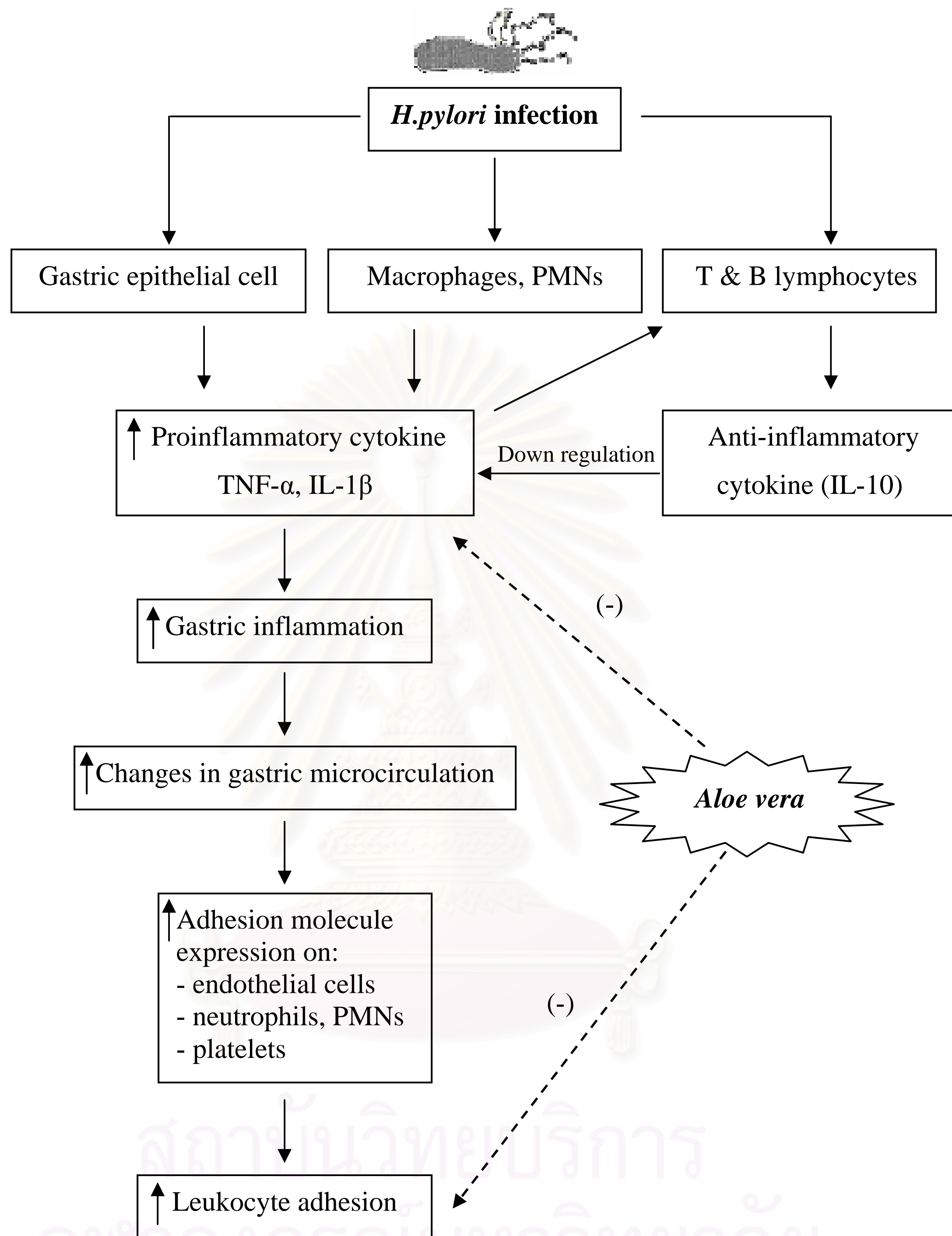


**Figure 30** The proposed mechanisms of *Aloe vera* as an anti-inflammatory agents.

# CHAPTER VI

# CONCLUSION

The overall results of this investigation indicated that infection with *H.pylori* in rat gastric mucosa induced increase of leukocyte adhesion,  and TNF-α levels.

Treatment with *Aloe vera* (200 mg/kg BW) by gavage twice daily could reduce amount of leukocyte adhesion and TNF-α level both on day 3 and day 8 after treatment. But *Aloe vera* had no effect on IL-10 level at two monitor time points. So, *Aloe vera* has an anti-inflammatory effect by reducing leukocyte adhesion through the suppress level of proinflammatory cytokine (TNF-α).

