Development of a prototype-reagent for inhibition of platelet function and anti-coagulation for commercialized purpose.

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Abstract

Introduction: Normally platelets can be activated by external environment. After activation they change the shape to be spherical and expose some neoantigens (CD62p & CD63). Study of *in vivo* platelet activation needs anti-coagulant and anti-platelet, to ensure that the activated platelets come from *in vivo* not *in vitro*. Therefore inhibition of platelet function must be immediately performed after blood collection. However the commercial anti-coagulant and anti-platelet reagent is imported and expensive.

Objective: To prepare a prototype-reagent for inhibition of platelet function and anti-coagulant mixture, then compare the quality of self-preparing reagents with the commercial reagent.

Materials and methods: Normal blood samples were collected in 4 types of reagents (3.2% sodium citrate, anti-platelet drugs and anti-coagulant 1 (APAC-1), APAC-2 and commercial CTAD (citrate, theophyline, adenosine & dipyridamol) reagent. The optimum ratio of anticoagulant in blood is 1 in 10. Platelet rich plasma were obtained by centrifugation and adjusted the concentration to 3 x 10^5 plt/cu.mm. Comparison of the results of platelet aggregation tests using epinephrine, ADP, collagen and ristocetin as agonists and activated platelet ($CD63^+$) count using flow cytometry was performed. Examination of the morphology of activated platelets using scanning electron microscopy was also done.

Results: The significant differences of percentages of platelet aggregation (% agg) were found between citrate vs APAC-1, citrate vs APAC-2 and citrate vs CTAD using ADP; citrate vs APAC-2 and citrate vs CTAD using collagen ($p<0.05$). While the significant differences of the percentages of activated platelets (% CD63 pos) counted by flow cytometry were found between citrate vs APAC-2 and APAC-1 vs CTAD ($p<0.05$) using collagen as an agonist. Aggregated platelets examined by SEM in citrate were found while in APAC-1, APAC-2 and CTAD was found in reduced degree.

Conclusion: APAC-1& APAC-2 are comparable to CTAD on platelet function inhibition using ADP and collagen as agonists. Therefore these 2 self-preparing reagents are encouraged to be used in stead of commercial CTAD for economical reason. The self-preparing reagents also have a potential in commercialization. However ristocetin can induce platelet aggregation in all reagents while epinephrine can not. So further study must be carried out for finding a complete inhibitor of platelet function, especially when ristocetin is used.

Key words: Prototype reagent, platelet function, Anti-platelet drug, Anticoagulant, flow cytometry.

1. Introduction

Platelets are the smallest anucleated blood cells in the blood circulation. They are derived from megakaryocytes in the bone marrow. They contain numerous granules that are the source of numerous mediators e.g. vasoactive amines, serotonin, ADP, etc. The human body does not want to face excessive blood loss. Therefore, the the coagulation system and platelets share an important role in stop bleeding. Platelets are activated, shape changed, released their contents, adhered and aggregated themselves to form a hemostatic plug. They also provide some vasoconstrictor and phospholipids that are needed for forming blood clots to reduce and stop bleeding.

The circulating resting platelets are discoid shape. After activation they change the shape to be spherical with long extensions and expose some neoantigens (CD62p & CD63). Normally platelets can be activated by external environment. Study of *in vivo* platelet activation needs anti-coagulant and anti-platelet to ensure that the activated platelets come truly from *in vivo* not *in vitro*. Therefore inhibition of platelet function must be immediately performed after blood collection. The commercial anti-coagulant and anti-platelet reagent is not only imported but also expensive.

2. Materials and Methods

1. Preparation of 3.2 % tri-sodium citrate solution: 3.2 gm of tri-sodium citrate was dissolved in 100 mL distilled water.

