Proposal

Serum thromboxane B2 Assay as a Measure of Platelet Production in Healthy Volunteers Taking Aspirin

Short study name: COX-1 and TxB2

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Overall objective:

To develop a valid method to quantify platelet production (without the use of radioactive isotopes) in order to examine the hypothesis that enhanced platelet production is a common cause of poor aspirin responsiveness in patients with atherothrombosis.

Specific objective:

To validate the Cayman Chemical serum thromboxane immunoassay as a measure of platelet production in healthy subjects who are treated with aspirin by demonstrating that the recovery of their serum thromboxane B2 levels occurs at a rate of about 10% per day after aspirin cessation.

Background and Rationale:

Cardiovascular disease is the most common cause of preventable death globally. Given once-daily, low dose aspirin reduces the risk of major cardiovascular events (MACE) by 25% in patients with atherothrombosis[1]. By irreversibly acetylating cyclooxygenase-1 (COX-1) in platelets, aspirin blocks the production of thromboxane (TX) A_2, a potent stimulator of platelet aggregation, thereby mediating its anti-thrombotic effect. Despite its rapid clearance (30 minutes) from the circulation, the effect of aspirin on the inhibition of TXA_2 synthesis lasts for the lifespan of the platelet, which is about 10 days[2]. In healthy subjects, a single 100-mg dose of aspirin inhibits almost 100 percent of TXA_2 synthesis within 30 to 60 minutes of its administration. TXA_2 synthesis then recovers slowly as the aspirin is cleared and new platelets with non-acetylated COX-1 ('unacetylated' platelets) are released from the bone marrow. Since only about 10% of platelets

are replaced daily by new platelets entering the circulation, at 24 hours after a dose of aspirin, TXA_2 synthesis is still suppressed by > 90%. At this level of suppression, TXA_2 mediated platelet aggregation remains maximally inhibited. So, provided that no more than 10% of 'unacetylated' platelets enter the circulation per day, once daily aspirin maintains an optimal antithrombotic effect.

Soon after aspirin was shown to be effective as an antithrombotic agent, several investigators reported that unlike healthy subjects, about a third of patients treated with aspirin showed incomplete suppression of TX synthesis or arachidonate-induced platelet aggregation[3]. These patients were called poor responders and subsequent studies showed that poor response to once daily aspirin, identified by either TX or by platelet aggregation assays, was associated with reduced antithrombotic effectiveness[4]. Several groups, including ours, proposed that, in certain conditions (e.g. post-coronary artery bypass grafting[5], diabetes[6], and essential thrombocythemia[7]), poor response to aspirin was caused by an increased rate of platelet production. Such a mechanism is a plausible cause of poor response to aspirin because an increased rate of platelet production would result in an increased proportion of 'unacetylated' platelets entering the circulation after aspirin is cleared, which would shorten the duration of platelet COX-1 suppression to <24 h. Because full suppression of TXA_2-dependent platelet function requires >90% inhibition of COX-1 activity, even a modest recovery of this activity can sustain a substantial platelet response[8]. Consistent with this proposed mechanism, we and others showed that a poor response to aspirin was reversed by giving aspirin twice daily (instead of once)[6,7]. It is possible that an increased rate of platelet production is responsible for most cases

of poor response, in which case, the response could be restored by twice daily aspirin administration. Our ultimate aim is to test this hypothesis, but to achieve this aim we need to develop an accurate test of platelet production to identify patient groups that would benefit from twice daily aspirin.

How can we identify patients in whom an increased rate of platelet production is responsible for poor response to aspirin? Previous methods to measure platelet production in healthy subjects, which were based on survival of radiolabeled platelets, have shown that platelets survive in the circulation for about 10 days. The ***specific objective*** of this study is to demonstrate that in healthy subjects in whom production of new platelets occurs at rate of 10% per day, the recovery of serum thromboxane B2 per day would be about 10%. Instead of labeling with radioactive isotopes, we will use the acetylation of platelet COX-1 by aspirin to identify the rate of platelet production. Our method is based on the observation that in normal subjects, after a single dose of aspirin, unacetylated COX-1 recovers at a rate of about 10 percent per day as new platelets enter the circulation[9]. However, measuring acetylation status of protein requires specialized proteomics assays. Instead, we intend to use an assay to quantify serum TXB2 as an indirect measure of the effect of unacetylated COX-1 from new platelets. If true, then the daily recovery of serum TXB2 would provide a readout of new platelet production.

The serum thromboxane B2 method has the additional advantage of providing information on both the rate of platelet production and the degree of platelet inhibition by aspirin. Such an advantage would facilitate the identification of non-responders whose reduced response is due

to an increased rate of platelet production, as well as the assessment of response following aspirin dose modification in future studies.

