CHAPTER III

MATERIALS AND METHODS

In this study, there are three experimental designs including :

1. An *in vitro* study to assess whether normal HDL and AP-HDL have inhibitory effects on the growth of gram-negative and gram-positive bacteria.

2. An *in vivo* study to assess the effects of both normal HDL and AP-HDL on LPS-induced leukocyte adhesion on endothelium.

3. An *in vivo* study to define the molecular components (apoHDL, lipids and apo A-I) of both normal HDL and AP-HDL which are responsible for those inhibitory effects on LPS-induced leukocyte on endothelium.

All protocols and procedures employed in this study were reviewed and approved by the Ethical Committee, Faculty of Pharmaceutical Science, Chulalongkorn University.

Experimental protocols

Protocol I. The inhibitory effects of normal HDL and AP-HDL on the growth of gram-negative and gram-positive bacteria

In this study the bacterial cultures were separated into 3 groups (group I : no HDL, group II : normal HDL, and group III : AP-HDL) (Figure 9 & 10). In each culture, 10 μl of bacterial suspension, containing 5 X 10^3 colony forming unit/ml (cfu/ml), was placed in a sterile test tube (Tada et al., 1993). 200 μg/ml protein of HDL or NSS was added. Liquid media was used to adjust the final volume to 1 ml in each tube. Test tubes were incubated at 37°C in a shaking incubator (Gerhardt and Drew, 1994). 50 μl of the media in the test tube was

collected at different time points (0, 0.5, 1, 2, 4, 6, 24 hours) and bacteria in the media was counted in duplicate or triplicate by a spread plate method (Koch., 1994) and calculated as percent of the no HDL group.

To evaluate the dose dependent manner of HDL in inhibiting bacterial growth, various HDL concentrations (50, 100, 200, 400, 800, 1,670 μg/ml protein of HDL) were used for incubation for 6 hours.

Protocol II. The effects of normal HDL and AP-HDL on LPS-induced leukocyte adhesion on endothelium

To examine the effects of LPS on leukocyte adhesion on endothelial cells, various LPS concentrations (0.1 μg/100g BW, 1 μg/100g BW and 10 μg/100g BW) were used (Woodman et al., 2000) (Figure 11). LPS was preincubated with NSS or various concentrations of normal HDL (Figure 12) or AP-HDL (Figure 13) at 37^{O}C for 3 hours before administration (Grunfeld et al.,1999). In addition, normal HDL or AP-HDL was preincubated with NSS at 37^{O}C for 3 hours before administration (Grunfeld et al.,1999). After administration, the experiment was performed following the procedure described below.

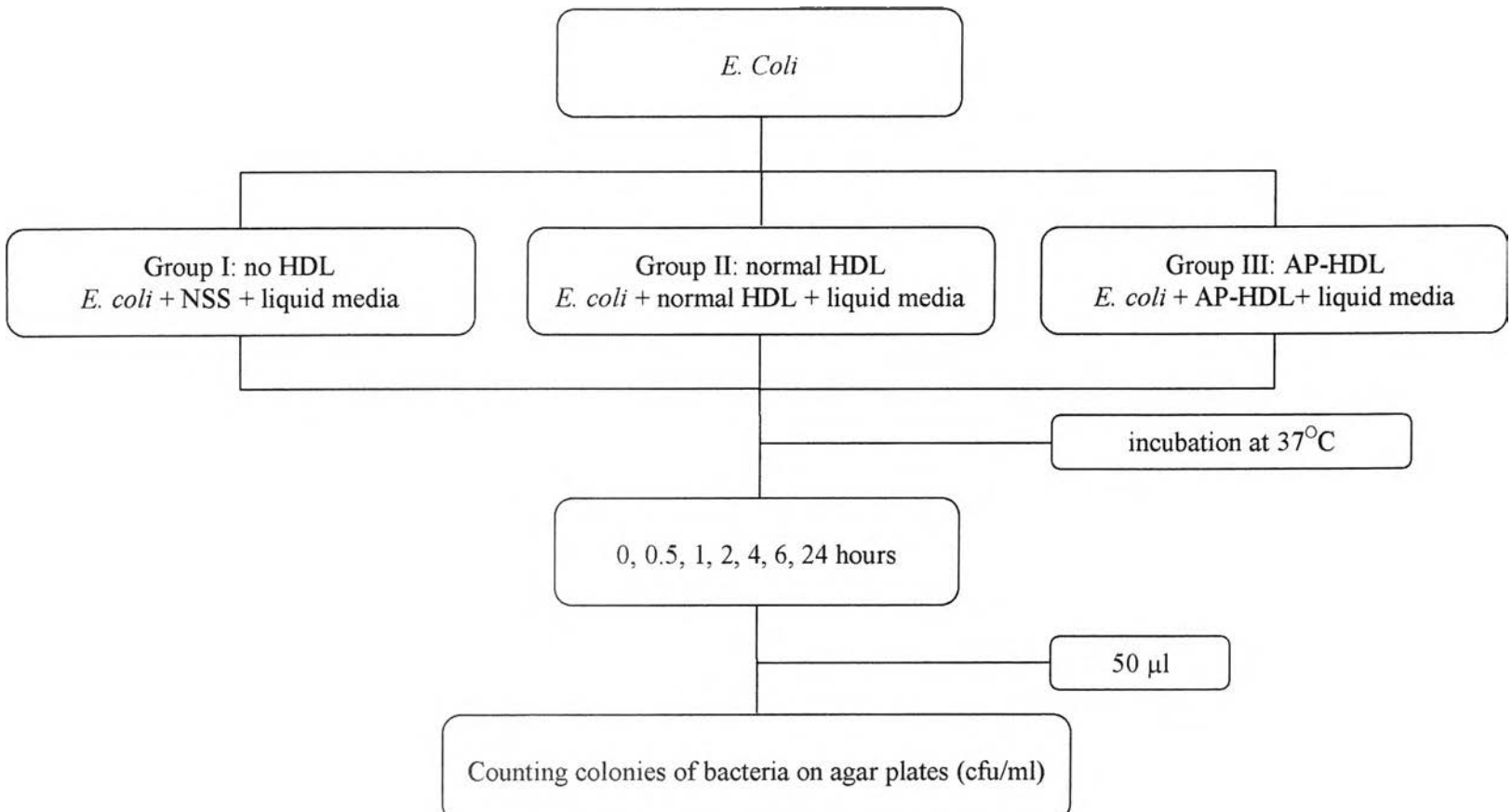


Figure 9 Diagram of experimental groups of the study of gram-negative bacteria.

