

II. MATERIALS AND METHODS

A. Materials

1. Subjects

Healthy subjects (control) were obtained from department of blood bank at Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine and medical technology students at Faculty of Associated Medical Sciences, Chiang Mai University. All of them must not take any medication for at least 14 days prior to the blood collection.

β -Thalassemia major and β -thalassemia/Hb E were recruited from out-patient department number 29 (OPD 29), Department of Pediatrics. They were all diagnosed as either β -thalassemia major or β -thalassemia/Hb E by electrophoresis. The criteria for sample collection were as follows:

1. Hb typing is A₂F for β -thalassemia major and EF for β -thalassemia/Hb E.
2. Hemoglobin levels between 3-11 g/dL with regular blood transfusion approximately once per three weeks for β -thalassemia major and once per six weeks for β -thalassemia/Hb E.
3. Either splenectomized or non-splenectomized patients were obtained.
4. Age between 3-18 years old.

2. Sample collection and anticoagulants

1. Whole blood samples were collected in 3 ml EDTA vacuum tube for complete blood count (CBC).
2. Whole blood samples for 9.0 mL were collected into plastic tube containing 1.0 mL of 3.2% tri-sodium citrate for the detection of *in vitro*

platelet aggregation and PS exposure on red blood cell membrane by flow cytometry.

3. Whole blood samples for 2.5 mL were collected into plastic tube containing 2.5 mL of 0.25% (v/v) glutaraldehyde in 0.2 M phosphate buffer for the detection of *in vivo* platelet shape change by scanning electron microscopy (SEM).
4. Whole blood samples for 9.0 mL were collected into plastic tube containing 1.0 mL of anti-platelet drug mixture (aspirin 83 mM, caffeine 32 mM, theophylline 17 mM and NaN_3 15 mM in 3.2% tri-sodium citrate) for the detection of plasma β -thromboglobulin (β -TG) and platelet factor 4 (PF4) levels.
5. Whole blood samples for 2.5 mL were collected into plastic tube containing 2.5 mL of fixative solution [0.2% glyoxal (v/v) and 0.4% paraformaldehyde (w/v) in phosphate buffer saline (PBS)] for the detection of *in vivo* platelet activation by flow cytometry.
6. Whole blood samples for 7.0 mL were collected into plastic tube containing 1.0 mL of citrate phosphate dextrose adenine solution formula 1 (CPDA-1) for co-culture experiments.
7. Clotted blood samples for 5 mL were collected for serum samples to be used in lymphocyte cross-matching by microlymphocytotoxicity test.

B. Methods

1. Preparation of anti-platelet mixture

Anti-platelet mixture contained: 1.5 gm aspirin (3 tablets of 500 mg/tablet) in 3.2% tri-sodium citrate 75 mL stirred at room temperature (RT) over night. After that added 0.625 gm caffeine, 0.313 gm theophylline and 0.1 gm NaN_3 stirred at RT for 2 hours (hr). Then adjusted the final volume to 100 mL with 3.2% tri-sodium and filtered with 0.45 μm membrane filter. It was kept in light protected bottle at RT. This mixture had been confirmed for the ability to suppress platelet activation *in vitro* induced by epinephrine on 10 healthy subjects.

2. Preparation of plasma samples for platelet aggregation test

The blood in 3.2% tri-sodium citrate at the ratio of 10:1 was centrifuged at 180g for 20 minutes (min) at 22°C and platelet rich plasma (PRP) was collected into a plastic tube. After that the remaining packed RBC was centrifuged at 1,000g, for 20 min at 22°C. The platelet poor plasma (PPP) was collected into another plastic tube. The platelet count of PRP was done on an automated blood cell analyzer (Hemacel). The final platelet concentration of PRP was adjusted to $2.5 \times 10^5/\mu\text{L}$ with auto-PPP.