Implications of this study:

Successful validation of serum thromboxane B2 assay performed in-house as a measure of platelet production will be critical to pursuing our multistep research program to: 1) investigate the frequency with which increased platelet production results in poor response to aspirin; 2) identify patient populations in whom increased production is a major cause of poor response to aspirin; 3) test the hypothesis that poor response in those populations can be overcome by twice daily aspirin; and 4) evaluate the effect of twice daily aspirin in those populations on reducing the risk of cardiovascular events.

Methods:

Study design: Healthy volunteers will take aspirin 81 mg daily for 5 days. Serum thromboxane B2 (sTxB2) will be measured before taking aspirin (baseline), at 4h after last dosing (daily peak level), and then daily for 5 days after aspirin cessation.

Population: 20 healthy volunteers. Enrollment criteria are listed below:

- Inclusion criteria:
    - Healthy non-smoking volunteers;
    - Age ≥ 18 years;
- Exclusion criteria:

- Allergy or intolerance to aspirin;
- Current pregnancy;
- Use of aspirin or drugs interfering with platelet function (NSAIDs, anticoagulants) within one week of study enrolment.

Assays:

- Serum Thromboxane B2: serum TXB2 concentrations were measured using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the instructions provided by the manufacturer. In-house controls were tested with each batch.

Blood collection: 2.5 ml of blood in a serum tube for sTxB2 assay, and 3 ml of blood in EDTA for platelet count at baseline.

Statistical considerations:

- Sample size: As a pilot study, we plan to enroll 20 patients as recruitment of this number is feasible. This study will provide estimates of mean/median and variability (e.g. standard deviation and range) of serum thromboxane B2 and recovery in healthy volunteers and will inform on sample size calculation of future studies with this assay.
- Proposed analyses:
  - Biological variability of serum TXB2 will be represented as mean/median and standard deviation/range, at each time point of sampling.

- Residual serum TXB2 will be plotted against time and recovery rate of serum TXB2will be estimated, and compared with 10% rate of new platelet production reported in historic studies using radiolabeling method for detection.

Anticipated result:

In healthy subjects in whom production of new platelets occurs at rate of 10% per day, we expect a 10% increase in serum thromboxane B2 per day;

Study coordination, recruitment and logistics

This study will be performed at PHRI & TaARI. Healthy volunteers will be screened and recruited by the investigators or research assistants. Informed consent will be obtained from participants before we conduct our study. Samples will be collected by our research assistant or investigators and then transferred to TaARI for testing.

Role of the principal investigator

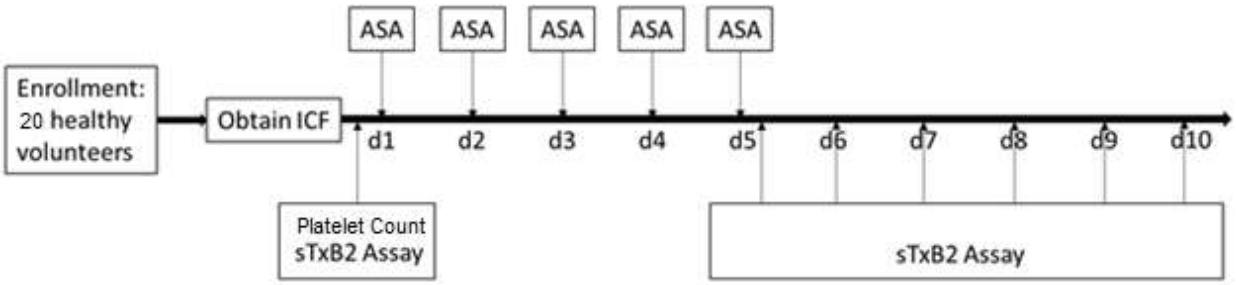
Dr Chan is the principal investigator, and will have lead roles in study oversight, protocol development, Research Ethics Board submission, creation of database, develop the detailed statistical analysis plan, and draft the manuscript for reporting study results.

Ethical considerations:

We will ensure that each participant fully understands the purpose and procedures of this study. Written informed consent will be obtained from each participant. The risk of aspirin is

well documented, and unforeseeable risk limited. We will keep in close contact with the participants for the 2 weeks of study. Participant data will only be used for the purpose of the study. We will ensure confidentiality by storing data in a keyword-secured computer in an ID badge-secured building and restricting data access to research personnel only.

Figure 1. Study procedure



* ASA, acetylsalicylic acid; sTxB2, serum thromboxane B2; ICF, informed consent form.

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