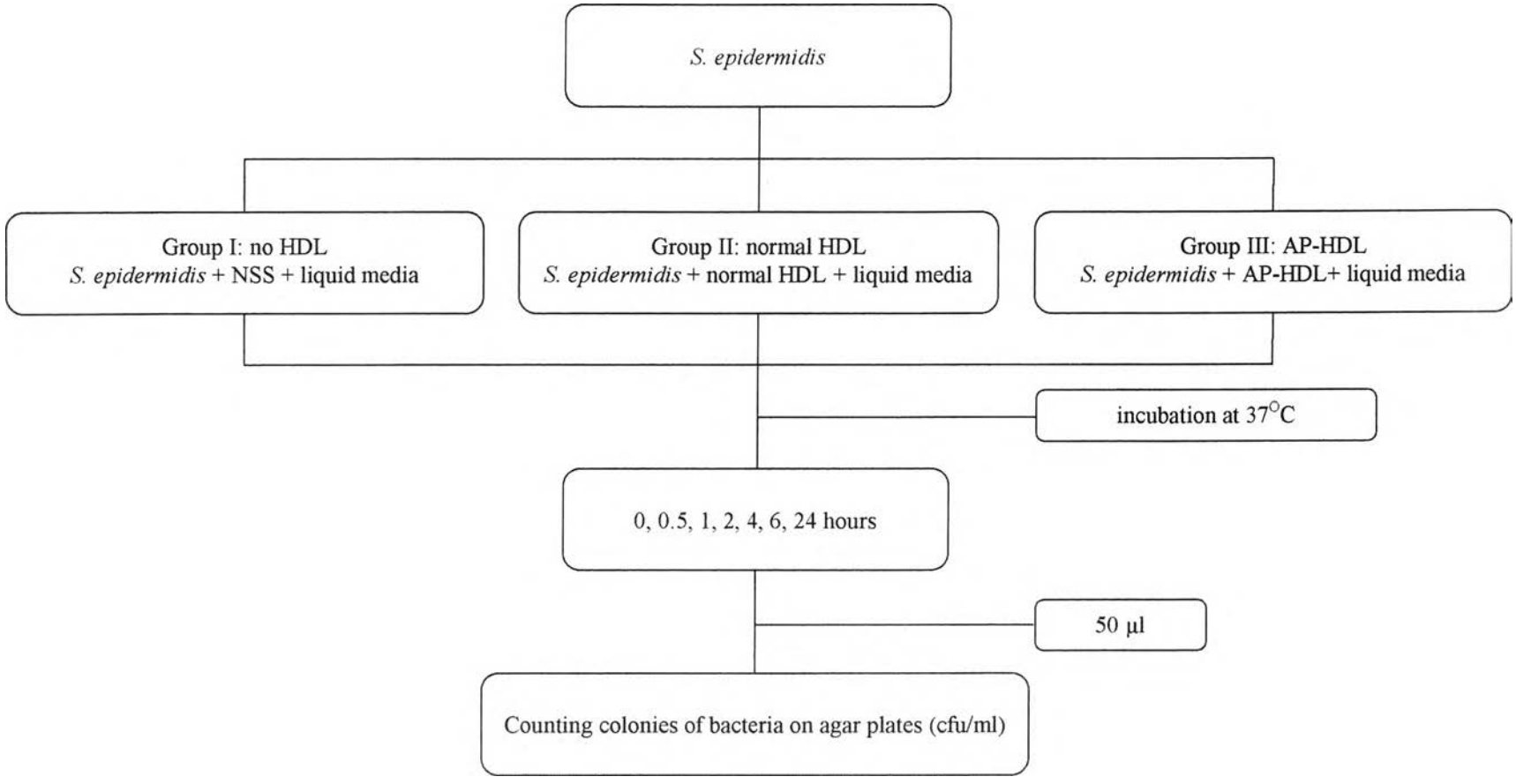


Figure 10 Diagram of experimental groups of the study of gram-positive bacteria.