Aggregation was measured at 37°C by turbidimetric method on Platelet Aggregometer II. A 575 μL of platelet suspension was stirred at 1,000 rpm and incubated at 37°C for 3 min, then activated by adding of 25 μL of either 1 mg/mL epinephrine, 0.2 mM ADP, 2 mg/mL collagen, or 30 mg/mL ristocetin agonists, for the final volume of 600 μL . Percent aggregation was recorded for 5 min.

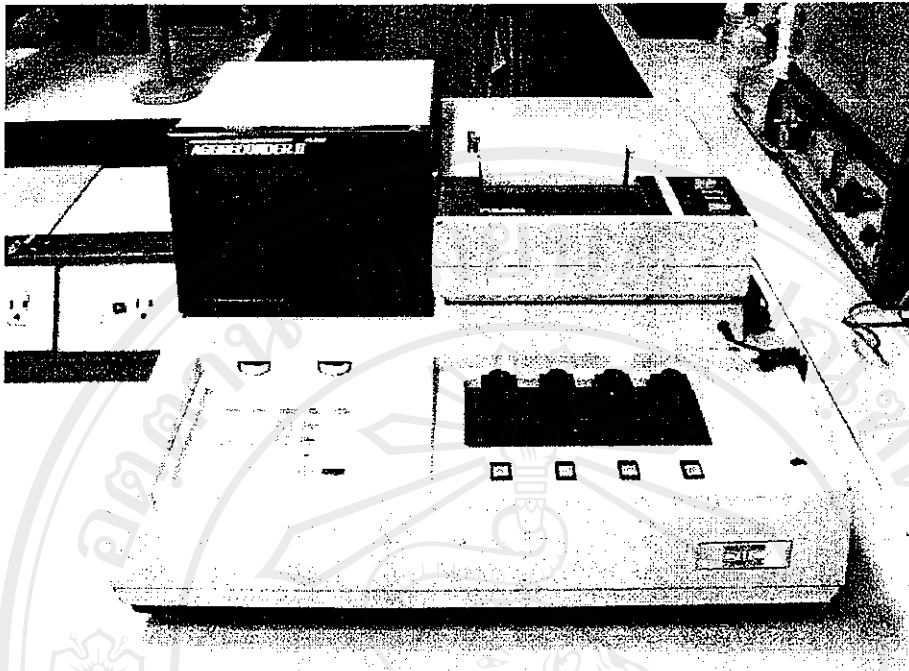
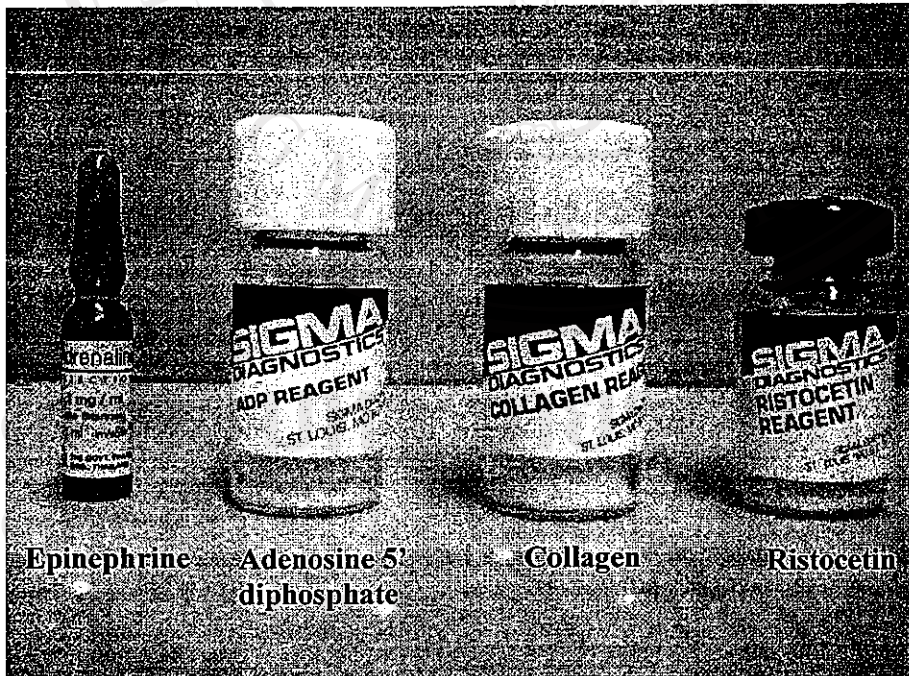