2. Preparation of anti-platelet drugs and anti-coagulant 1 (APAC-1): 1.5 gm aspirin was dissolving in 75 mL of 3.2% tri-sodium citrate solution, stirred at room temperature (RT) over night. After that 0.625 gm caffeine, 0.313 gm theophylline and 0.1 gm NaN_3 were added, stirred at RT for 2 hr. Then adjusted the final volume to 100 mL with 3.2% tri-sodium citrate solution and the mixture was filtered with 0.5 nm membrane filter. It was kept at RT for months.

3. Preparation of anti-platelet drugs and anti-coagulant 2 (APAC-2): 0.025 gm dipyridamol was dissolving in 75 mL of 3.2 % tri-sodium citrate solution, stirred at RT over night. After that 0.778 gm theophylleine, 0.247 gm adenosine and 0.243 gm NaN_3 were added, stirred at RT for 2 hr. Then adjusted the final volume to 250 mL with 3.2% tri-sodium citrate solution and the mixture was filtered with 0.5 nm membrane filter. It was kept at 4 ºC for months.

4. Normal whole blood samples were obtained from a peripheral vein and collected into 4 types of reagents (3.2 % sodium citrate, anti-platelet drugs and anti-coagulant 1 (APAC-1), APAC-2 and commercial CTAD (citrate, theophylline, adenosin & dipyridamol)

reagent. The optimum ratio of anticoagulant in blood is 1 in 10. Platelet rich plasma (PRP) were obtained by centrifugation at 1,000 rpm for 10 min and adjusted the concentration to 3 x 10^5 plt/cu.mm. and then comparison of the results of platelet aggregation tests by platelet aggregometer II using epinephrine, ADP, collagen and ristocetin as agonists in all samples was done. Percent platelet aggregation (% agg) was recorded for 5 min.

After activation the platelet suspension was fixed with 0.2% glyoxal and 0.4% paraformadehyde solution immediately. Ten L of activated platelet suspension was directly stained with 2.5 L of monoclonal antibody to CD42b conjugated rhodamine phycoerythrin (MoAb-CD42b-RPE) and 2.5 L of MoAb-CD63 conjugated fluorescein isothiocyanate (FITC) simultaneously. In addition mouse IgG isotype controls both RPE and FITC conjugates for 2.5 L were applied to control a non-specific binding. After incubation for 30 min at RT in the dark, the samples were resuspended in 1 mL of 0.1% BSA-PBS and store at 4 ^{o}C for a maximum of 2 hr prior to flow cytometric analysis.

Morphology change of activated platelets was examined by scanning electtron microscopy. Fixed platelets immediately after activation were serially fixed with 0.25% and 2% glutaraldehyde at RT for 30 min each. The fixed platelets were washed 3 times with PBS. The platelets were then smeared on coverslip pre-coated with poly-L-lysine and left for air-dry. The smears were then dehydrated with serial concentration of ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90% and absolute ethanol) and acetone for 15 min each. The critical point drying (CPD) was performed under liquid carbondioxide followed by gold coating. The specimens were then examined with SEM at 3000x and 5000x magnifications. Electron micro-graphs were developed and printed.

3. Results

3.1 Platelet aggregation test

Table 1. Mean % plateletaggregation in each reagent, activated by each agonist

Agonist	Reagents			
	Citrate	1-APAC	2-APAC	CTAD
Epinephrine	18.1	5.4	7.9	4.4
ADP	61.4	0.4	22.4	6.2
Collagen	78	5.2	34	13.1
tocetinsiR	73	83.6	55	51

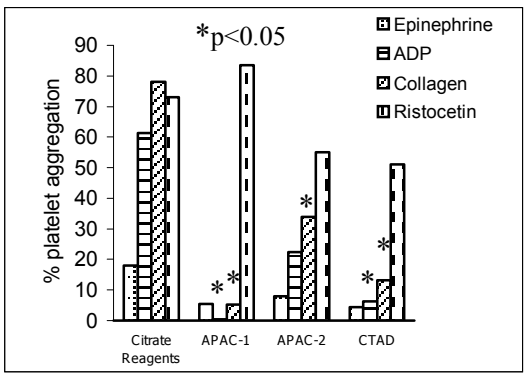

Figure.1. Comparison of mean % plateletaggregation in each reagent, activated by each agonist

The significant differences of platelet aggregation (% agg) were found between citrate vs APAC-1, citrate vs APAC-2 and citrate vs CTAD using ADP; citrate vs APAC-2 and citrate vs CTAD using collagen ($p<0.05$).