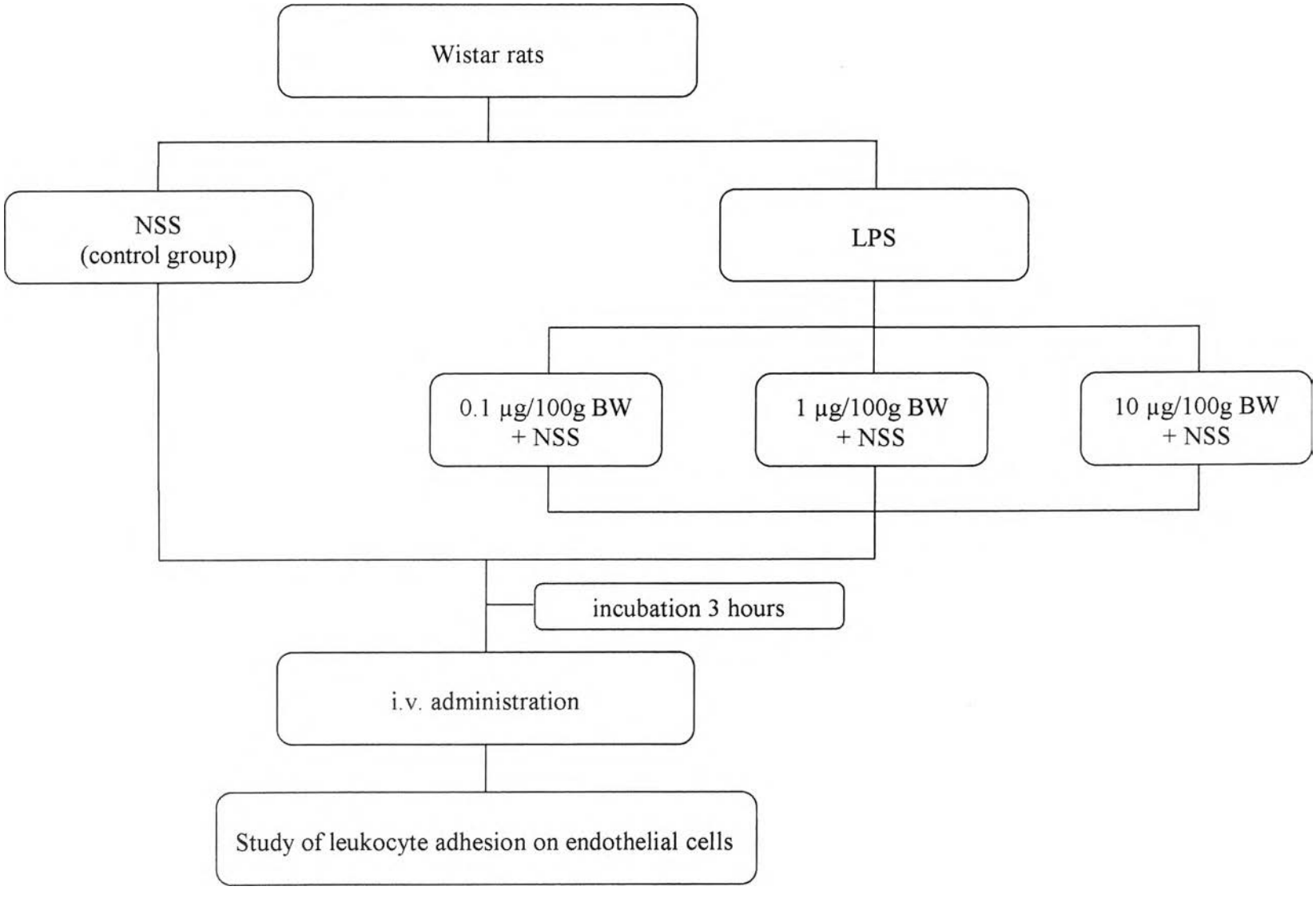


Figure 11 Diagram of experimental groups of the study of LPS – induced leukocyte adhesion on endothelium

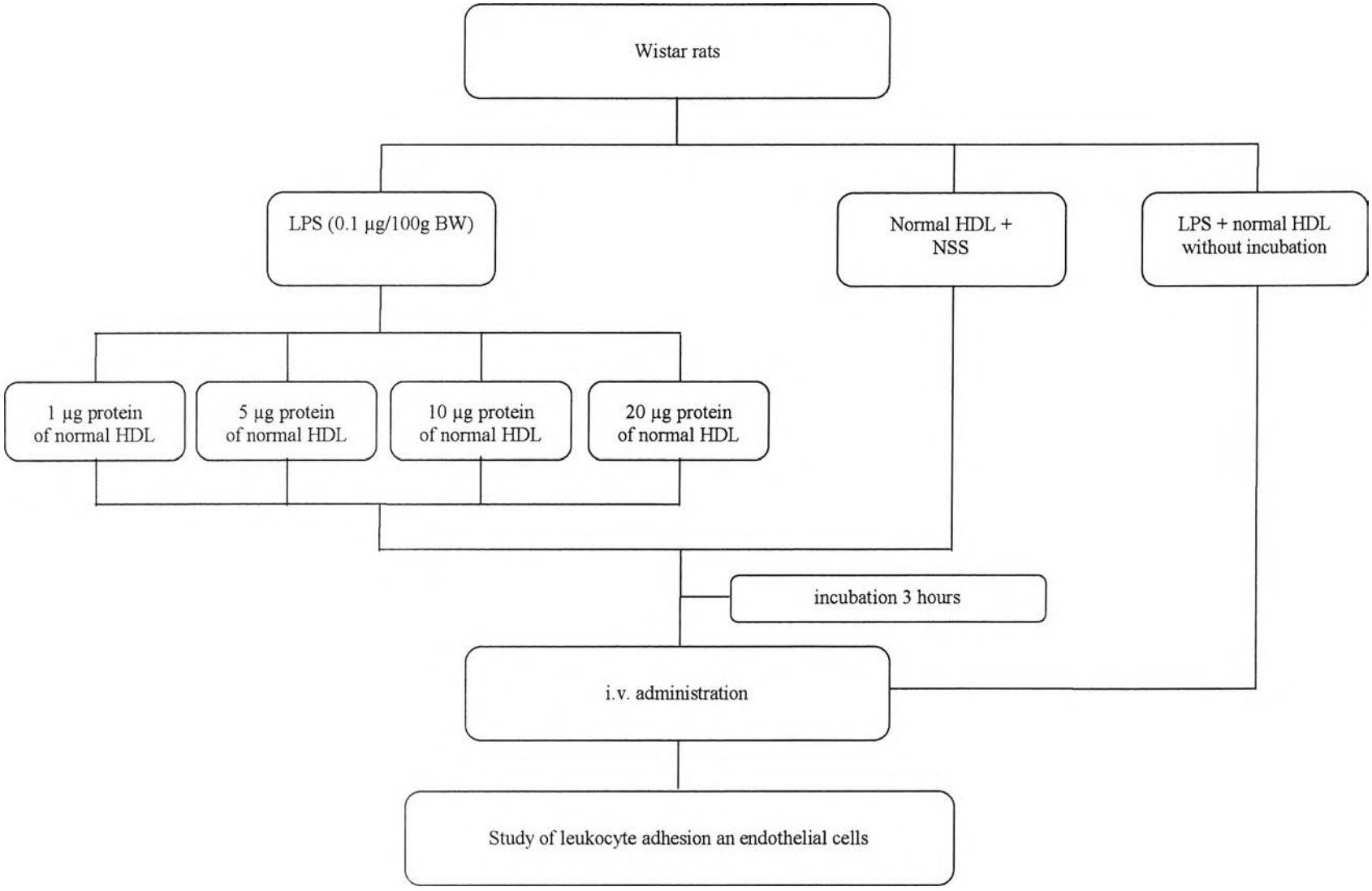


Figure 12 Diagram of experimental groups of the study of normal HDL on LPS – induced leukocyte adhesion on endothelium