Figure 9 Platelet aggregometer model Aggrecoorder II (PA-3220)



Epinephrine Adenosine 5' diphosphate Collagen Ristocetin

Figure 10 Agonist reagents for platelet aggregation test

3. Preparation of red blood cells for flow cytometric analysis

Pack red cells from 3.2% tri-sodium citrate was washed 3 times with 0.9% sodium chloride (0.9% NaCl, which was centrifuged at 1,200g for 20 min at 22°C before use, to remove dust contaminants) and washed once with annexin V buffer (AVB, which was also centrifuged at 1,200g for 20 min at 22°C before use, to remove dust contaminants) by centrifuge at 1,200g for 5 min at 22°C. After that, equal volume of AVB was added to give approximately 20,000 cells/ μ L. Dispensed 20 μ L of washed red blood cells into new plastic tubes for staining. Anti-GPA-RPE 1 μ L and Annexin-V-FITC 2.0 μ L were added at undiluted or original concentrations. IgG isotype control for 1.0 μ L was applied to control a non-specific binding. After incubation for 15 min at RT the samples were resuspended in 1 mL of 0.05% formaldehyde in AVB prior to flow cytometric analysis.

3.1 Preparation of phosphatidylserine exposing red blood cells (positive control)

Erythrocyte suspensions were prepared from normal samples as indicated above using normal fresh human venous blood collected in 3.2% tri-sodium citrate. Erythrocyte were washed twice with 0.9% NaCl and once with incubation buffer, and finally diluted with incubation buffer (10 mM Tris/HCl buffered saline, pH 7.4; TBS) to approximately 30% hematocrit. Erythrocyte at 30% hematocrit were incubated in buffer containing 10 mM N-ethyl maleimide (NEM) for 30 min at RT and subsequently washed twice with buffer without NEM. The erythrocytes were treated with Calcium ionophore A23187 to induce membrane lipid scrambling. Erythrocytes at 16% hematocrit were equilibrated in incubation buffer with 1 mM calcium chloride for 3 min at 37°C. Subsequently, calcium ionophore A23187 was added to the erythrocyte suspension to a final concentration of 4 μ M. The suspension was incubated for 1 hr at 37°C, the cells was washed twice with 5 mM EDTA and 3 times with buffer containing 1% BSA, and once with AVB. After that an equal volume of

AVB was added to give an approximately 20,000 cells/ μ L. The further steps were as same as the preparation of platelets for flow cytometric analysis above.



Figure 11 Flow cytometer model Becton Dickinson FACSsort

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4. Preparation of platelets for scanning electron microscopy (SEM)

The blood in 0.25% glutaraldehyde was centrifuged at 180g for 10 min at 22°C and platelet rich plasma was collected into a plastic tube. After another centrifugation at 180g for 20 min at 22°C a platelet pellet was suspended in 4 mL of 2% glutaraldehyde in 0.1 M phosphate buffer (which was centrifuged at 1,200g for 20 min at 22°C before use, to remove dust contaminants). After 30 min of fixation, the platelet suspension was centrifuged at 1,200g for 5 min at 22°C, the pellet was washed 3 times with 0.1 M phosphate buffer and suspended in 0.5 mL of 0.1 M phosphate buffer. Fifty μL of suspension were dispensed on poly-L-lysine pre-coated glass cover slips and smear to cover the area of 1 cm^2 . Then incubated in RT for 15 min. The smears were dehydrated in an ethanol series (10%, 30%, 50%, 70%, 80%, 85%, 90%, 95%, and 100% respectively) for 15 min each and in acetone twice for 15 min each. Then the samples were further dried by the critical point drying (CPD) method in CO_2 atmosphere, then mounted into the stubs and coated with gold for 10 min in a sputter coater, before examining by SEM.

