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Platelet inhibition by single low-dose aspirin, using the newly developed aggregometry with the laser light scattering method

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Low -dose aspirin (acetylsalicylic acid 81 mg/day, LDA) is often used as an antiplatelet drug in the treatment of cardiovascular and cerebrovascular diseases as well as for patients with anti-phospholipid antibody syndrome. In this study, we explored the duration of the inhibitory effect of a single LDA on platelet aggregation, using the newly developed aggregometry with the laser light scattering method.
Five healthy volunteers (females between 23 and 30 years old) ingested 81 mg of buffered aspirin. Platelet aggregation was measured with adenosine 5'-diphosphate before the ingestion and at the 1st, 2nd, 4th, 6th, and 8th day thereafter.
The results showed that the effect of 81 mg of aspirin continues for at least 8 days, which suggested that the intermittent administration of 81 mg of aspirin (a few times a week) might be an alternative way to induce the anti-platelet effect.

Key words : aspirin, platelet aggregation, aggregometry, laser light scattering

INTRODUCTION

Pregnancy loss has been one of the associated risks of autoimmune diseases, such as systemic *lupus erythematosus* [1], and extensive studies have revealed a disease, anti-phospholipid antibody syndrome (APS), to occur in conjunction with recurrent fetal loss [2, 3]. It is likely that a deregulated placental circulation and a resultant vascular coagulopathy are the causes of the fetal loss in APS pregnancies, while an impaired implantation of the embryo has been also suggested as a cause. Anticoagulation treatment was proposed to prevent these pathological consequences, and a regimen of low-dose aspirin (acetylsalicylic acid, LDA) with post-conception heparin has been demonstrated to successfully maintain APS pregnancies after recurrent abortions [4-6], as well as to induce a high fecundity rate of *in vitro* fertilization and embryo transfer in APS [7]. As LDA, defined as daily intake of 75 to 150 mg [4], 81 mg of buffered aspirin is commonly used, which is originally the dose for children, as one of the antipyretic analgesics and commercially available preparation in Japan [3].

As mentioned above, nowadays aspirin is clinically used as a platelet inhibitor. Aspirin irreversibly acetylates platelet cyclooxygenase (COX) and permanently inhibits thromboxane A2 production. Platelets do not resynthesize COX, because platelets do not have nuclei. Therefore, thrombotic activity recovers within a time period reflecting the regeneration of the platelet, the life span of which is approximately 1 week [8-11]. According to the pharmacological mechanism, an intermittent administration of aspirin (1-2 times

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a week) may be sufficient to induce the anti-platelet effect. In addition, if this regimen grows popular, some adverse effects may be reduced and emergency surgeries may be made possible.

In this context, our study was undertaken to investigate the duration of the anti-platelet effect of 81 mg of aspirin by using the newly developed aggregometry with the laser light scattering (LS) method.

As a preliminary experiment, various needle sizes were examined for their appropriateness in the blood drawing procedure, since the 21G needle is predominantly used for venipuncture as per conventional clinical practice. We speculated that the rate of contact between platelets and inner surface of the needle would increase the smaller the girth of the needle, and that platelet aggregation would increase. Based on the results of this preliminary experiment, the main experiment was finally attempted.

SUBJECTS AND METHODS

Five healthy female volunteers (23-30 years old) with written informed consent were enrolled for this study. They are non-smokers without any past history of pregnancy and particular disease.

Blood sampling

To investigate platelet aggregation for different gauges (G) of needles, needles of three sizes (18G, 21G, 25G) were used in the collection of blood from the five female volunteers by the same medical doctor, who applied an intentionally low retracting pressure. To evaluate the effective duration of aspirin on platelet inhibition, all subjects were asked to ingest 81 mg of buffered aspirin at 9 PM. Venous blood was collected at 9 AM in fasting conditions for more than 12 hours before the aspirin ingestion, and on the 1st, 2nd, 4th, 6th and 8th day thereafter. Blood was transferred into tubes containing sodium citrate, and then centrifuged at 150×g for 10 min in order to obtain platelet-rich plasma (PRP). PRP aggregation was immediately determined by the new aggregometry using the LS method, with the help of the PA 20 (Kowa Company Ltd., Tokyo, Japan). Platelet-poor plasma (PPP) was produced by another centrifugation (1500 × g for 15 min) of PRP. ADP (adenosine 5' -disphosphate; Sigma Chemical Co., Missouri, U.S.A) was used in the two final doses (0.5 μM and 4 μM) as an aggregating reagent [12, 13].