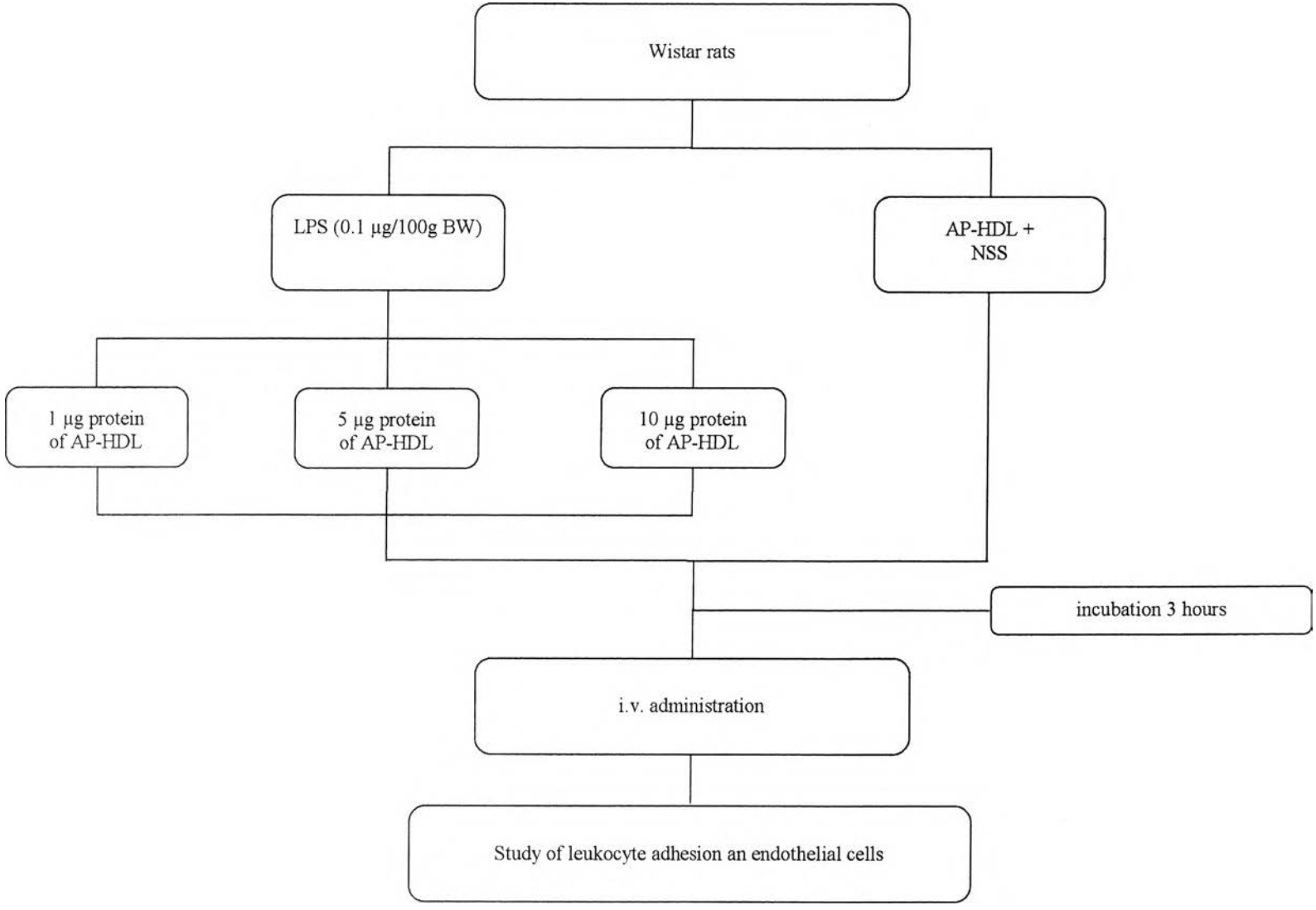


Figure 13 Diagram of experimental groups of the study of AP-HDL on LPS – induced leukocyte adhesion on endothelium

Protocol III. The effects of the molecular components (apoHDL, lipids and apo A-I) of normal HDL and AP-HDL on LPS-induced leukocyte adhesion on endothelium

In order to investigate whether the effect of HDL on inhibiting LPS-induced leukocyte adhesion was due to protein or lipid component in HDL, protein and lipid component were isolated from HDL as described below. The concentration of lipid used was similar to the concentrations described previously (Grunfeld et al.,1999).

LPS was preincubated with apoHDL or lipids (Figure 14) or apo A-I (Figure 15) at 37^{O}C for 3 hours before administration (Grunfeld et al.,1999). Lipid solvent was also preincubated with NSS at 37^{O}C for 3 hours before administration (Grunfeld et al.,1999). After administration, the experiment was performed following the procedure described below.

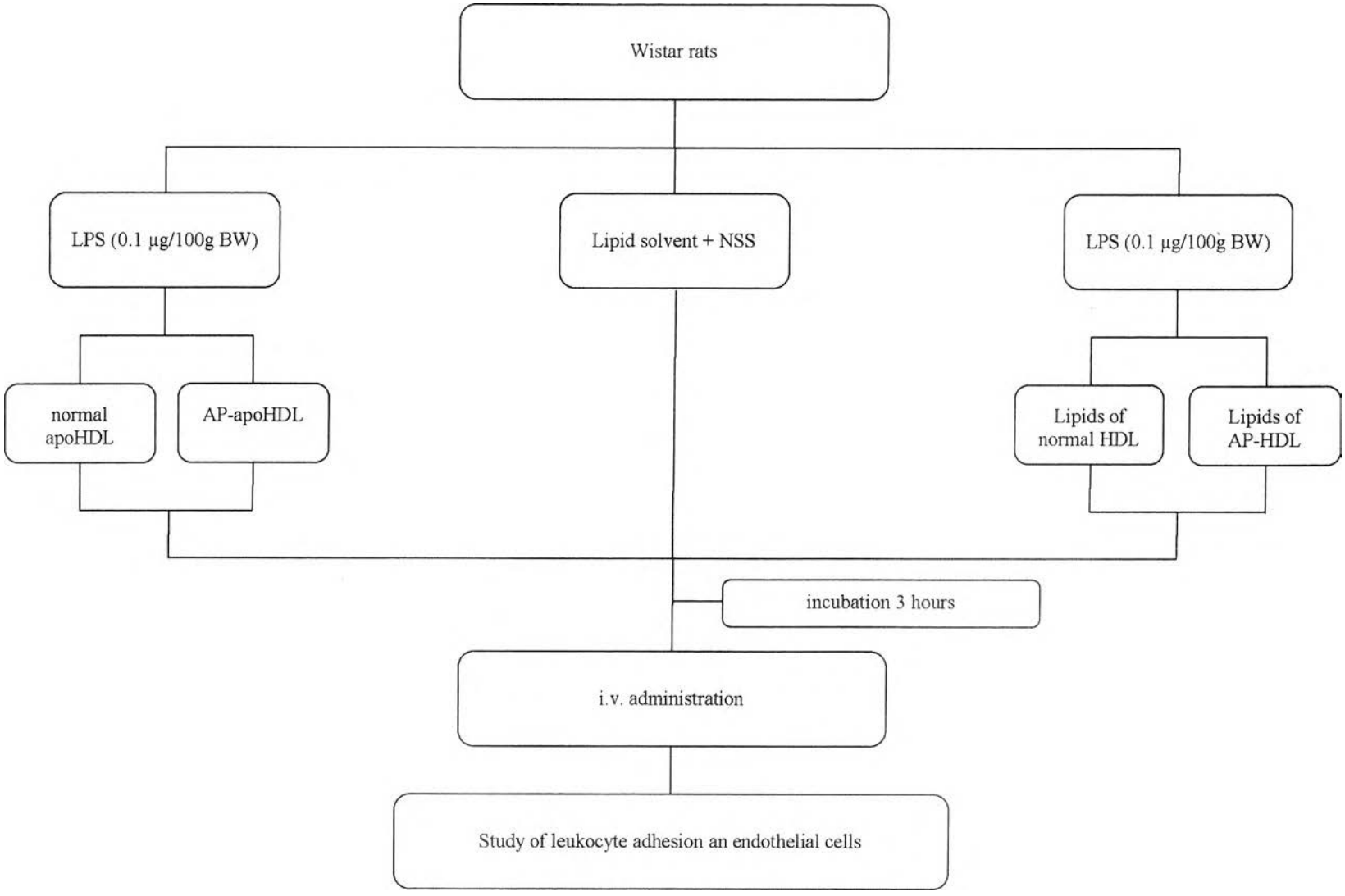


Figure 14 Diagram of experimental groups of the study of apoHDL and lipids of HDL on LPS – induced leukocyte adhesion on endothelium