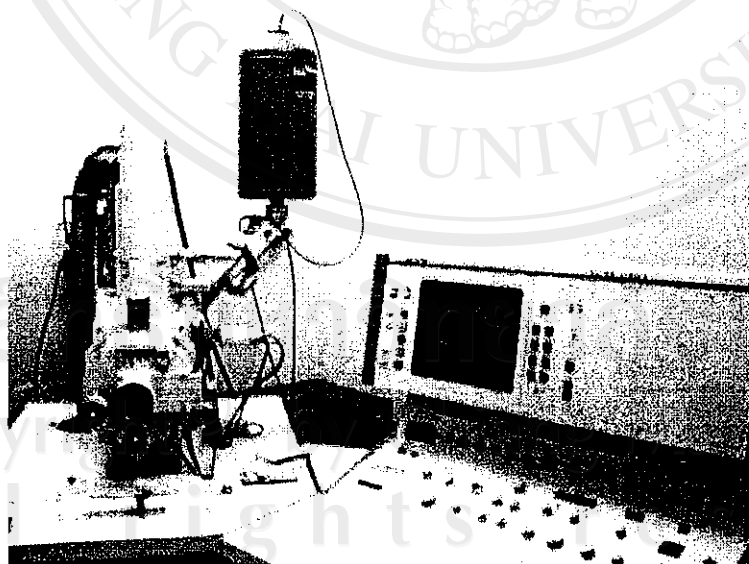


Figure 12 Scanning electron microscope model JSM-840A

5. Preparation of plasma for enzyme linked immunosorbent assay (ELISA)

The blood in 3.2% tri-sodium citrate with anti platelet mixture was centrifuged at 1,200g for 20 min at 22°C. As soon as centrifugation was completely stop, collected one-third volume of the plasma supernatant, making sure not to aspirate some light platelets that may be found next to the platelet layer. The plasma was aliquot into 5 vials, kept at -60 °C for not more than 3 months before assay.

5.1 Principle of β -thromboglobulin (β -TG)

A plastic support coated with specific rabbit anti-human β -thromboglobulin antibodies captured the β -TG to be measured. Rabbit anti- β -TG antibody coupled with peroxidase binds to the remaining free antigenic determinants of the captured β -TG, forming the “sandwich”. The bound enzyme peroxidase was then revealed its activity within a predetermining time using the ortho-phenylenediamine dihydrochloride (OPD) substrate in the presence of hydrogen peroxide. After stopping the reaction with a strong acid, the intensity of the produced color had a direct relationship with the β -TG concentration initially present in the plasma sample.

5.2 Assay of β -thromboglobulin (β -TG)

Dispensed 200 μ L of calibrator, control or diluted sample into precoated wells, then cover the wells and incubated 1 hour at RT (18-25°C). After washing all wells for 5 times with washing solution, immediately added 200 μ L of specific rabbit anti-human β -TG antibodies coupled with peroxidase, cover the wells and incubated 1 hr at RT. After that washed all wells for 5 times with washing solution, then immediately added 200 μ L of OPD/H₂O₂ substrate, incubated at RT for exactly-3 min, then added 50 μ L acid (3M H₂SO₄) into each wells and waited for 10 min, then measure the absorbance at 490 nm within 2 hr (adjust reader to zero on reagent blank).