3.2 Activated platelet counted by flow cytometry

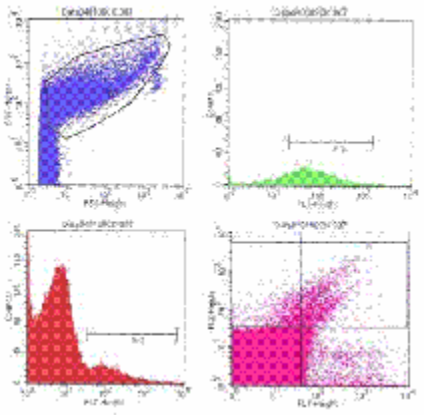

Figure 2 Flow cytometric histogram of platelet in citrate activated with collagen

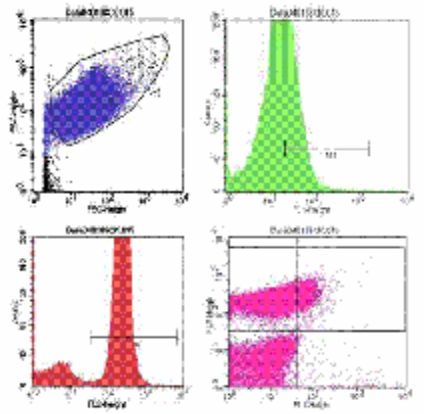

Figure 3 Flow cytometric histogram of platelet in APAC-1 activated with collagen

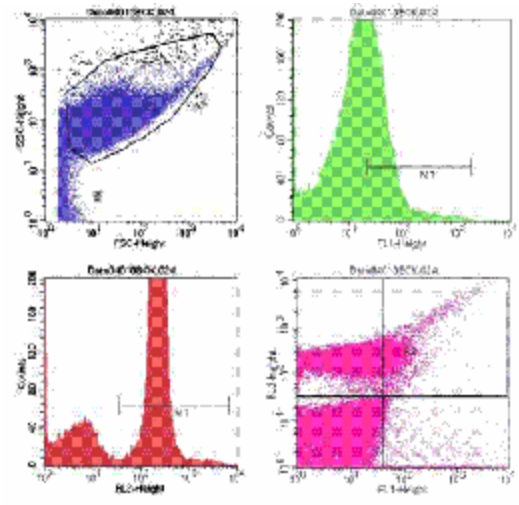

Figure 4 **Flow cytometric histogram of platelet in APAC-2 activated with collagen**

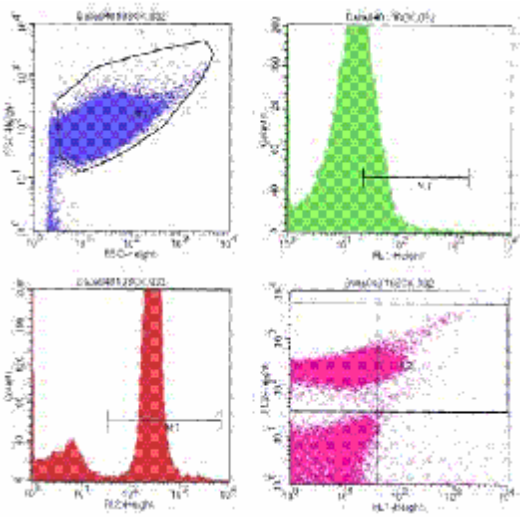

Figure 5 **Flow cytometric histogram of platelet in CTAD activated with collagen**

Table 2. **Mean % activated platelets in each reagent, activated by each agonist**