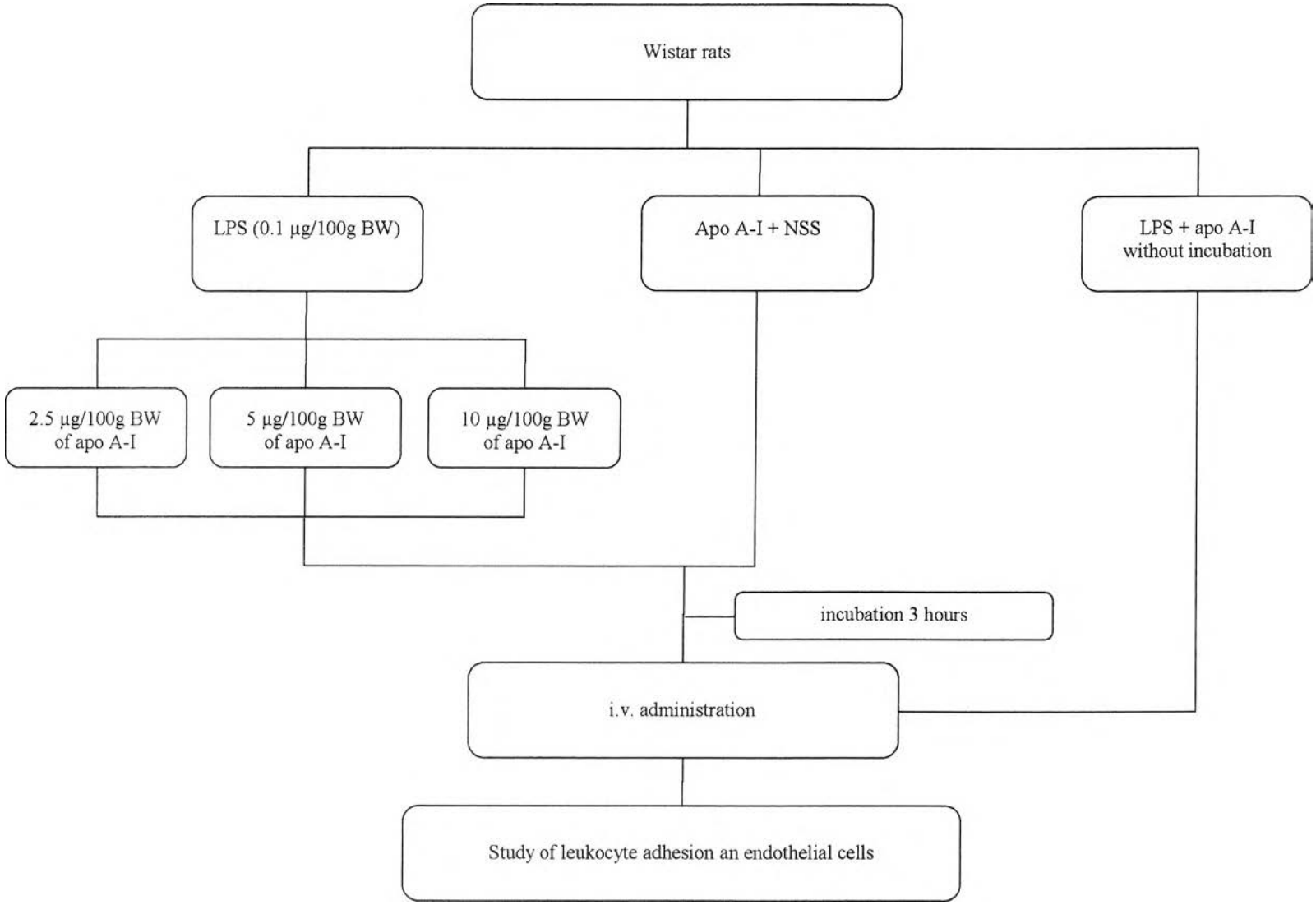


Figure 15 Diagram of experimental groups of the study of apo A-I on LPS – induced leukocyte adhesion on endothelium

Chemicals

Lipopolysaccharide (LPS, Escherichia coli 055:B5) was purchased from Sigma (USA). Centrifugal filter devices (molecular weight cutoff 10,000 Da) and 0.22 um pore size filter unit were from Millipore (Ireland). Quick-seal polyallomer tubes were from Beckman Coulter Inc. (USA). Modified Lowry assay kit was purchased from Pierce (USA). Various chemicals were purchased from Asia Pacific Specialty Chemicals Limited (Australia), Merck (Germany), and Sigma (USA). Normal saline solution (NSS) and sterile water were from General Hospital Product Public Co.,Ltd. (Thailand).

Animals

Syrian hamsters were used in these experiments because lipoprotein metabolism of hamsters closely resembles that of humans than other rodents (Ly et al., 1995; Woollett and Spady, 1997; Khovidhunkit et al., 2001).

Male Syrian hamsters weighing 100-130 g and male Wistar rats weighing 180-220 g, 6 – 8 weeks of age, were purchased from the National Animal Center at Mahidol University, Salaya, Thailand. They were maintained on standard laboratory chow and tap water ad libitum 5 – 7 days before the experiments at the Experimental Animal Center, Faculty of Medicine, Chulalongkorn University. The temperature at the animal center is kept at 25^{O}C with dark-light cycle 12-12 hours.

Purification of normal HDL and acute-phase HDL (AP-HDL)

On the experimental day, hamsters were divided randomly into two groups ; one group received 100 μg/100g body weight (BW) of LPS and the other received NSS. Because LPS can cause anorexia, food was withdrawn after the injection in both groups. Sixteen hours after the injection, animals were anesthetized with ethyl-ether (Perez-Llamas et al., 1992) and blood samples were collected from inferior vena cava with sterile syringes and

allowed to clot in a sterile test tube. Serum was separated by centrifugation at 3,000 g for 30 minutes at $4^{O}C$ and used for HDL isolation (Havel et al., 1955).