5.3 Principle of platelet factor 4 (PF4)

A plastic support coated with specific rabbit anti-human PF4 antibodies captured the PF4 to be measured. Rabbit anti-PF4 antibody coupled with peroxidase binds to the remaining free antigenic determinants of the captured PF4, forming the “sandwich”. The bound enzyme peroxidase was then revealed its activity within a predetermining time, using the OPD substrate in the presence of hydrogen peroxide. After stopping the reaction with a strong acid the intensity of the color produced had a direct relationship with the PF4 concentration initially present in the plasma sample.

5.4 Assay of platelet factor 4 (PF4)

Dispensed 200 μL of calibrator, control or diluted sample into precoated wells, then cover the wells and incubated 1 hr at RT. After washing all wells for 5 times with washing solution, then immediately added 200 μL of specific rabbit anti-human PF4 antibody coupled with peroxidase, cover the wells and incubated 1 hr at RT. After washing all wells for 5 times with washing solution, then immediately added 200 μL of OPD/ H_2O_2 substrate, incubated at RT for exactly 3 min, then added 50 μL acid (3M H_2SO_4) and waited for 10 min, then measure the absorbance at 490 nm within 2 hr (adjust reader to zero on reagent blank).

6. Preparation of platelets for flow cytometric analysis

The blood in 0.2% glyoxal and 0.4% paraformaldehyde was centrifuged at 100g for 20 min at 22^oC and platelet rich plasma was transferred into a plastic tube. It was washed 3 times with 0.1% bovine serum albumin in PBS (0.1% BSA-PBS, which was centrifuged at 1,200g for 20 min at 22^oC before use, to remove dust contaminants). After that the pellet was resuspended with 0.1% BSA-PBS to give an approximately 20,000 cells/ μ L. Twenty μ L of wash platelets was dispensed into new plastic tubes then directly stained with monoclonal antibodies. Anti-CD42b-FITC for 2.5 μ L, anti-CD63-RPE for 2.5 μ L was added at undiluted or original concentrations. IgG isotype controls for 2.5 μ L were applied to control a non-specific binding. After incubation for 30 min at RT, the samples were resuspended in 1 mL of 0.1% BSA-PBS and stored at 4^oC for a maximum of 2 hr prior to flow cytometric analysis.

6.1. Preparation of activated platelets (positive control)

An aliquot of 500 μ L normal platelet rich plasma (250,000 cells / μ L) was reacted with 10 μ L of ADP (adenosine 5'diphosphate at the final concentration of 1 μ M) and incubated at RT for 2 min. After that fixative reagent (0.2% glyoxal & 0.4% paraformaldehyde) was added and incubated for 10 min. The platelets were washed 3 times with 0.1% BSA-PBS. After which an equal volume of 0.1% BSA-PBS was added to give an approximately 20,000 cells/ μ L. The further steps were as same as the preparation of platelets for flow cytometric analysis above.

7. Preparation of platelet rich plasma, plasma and red blood cells for co-culture

7.1 Preparation of normal platelet rich plasma (PRP) as a responder

Whole blood containing CPDA-1 was centrifuged at 200g for 20 min at 22⁰C. After transfer platelet rich plasma to another plastic tube and measure the platelets count by an automatic blood cell analyzer (Hemacel). The final platelet concentration was adjusted to $4.0 \times 10^5/\mu\text{L}$ with auto-PPP.

7.2 Preparation of plasma as a stimulator

Whole blood containing CPDA-1 was centrifuged at 200g for 20 min at 22⁰C. After carefully transfer platelet rich plasma into another plastic tube, then centrifuged at 300g for 20 min at 22⁰C, transferred the supernatant into another plastic tube then repeat this once. The supernatant was transferred into another plastic tube and was centrifuged at 400g for 20 min at 22⁰C transferred the supernatant in to another plastic tube then repeat the centrifugation at 500g for 10 min at 22⁰C and did it again at 600g for 10 min at 22⁰C, after that transfer the plasma to another plastic tube. It was kept in -20⁰C.

