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1

Effects of Chronic *Helicobacter pylori* Infection on Changes of Leukocyte Adhesion and TNF-Alpha Levels in Rats

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ABSTRACT

Background: *Helicobacter pylori (H. pylori)* infection causes gastric inflammation and the release of cytokines.

Aims: To investigate the effects of chronic *H. pylori* infection on leukocyte adhesion and serum TNF-alpha levels.

Materials and Methods : Twelve male Spraque-Dawley rats were used in this study (control group = 6 and *H. pylori* infection group = 6). Intravital fluorescence microscopic technique was performed to examine leukocyte adhesion on postcapillary venules. Serum TNF-alpha levels were analyzed using ELISA technique.

Results: In *H. pylori* infection groups, the leukocyte adhesion were 13.4 ± 1.0 cells/field and TNF-alpha were 76.8 ± 23.2 pg/ml, those were significantly ($p < 0.05$) increased when compared with the control groups (leukocyte adhesion 2.5 ± 0.6 cells/field and TNF-alpha = 9.9 ± 2.6 pg/ml, respectively).

Conclusions: *H. pylori* infection induces increase of leukocyte adhesion, as well as TNF-alpha. These results are helpful to explain the pathophysiology of *H. pylori* that causes gastric inflammation.

Key words : *H. pylori*, gastric microcirculation, TNF-alpha

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BACKGROUND

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[1]. Infection by this bacterium, patients were suffering from gastric ulcer, duodenal ulcer, or chronic gastritis.

Chronic gastritis associated with *H. pylori* is characterized by infiltration of lamina propria with inflammatory cells, enhanced release of proinflammatory

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cytokines such as interleukins (IL), for instance IL-1β and IL-8, and tumor necrosis factor-α (TNF-α)[2,3] as well as the generation of reactive oxygen species[4]. TNF-α and IL-1β are potent inducers of IL-8 production and up regulation of neutrophil expression of CD11b/CD18[5]. 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[6].

The cell envelope of gram negative bacteria (H. pylori), lipopolysaccharide (LPS) or endotoxin, 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[7-9]. Early studies in vivo on the rat mesentery have demonstrated that *H. pylori* extracts cause leukocyte adherence in and emigration from the microcirculation[10]. Currently studies, they have demonstrated, using the technique of fluorescent in vivo microscopy, that short-term exposure to water extracts of *H. pylori* induces significant platelet aggregation and macromolecular leakage of labeled albumin and chronic oral administration of water extracts of *H. pylori* induces leukocyte rolling and adhesion in the rat gastric mucosal microcirculation[11,12]. It was obvious that those previous studied 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, we studied the effects of chronic *H. pylori* infection on changes in rat gastric microcir - culation by using intravital fluorescent microscopic technique and on TNF-α to understand the pathogenic mechanism of inflammation.

Materials and Methods

The 12 male Spraque-Dawley rats weighing 200 to 250 grams were used in this study.

Control group

The animals were not inoculated with *H. pylori*. There were housed with free access to water and standard chow until fourteen day. Then these animals were treated with distilled water at a volume of 1 ml/rat by gavage twice daily at an interval of four to six hours. Animals were treated with distilled water continuously until the day of performing intravital fluorescent microscopy.

***H. pylori* infection group**

The animals were inoculated with *H. pylori* by using the model of Thong-Ngam D, *et al*[13]. Briefly, the *H. pylori* suspension (about 10^8 to 10^{10} CFU/ml; 1 ml/rat) were given to the rats by gavage twice daily, with an interval of four hours, 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. The present of *H. pylori* was determined by positive rapid urease test and positive histopathology.

Experimental protocol for intravital fluorescence microscopic study of gastric microcirculatory changes

The animal was anesthetized with intraperitoneal injection of 45 mg/kg BW of sodium pentobarbital. A constant level of anesthesia was maintained throughout the experiment by supplement dose (20% of original dose) every 30-45 minutes[14]. The tracheostomy was performed. The common carotid arterial pressure (CAP) was recorded via pressure transducer (Nikon model RM 6000). The jugular vein was canulated for injection of fluorescent-labeled leukocyte (fluorescein marker acridine orange).