Normal HDL and AP-HDL (d = 1.063 – 1.21 g/cm^3) were isolated by differential ultracentrifugation from pooled sera of hamsters injected with NSS and LPS, respectively (five hamsters for each group) (Havel et al., 1955). Potassium bromide (KBr) was used to adjust for the desired density, and ultracentrifugation was performed using a Beckman ultracentrifuge. Briefly, VLDL, IDL and LDL were isolated by centrifugation at density of 1.063 g/cm^3. In order to adjust the density of serum to 1.063 g/cm^3, the appropriate volume of KBr stock solution of density of 1.346 g/cm^3, which was calculated from formula belowed, was added to serum and mixed. Serum of appropriate density was pipeted into polyallomer tubes and they were sealed. They were centrifuged at 100,000 g at $10^{O}C$ for 24 hours using a 50.4Ti rotor. After centrifugation, lipoproteins with the density less than 1.063 g/cm^3 were concentrated in a layer at the top of the tube. Beneath this layer was a clear region. Then, tubes were placed in a Beckman tube slicer and sliced. Infranates were collected for HDL isolation. HDL were centrifuged at the density of 1.063-1.21 g/cm^3 fraction after adjustment of density by addition of pre-heated solid KBr (1.171 g/ 5 ml. fraction) at 100,000 g at $10^{O}C$ for 48 hours using a 50.4Ti rotor. After centrifugation, HDL were concentrated in the top layer. Tubes were sliced and HDL were collected. Normal HDL and AP-HDL were dialyzed three times against normal saline, filtered with a 0.22 μm sterile filter, kept at $-20^{O}C$ and used within 2 weeks. Protein concentrations of HDL were determined by a modified Lowry method. Special precautions during isolation and handing of HDL were used to avoid the contamination with bacteria or LPS (Khovidhunkit et al., 2001).

The density of serum was raised by the addition of concentrated salt solution. The stock salt solution that was density of 1.346 g/cm^3 contained 153 g of sodium chloride and 354 g of potassium bromide per liter. Solutions of

lower density or serum or infranates were prepared to the desired non-protein solvent density by the addition of a salt solution according to the formula :

$$(A \times Y) + (B \times Z) = (A + B)X$$

Where A was the volume of serum or serum infranates, B was the volume of salt solution, Y was the non-protein solvent density of the sample whose density was to be raised, Z was the density of the salt solution, and X was the desired non-protein density of the mixture.

Purification of apoHDL and lipids of HDL

Removal of lipids from HDL in the process of delipidation results in lipid-free protein of HDL called apoHDL as briefly described below. Purified HDL was extracted with 10 volume of 3:1 (vol/vol) cold ethanol/diethyl ether and stored overnight at -20^{O}C. Then, the solution was centrifuged at -5^{O}C for 15 minutes and the apoHDL protein pellet and the lipid phase were separated. ApoHDL was washed with cold diethyl ether once. Both apoHDL and the lipid phase were dried under N_2 gas. ApoHDL was solubilized in phosphate buffer solution (pH 7.4), filtered with a 0.22 μm sterile filter and protein concentration was measured by modified Lowry assay. Lipids were dissolved in 2:1 (vol/vol) chloroform-methanol (Sperry et al.,1955).

Purification of human apolipoprotein A-I (apo A-I)

Apo A-I was purified from normal human HDL because human HDLcan be easily obtained in high amount. In addition, lipoprotein metabolism of hamster closely resembles that of human than other rodents (Ly et al., 1995, Woollett and Spady, 1997).

Human HDL from normal healthy volunteers was isolated by ultracentrifugation as described above and electrophoresed in polyacrylamide gels (The Laemmli system) (Laemmli, 1970). It consisted of resolving or separating (lower) gels and stacking (upper) gels. The stacking gels act to concentrate large sample volumes, resulting in better band resolution than is

possible by using the same volumes on a gel without a stack. The gel recipes were as follows :

The 11.25% acrylamide resolving gel contained (41 ml)

1. deionized water 23.59ml.
2. 3M Tris-HCl<pH 9 5 ml.
3. 20% (w/v) SDS (sodium dodecyl sulfate) stock 250 µl.
4. glycerol 960 µl.
5. acrylamide/Bis (40% stock) 11 ml.
6. 10% ammonium persulfate 150 µl.
7. TEMED (*N, N, N', N'*-tetramethylethylenediamine) 50 µl.

10% ammonium persulfate and TEMED were added to the monomer solution before pouring. Two resolving gels were cast simultaneously and were immediately covered with deionized water until gels had polymerized. Then, deionized water was removed, and the stacking gel that used agarose gel was poured just after a comb was placed above resolving gel.

Electrode (running) buffer was prepared by combining 100 ml of 10X electrode with 900 ml of deionized water. 10X electrode buffer was composed of

1. Tris-base 30.3 g
2. glycine 144 g
3. SDS 10 g

Deionized water was added to the final volume of 1 L. Electrode buffer was added to the lower buffer chamber and upper buffer chamber. Standard marker and sample were loaded into the wells under the electrode buffer. Samples had to be diluted with a sample buffer at least 1:4 (4 volumes of sample : 1 volumes of sample buffer) before loading. The sample buffer contained :

1. deionized water	3.8	ml
2. 0.5M Tris-HCl, pH 6.8	1.0	ml
3. glycerol	0.8	ml
4. 10% (w/v) SDS	1.6	ml
5. 2-mercaptoethanol	0.4	ml
6. 1% (w/v) bromophenol blue	0.4	ml

The electrophoresis apparatus was connected to a power supply and current should be approximately 70 mA for 3-3.5 hours.

After termination the running, Apo A – I bands identified by copper staining (0.3M $CuCl_2.2H_2O$) were cut and proteins were eluted using an electroeluter (Bio-Rad, Hercules, CA) as previously described (Khovidhunkit et al., 2005). Briefly, after cutting apo A-I band from the gel, it was destained with 0.25M EDTA/0.25M Tris-HCl, pH 9 for 3 times 10 minutes and then 20nM Tris-base/150mM glycine/0.01% SDS for another 10 minutes. Then, apo A-I band was cutted into small pieces and loaded into the electroeluter apparatus and eluted at 8-10 mA/glass tube constant current for 3-5 hours. The elution buffer contained 0.05M NH_4HCO_3 and 0.1% SDS. After the elution was completed, the eluted apo A-I was in the solution in the glass tubes. Then, the solution containing eluted apo A-I was collected and dried in the speed vacuum machine for 2 hours. Purified apo A-I was dialyzed in NSS and filtered with a sterile filter. Protein concentrations of apo A-I were determined by a modified Lowry method. Special precautions during purification and handing of apo A-I were used to avoid the contamination (Khovidhunkit et al., 2001).