7.3 Preparation of absorbed plasma as a stimulator

The pool platelet concentrate was obtained from 15 blood group "O" donors keeping in anti-platelet mixture. The individual platelet concentrate was washed 5 times with CPDA-1-0.1% BSA-PBS. The washed platelet concentrate from 15 donors were then pooled together.

Two mL of CPDA-1 plasma was incubated at 37⁰C overnight (shacking regularly) with a platelet pellet, originally came from the centrifugation of 2 mL pool platelets ($10^6/\mu\text{L}$). After that they were centrifuged at 1,500g for 20 min and transfer the plasma to another plastic tube. It was kept in -20⁰C.

7.4 Preparation of peripheral blood mononuclear cells for lymphocytotoxicity

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-hypaque density gradient method (density = 1.077 gm/mL). The diluted 5 mL whole blood containing 0.5 mL heparin (500 IU/mL) in 1:1 with PBS was underlaid with the Ficoll-hypaque then was centrifuged at 400g for 10 min at 22°C. The PBMCs were collected into another tube and washed twice with PBS (pH 7.3) and washed once again with RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum and 5,000 unit/mL of penicillin and 5 mg of streptomycin. After that resuspended in RPMI-1640 medium then counted and stained with trypan blue for viability test. The viable PBMCs should be 90% or more in the cell suspension.

7.5 Lymphocyte cross matching by microlymphocytotoxicity assay

Five μL of mineral oil was dispensed into every wells of a terazaki tray, followed by 1 μL of patient or normal serum and 1 μL of responder lymphocyte (pre-adjusted to 2,500 cells/ μL). They were mixed and incubated at RT for 30 min. After that 5 μL of rabbit complement was added and incubated at RT for 60 min. After that 5 μL of 5% Eosin Y was added and incubated at RT for 2 min. Then 5 μL of neutral formalin was added, incubated at RT for 15 min and finally the cells were analyzed by inverted phase contrast microscopy.

7.6 Preparation of red blood cells as a stimulator

Pack red cell form CPDA-1 blood was washed 5 times with CPDA-1-0.1% BSA-PBS (pH 7.3), then resuspended in 1% formaldehyde in CPDA-1-0.1% BSA-PBS (pH 7.3). They were washed 5 times with CPDA-1-0.1% BSA-PBS before use.

7.7 Co-culture assay

Co-culture between normal platelet rich plasma (PRP; responder) with red blood cells, unabsorbed plasma or absorbed plasma stimulators were performed as follows:

- Tube No.1 Normal PRP 1 mL with auto pack red blood cells 1 mL
- Tube No.2 Normal PRP 1 mL with auto plasma 1 mL
- Tube No.3 Normal PRP 1 mL with another normal pack red blood cells 1 mL
- Tube No.4 Normal PRP 1 mL with another normal unabsorbed plasma 1 mL
- Tube No.5 Normal PRP 1 mL with another normal absorbed plasma 1 mL
- Tube No.6 Normal PRP 1 mL with patient pack red blood cells 1 mL
- Tube No.7 Normal PRP 1 mL with patient unabsorbed plasma 1 mL
- Tube No.8 Normal PRP 1 mL with patient absorbed plasma 1 mL

They were incubated at 37⁰C, in 5% CO₂ and harvested the cells at 0 min and 30 min. The harvested cells were immediately fixed with 0.2% glyoxal and 0.4% paraformaldehyde in PBS (pH 7.3). The staining with monoclonal antibodies (MoAb anti CD42b and MoAb anti CD63) was employed before flow cytometric analysis. The procedure was as same as the flow cytometric CD63⁺ platelets assay describing before.