After laparotomy, the stomach was gently extended and held in place by a stay suture. The body temperature of the animal was kept constant at 36-37 ˚C by mean of heating pad. The stomach was placed on microscopic stage of the fluorescent microscope equipped with transillumination and epiillumination optics (Nikon Optiphol-2). 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).

Studies of leukocyte-endothelium interaction in postcapillary venules

For visualization of the leukocyte, the fluorescent marker, acridine orange was infused intravenously (Sigma chemical Co., USA, 0.5 mg/kg BW/min) for 5 minutes[15]. During experiment, leukocytes were recorded on videotape for further observation of leukocyte adhesion. The adherent leukocytes on postcapillary venules (diameter 15 to 30 μm) were counted by visual observation. The adherent leukocyte was defined when the cell adhere to endothelium of postcapillary venules remained stationary for at least 30 seconds and expressed as the number of cells per field of view. Whereas rolling leukocytes were defined as nonadherent leukocytes passing through the observed vessel segment within the observation period[16].

Enzyme-Linked Immunosorbent Assay (ELISA)

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 2000 *g. Then serum was removed and stored at -70 ˚C until the day of analysis. The enzyme-linked immunosorbent assay (ELISA) kit of R & D systems, Inc. (Minneapolis, MN, USA) was used to determine level of TNF-α in serum.

Statistical analysis

Results were expressed as means ± standard error. Unpaired t-test was performed to examine the different of each parameter. The data were analyzed by using SPSS program version 11.5 for window.

RESULTS

The leukocyte adhesion was significantly increased in *H. pylori* infection group (13.4 ± 1.0 cells/field) when compared to control group (2.5 ± 0.6 cells/field, $p < 0.01$) (Figure 1).

For TNF-α, the results indicated that in *H. pylori* infection group (76.8 ± 23.2 pg/ml) were significant higher than control groups (9.9 ± 2.6 pg/ml, $p < 0.05$) (Figure 2).

DISCUSSIONS

The technique of induction *H. pylori* infection to rat's stomach in the present study was concordance to the previous study[13]. There was the successful rate 82.6% in this study by positive both rapid urease test and histopathology. In our experiment, we observe the leukocyte activity in gastric microcirculation through intravital fluorescence microscope by using acridine orange to label leukocyte. Two weeks after *H. pylori* inoculation, the present experiment exhibited marked enhancement of leukocyte adhesion. The results of this study revealed that *H. pylori* may release some toxicity or toxicity from cell envelope of gram negative bacteria to activate those cells. *H. pylori* water extract could exhibit chemotactic substance to activate inflammatory cells to interact with venules endothelium[10,17]. Generally, gastrointestinal inflammation is accompanied with vasodilatation followed by vasocon-

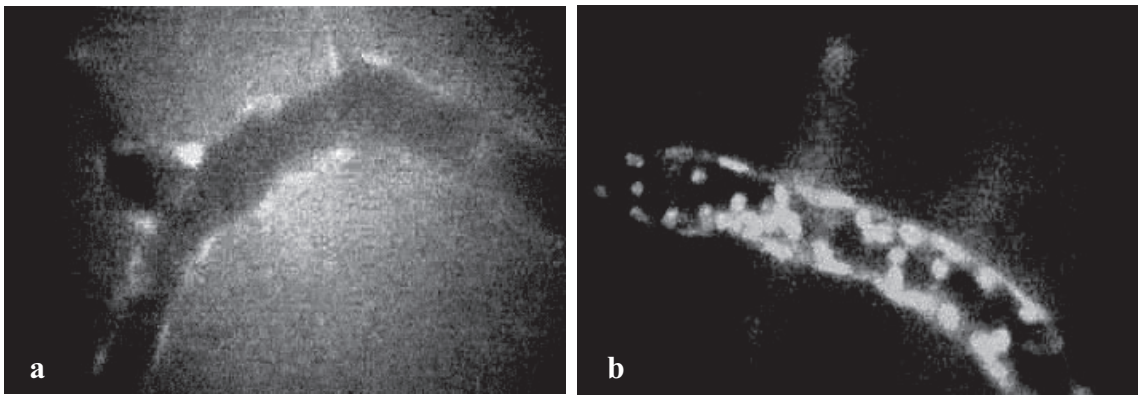
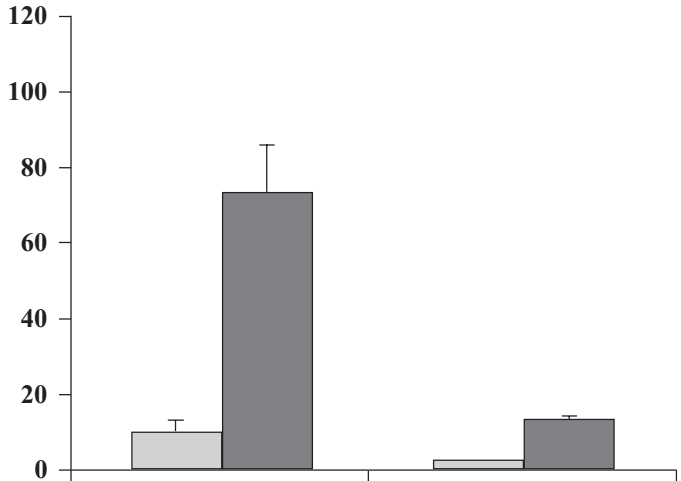