Based on these findings, it is reasonable to suggest that *Aloe vera* is a useful agent to reduce gastric inflammation in *H.pylori* infected rats and it might be beneficial for application as an anti-inflammatory agent by using combination with another antibiotic drug for treatment in *H.pylori* infected patients.

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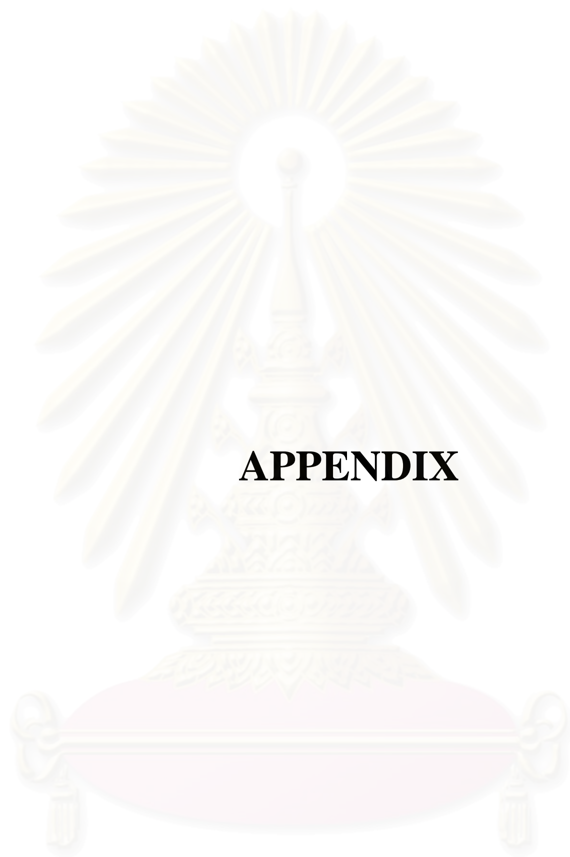

# APPENDIX

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# APPENDIX

## I. *H.pylori* growth condition in culture plate

The *H.pylori* organisms used in this study were originally obtained from peptic ulcer patients. The organisms were growing in Brucella broth (pH 7.0) supplemented with 10% goat serum throughout 24 hr at 37 $^{o}C$ for 1-2 weeks in an automatic $CO_2$-$O_2$ incubator under microaerophilic conditions (85% $N_2$, 10% $CO_2$, and 5% $O_2$), the same condition was used in the following culture.

## II. Chemical substance

### 1. Streptomycin (M&H Manufacturing Co., Ltd)

Streptomycin 1 gram in powder form was dissolved in 200 ml of tap water for prepare the dose of 5 mg/ml. Give the animal drink freely for three consecutive days for get rid of organism that may live in rat stomach.

### 2. *Aloe vera*

***Aloe vera* spray dried powder** (Lipo Chemical Co, Florida, USA.)

| Analysis | Specification |
|---|---|
| Appearance | free flowing powder |
| Color | 1 Gardner (MAX) extended |
| pH (extended 1:199) | 4.0-6.0 |
| ODOR | Characteristic vegetable |

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| | |
|---|---|
| I.R. scan | Match standard |
| Microbiology | less than 100 CFU/gm |
| Aloin | 10.0 ppm max @ 1:199 |

## 3. ELISA reagents

**For TNF-α assay**

- Rat TNF-α microplates
- Rat TNF-α conjugate concentrate
- Type 2 conjugate diluent
- Rat TNF-α standard
- Rat TNF-α control
- Assay diluent RD1-41
- Calibrator diluent RD5-17
- Wash buffer concentrate
- Color reagent A
- Color reagent B
- Stop sollution
- Plate covers

**For IL-10 assay**

- Rat IL-10 microplates
- Rat IL-10 conjugate concentrate
- Type 11 conjugate diluent
- Rat IL-10 standard
- Rat IL-10 control
- Assay diluent RD1-21
- Calibrator diluent RD5-3
- Calibrator diluent RD5T

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- Wash buffer concentrate
- Color reagent A
- Color reagent B
- Stop solution
- Plate covers

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# BIOGRAPHY

Miss Ratsamee Prabjone was born on February 27th, 1980, in Trang. She graduated Bachelor Degree of Science in Physical Therapy from Thammasat University in 2002.

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