Whole blood in CPDA-1 or 3.2% Tri-sodium citrate (0.106 M) with or without

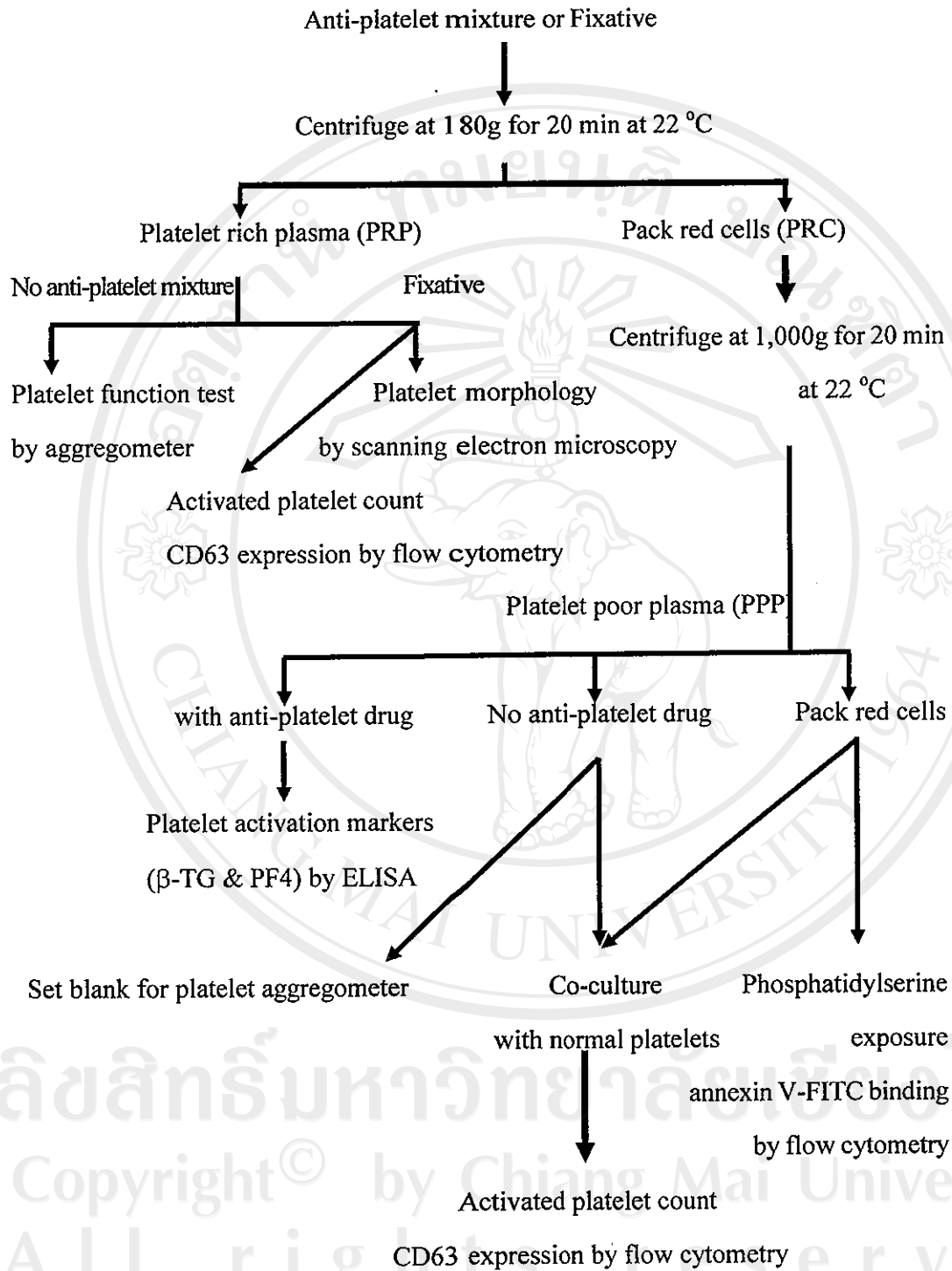
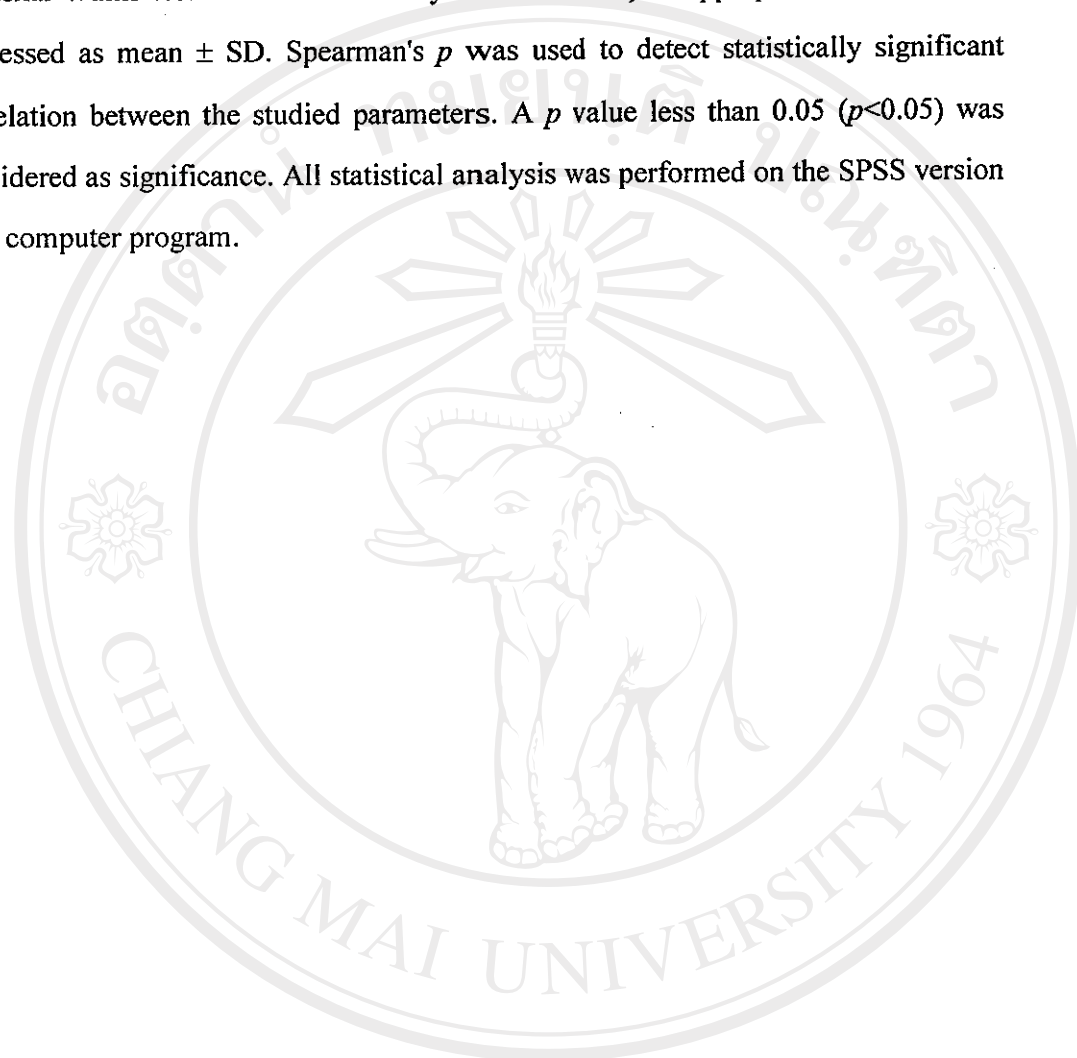


Figure 13 Processing of specimens for all analyses

Statistical analysis

Comparisons of quantitative data were performed using non-parametric test (Kruskal-Wallis test and Mann-Whitney rank sum test) as appropriate. Results were expressed as mean \pm SD. Spearman's ρ was used to detect statistically significant correlation between the studied parameters. A p value less than 0.05 ($p < 0.05$) was considered as significance. All statistical analysis was performed on the SPSS version 10.0 computer program.



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