Agonist	Reagent			
	Citrate	1-APAC	2-APAC	CTAD
Epinephrine	32.2	26.6	35.5	26.5
ADP	27.3	35.7	26.6	27.5
Collagen	45.3	35.4	18.7	25.6

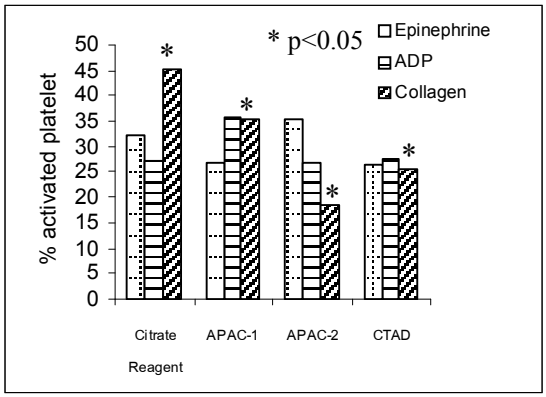

Figure.6 **Comparison of mean % activated platelet in each reagent, activated by each agonist**

The percentages of activated platelets (% CD63 pos) counted by flow cytometry, were found to be significant differences between citrate vs APAC-2 and APAC-1 vs CTAD ($p<0.05$) using collagen as an agonist.

3.3 Morphology of shape changed platelets by scanning electron microscopy

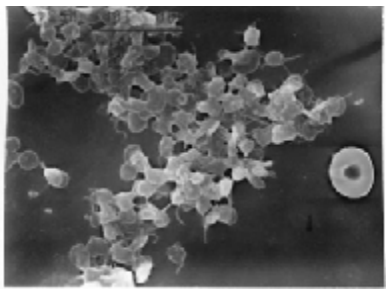

Figure. 7 **Morphology of activated platelets in citrate** (no anti-platelet drugs) using ADP as agonist.

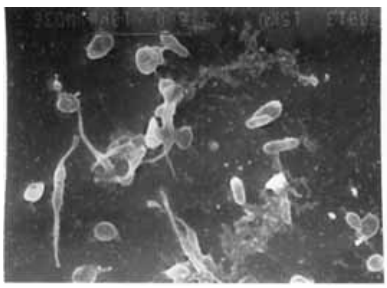

Figure. 8 **Morphology of activated platelets in APAC-1 (with anti-platelet drugs) using ADP as agonist**

Activated platelets with clear shape changes and aggregation were found in citrate (no anti-platelet drugs). While the shape changes and aggregation were found in reduced degree in APAC-1 (with anti-platelet drugs).

4. Discussion

4.1 Platelet aggregation test

- Percentages of platelet aggregation were decreased when the platelets were activated with epinephrine in all reagents.

- Percentages of platelet aggregation were increased when the platelets were activated with ristocetin in all reagents.

- APAC-1 & APAC-2 show a clear ability to suppress platelet function comparable with CTAD in all agonists except ristocetin. APAC-1 shows a little higher ability to suppress platelet function than APAC-2.

4.2 Activated platelet counted by flow cytometry

- CTAD shows the ability to suppress platelet function in all agonists except ristocetin. However ristocetin can induce platelet aggregation in all reagents. APAC-2 also can suppress platelet function with ADP and collagen as agonists. While APAC-1 can suppress only when ADP agonist was used. The quality of APAC-2 is comparable to CTAD. Therefore it can be used in platelet study.

4.3 Morphology of shape changed platelets by scanning electron microscopy

- Aggregated platelets in citrate were clearly found while in APAC-1, APAC-2 and CTAD was found in reduced degree.

5. Acknowledgement

:The authors thank

,Department of Clinical Microscopy
,Department of Clinical Immunology
.Faculty of Associated Medical Sciences
,Medical Science Research Equipment Center
.Faculty of Medicine
.Chiang Mai University
for Undergraduate sIndustrial and Research Project
.(TRF) Thailand Research Fund ,(IRPUS) sdentStu
.All blood donors

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