Bacteria

Preparation of bacterial stock

Gram-positive bacteria, *Staphylococcus epidermidis* (*S. epidermidis*), and Gram-negative bacteria, *Escherichia coli* (*E. coli*), were used. These

bacteria are the common causes of infection in the hospital. *S. epidermidis* (ATCC 12228) and *E. coli* (ATCC 25922) were obtained from the Ministry of Public Health, Thailand.

A pure strain of bacteria was streaked on an agar plate and incubated overnight at 37°C. 3 - 4 colonies were collected to subculture in liquid media until reaching a stationary phase of bacterial growth cycle in which the numbers of new organisms produced are equal to the numbers of cells died. Cells were resuspended in a smaller volume of the same growth media, and an equal amount of 20% (vol/vol) glycerol was added. Bacterial suspension was aliquoted into small plastic vials and frozen at –80°C for long term use.

Preparation of bacterial suspension

To recover a bacterial strain from the –80°C glycerol stock, a sterile loop was used to streak out the stock culture on an agar plate (Heckly, 1978). The plate was then incubated in an incubator overnight at 37°C. Three to four colonies were subcultured in liquid media (Smith et al., 2003) and incubated in a shaking incubator at 37°C until reaching the log phase of bacterial growth cycle of which cell growth and cell division are occurring at their maximal rates. Cells in the log phase were used in the study.

Determination of the log and stationary phases (Smith et al., 2003)

Three to four colonies of a pure overnight culture were subcultured in liquid media and incubated in a shaking incubator overnight at 37°C. Then, dilution of subcultured bacteria in a liquid media was incubated in a shaking incubator at 37°C. Sampling was performed at a 30-minute interval. Aliquots containing 50 µl were subcultured by using serial dilutions on an agar media in triplicate, and colonies were counted at each time interval. The number of colonies and time were plotted to determine the log and stationary phase of the bacterial growth cycle (Figure 16 & 17). *E. coli* was incubated in liquid media for 3 hours and 3.5 hours for *S. epidermidis* before using in the protocol I.

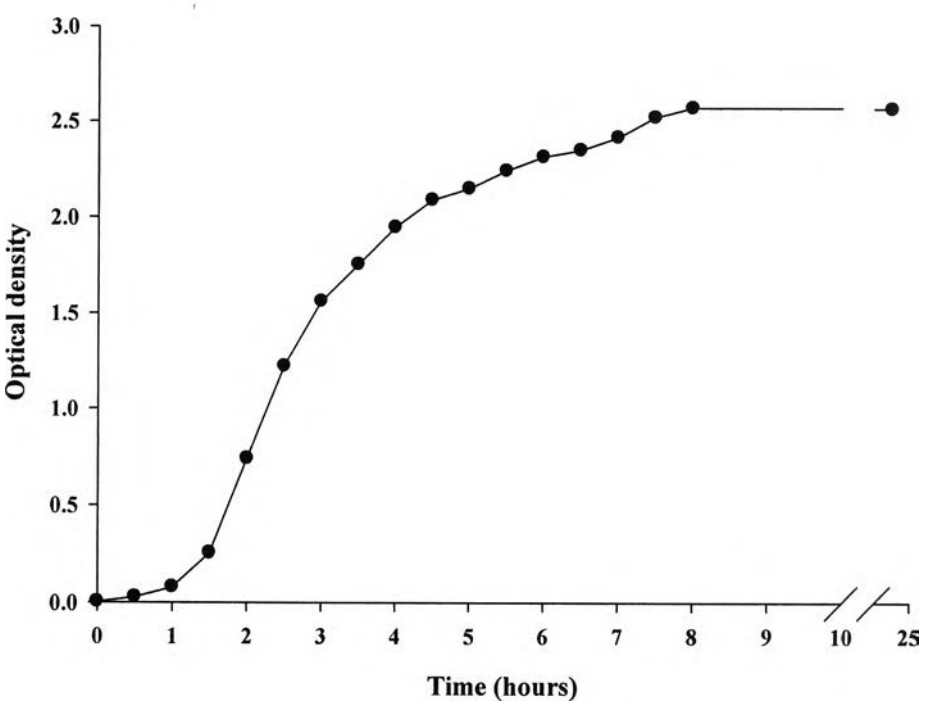


Figure 16 Growth curve of *E. coli*

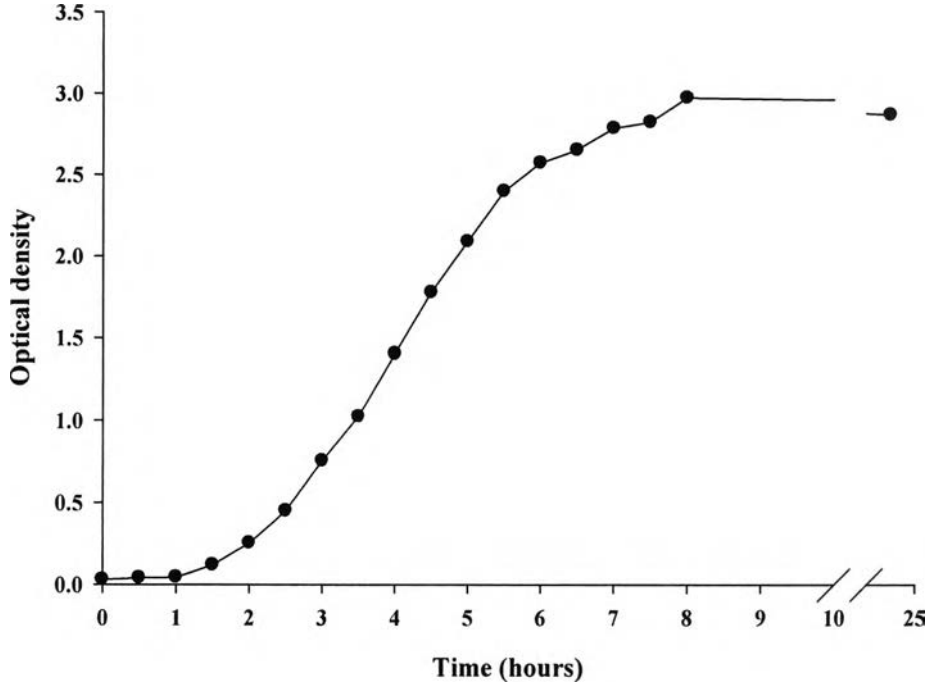


Figure 17 Growth curve of *S. epidermidis*

Leukocyte-endothelial cell interaction

Leukocyte adhesion on endothelial cells of the mesenteric microcirculation

In this study the mesenteric microcirculation was used to observe the interaction between leukocyte and endothelial cells. This tissue model is easy to induce inflammation and enable us to observe this interaction *in vivo* using a fluorescence microscope.