Figure 1 Intravital microscopy demonstrated leukocyte adhesion in control group (a), and *H. pylori* infection group (b). (× 40)



	TNF alpha (pg/ml)	Leukocyte adhesion (cells/field)
control	9.9	2.5
HP infected	76.8	13.4

Figure 2 Bar graph is showing the means ± SE of adherent leukocytes and TNF-alpha of control group compare with *H. pylori* infection group.
*TNF-α in *H. pylori* infection group was significant higher than control group ($p < 0.05$).
**The leukocyte adhesion in *H. pylori* infection group was significantly increased than control group ($p < 0.01$).

striction, changes in vascular endothelium, blood flow, shear rate, platelet activity, and especially polymorphonuclear leukocyte activity[18]. The PMN is one of the main inflammatory cells. PMN-EC adhesion resulted in the formation of a microenvironment between the PMNs and the endothelial cells[19]. 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 studied that reported in acute *H. pylori* gastritis could found the neutrophils activation detected by histopathology[20,21]. 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[22]. Aggregation of inflammatory cells in gastric mucosa was caused from many sequence of leukocyte activity including leukocyte activation, rolling, adhesion, extravasation, and emigration to site of inflammation or to ingest the bacteria by phagocytosis[23].

Moreover, our data was according to those of previous studies that using topical application of *H. pylori* extracts with cag A positive, vac A s1m1 toxigenic strain on mesenteric or gastric mucosa could significantly increased adherent leukocyte. They also found the increase of leukocyte emigration, platelet activation, and macromolecular leakage (MML)[17,18]. 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 endothelium[17]. The increased leukocyte chemotactic activity elicited an oxidative burst that responded from leukocyte to damage tissues.

In our experiment, the increase of leukocyte adhesion in *H. pylori* infected rat as we showed in Figure 1 indicated that it followed from a chronic *H. pylori* infection in gastric mucosa. *H. pylori* toxigenic strain could release the various of bacterial toxins to enhance the synthesis of chemokines in the gastric epithelium[18]. Those induced infiltration of inflammatory cells, enhance the release of proinflammatory cytokine such as IL-1β, IL-6, and TNF-α, as well as the generation of ROS[2]. 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.

Results obtained in our study showed that there was marked increase in serum TNF-α in *H. pylori* infection 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 were further challenged by infection, the over production 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 to the ulcer group without *H. pylori* extract (TNF-α at day 9 = 3 ± 0.6 pg/ml, day 15 = 5 ± 1.2 pg/ml). It indicated that *H. pylori* extract provoke the production of cytokine.[2] According to the studied of Perez-perez, *et al*. (1996)[24] 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 mucosal 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)[25]. All of these studies were concordance to our results. Therefore, we concluded that, *H. pylori* infection induces increase of leukocyte adhesion, as well as TNF-alpha. These results are helpful to explain the pathophysiology of *H. pylori* that causes gastric inflammation.

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