Male Wistar rats (180-220g) were used in this study. After an overnight fast, rats were anesthetized with sodium pentobarbital (60 mg/kg BW i.p.). Rats were prepared for intravital microcirculation study according to a technique described previously (Chakraphan et al., 2005). The carotid artery and jugular vein were cannulated for measuring mean arterial blood pressure (MAP), and for agent administration, respectively. A midline laparotomy was made and a loop of mesentery was exteriorized and spreaded onto a Plexiglass chamber for microscopic observation. The mesentery was fixed with a 37^0C–Krebs Ringer Solution (pH 7.4)–soaked gauze and superfused continuously with 37^0C Krebs Ringer Solution (pH 7.4) to avoid dehydration throughout the experiment.

The animal was then placed on the stage under a fluorescence microscope. A fluorescence videomicroscopic system (Nikon, Tokyo, Japan) were equipped with a videocamera (MTI SIT68), a videorecorder (Sony GUM-1411QM) and a videotimer (Sony, Japan) (Figure 18). An 20X objective len was used, and video-images were recorded on videocassettes for off-line analysis.

After the rats were stabilized for 20-30 minutes, a 25-45 μm diameter postcapillary venule was chosen for observation. Acridine orange at the concentration of 1.8 mg/ml was i.v. injected as a bolus (0.25 ml) through the cannulated jugular vein. Twenty minutes after acridine orange injection, baseline quantification of leukocyte adhesion was recorded. Then, LPS was

administered and leukocyte adhesion was recorded at time zero. In some studies, LPS was preincubated with normal HDL, AP-HDL, normal apoHDL, AP apoHDL, lipids of normal HDL, lipids of AP-HDL, or normal human apo A-I at 37^{O}C for 3 hours before use (Grunfeld et al., 1999). Leukocytes which remain stationary for at least 30 seconds on endothelial cells were counted at 1, 3, 5, 10, 15, 30, 45, 60, 75, and 90 minutes along the 100 μm of the venule length (Davenpeck et al., 1998, Chakraphan et al., 2005). Videotape of each experiment was played back and then adherent leukocytes on the post-capillary venules were counted and expressed as number of cells per 100 μm vessel length as shown in figure 19.

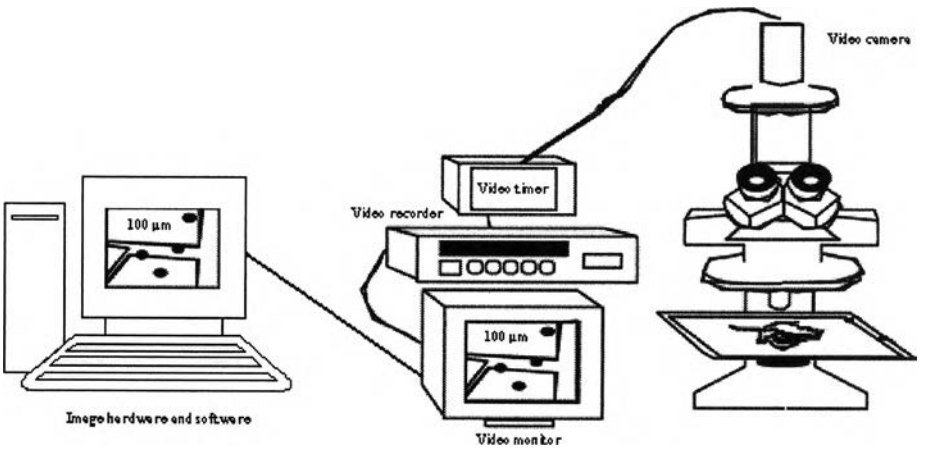
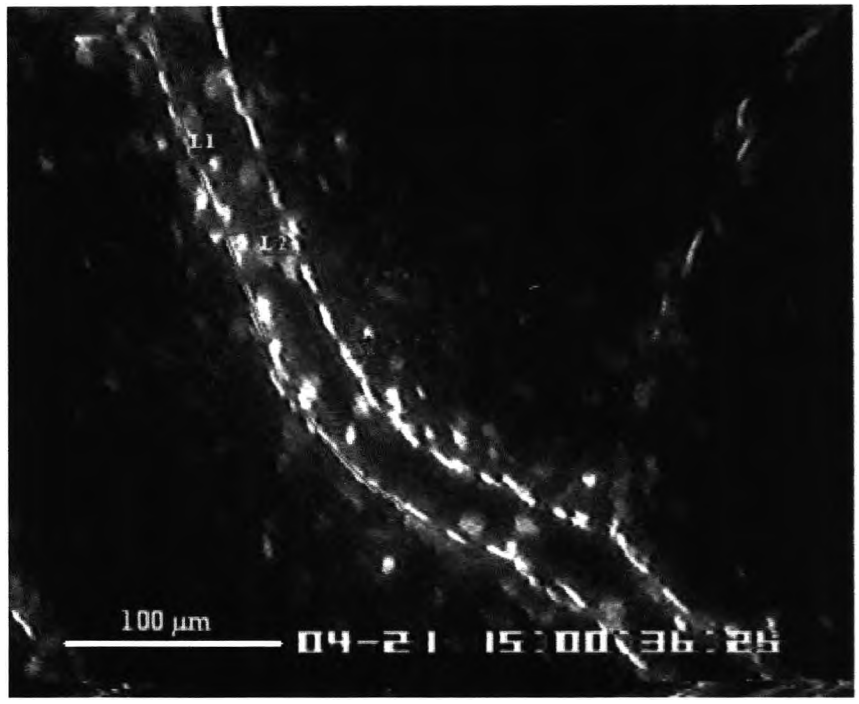


Figure 18. Intravital fluorescence microscope and instruments used for quatitative studies of leukocyte adhesion on endothelial cells of the mesenteric post-capillary venule.

Videoimage at each time was captured for counting the number of adherent leukocytes which was defined as it adhered on endothelium of a venule for a period at least 30 seconds.



L1 : Length of vessel = a (µm) measured from branch to branch

b : Number of adherent leukocytes along the L1 vessel

Number of leukocyte adhesion = $\frac{100 \times b}{a}$ = cells/100 µm of vessel length

Figure 19 Image analysis for determination of leukocyte adhesion on endothelial cells at each time.

Data analysis

All data were presented as mean and standard errors of the mean (mean ± SEM). Comparison among groups was done by ANOVA and the differences in pairs of means among groups were defined by Bonferroni test. The p-value of less than 0.05 indicated a significant difference between groups.