Platelet Aggregation Study

The principles of the LS method have been developed recently [14]. Briefly, a laser beam 40 μm in diameter was generated using a 20 mW diode laser (675 nm, Toshiba, Japan), and was passed through the PRP (300 μL) stirred in a cylindrical glass cuvette with a 5 mm internal diameter. The light scattered from the observation volume (48 × 140 × 20 μm) was detected by a photocell array. The light intensity corresponds to the particle size. The signal frequency was recorded at 10 s intervals. Data were expressed as change over time (s) in the number of aggregates (counts/s) of individual sizes (determined by light intensity, expressed in volts). Data were recorded in a two-dimensional graph showing the change over time of total light intensity expressed as a cumulative summation at 10 s intervals of scattered light intensity (I) and the number of particles corresponding to that intensity (N_i) in terms of particle size (intensity) (sI_I N_i) (volt×count/10 s). The total light intensities of small, medium, and large aggregates were determined: particles with an intensity of 25 to 400 mV represented small aggregates (9-25 μm), those with 400 to 1,000 mV represented medium aggregates (25-50 μm), and those with 1,000 to 2,048 mV represented large aggregates (50-70 μm). The platelet volume in the normal human is 5.8 μm^3. This volume increases to 7.2 μm^3 in the presence of 0.5 to 4.0 μM ADP. Small aggregates contain approximately 70-1,400 platelets (50-1,100 in the presence of ADP), medium aggregates contain approximately 1,000-11,000 platelets (1,000-9,000 in the presence of ADP), and large aggregates contain approximately 11,000-31,000 platelets (9,000-25,000 in the presence of ADP) [15]. Generally, aggregates smaller than 10 μm are formed in the first phase of aggregation, and large aggregates are formed in the second phase. Changes in total intensity were recorded at 10 s intervals for 5 min. Quantitative estimation was performed by determining the area under the curve (AUC) representing the sum of the 30 measurements of the LS intensity (LSI).

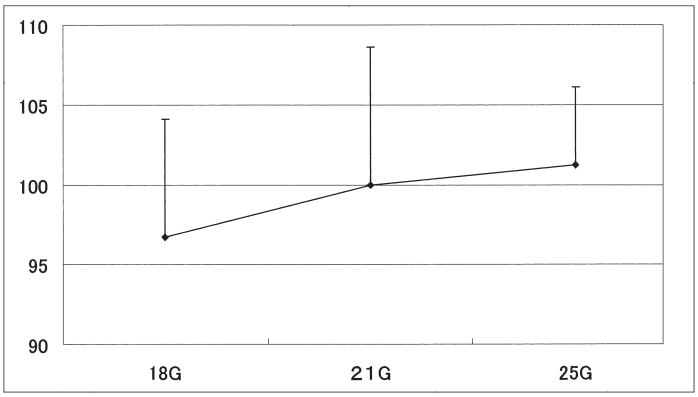

Fig. 1 Difference in platelet aggregation rate (%) as established by the conventional method, after using 18G, 21G or 25G needles for venipuncture.

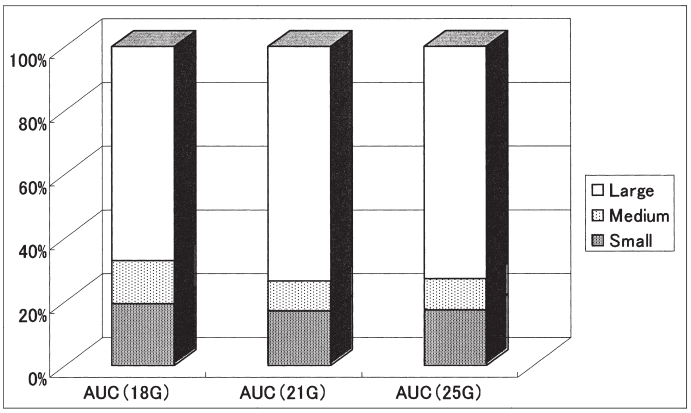

Fig. 2 Profiles (%) of aggregates of three sizes during platelet aggregation, when 18G, 21G or 25G needle was used for venipuncture, as established by laser light scattering aggregometry.

Statistical Analysis

The results were analyzed using one-way ANOVA with the *post-hoc* test of Fisher and PLSD. Statistical significance denotes $P<0.05$.

RESULTS

Effects of needle size in blood sampling

Figure 1 illustrates the platelet aggregation rate, when collected with needles of three sizes (18, 21, and 25 G). The rates were expressed as % changes, in comparison with the value at 21 G as 100 %, and showed no significant difference among the three sizes: 99.7 ± 7.4 % for 18 G, and 101.2 ± 4.9 % for 25 G. Additionally, the profiles of aggregate size for each size of needle (Fig. 2) showed no significant changes.

Effects of Aspirin

Five healthy female volunteers (23-30 years old) ingested 81 mg of buffered aspirin. Platelet aggregation was measured before aspirin ingestion and on the 1st, 2nd, 4th, 6th, and 8th day thereafter. The aggregation rate of the platelets after aspirin ingestion during the 8 days is shown in Table. The mean inhibition rate of platelet aggregation was 22.3 ± 12.9 % on the 1st day ($P=0.0001$), 20.1 ± 6.1 % on the 2nd day ($P=0.0004$), 17.6 ± 8.5 % on the 4th day ($P=0.0021$), 16.1 ± 2.7 % on the 6th day ($P=0.0076$), and 11.7 ± 4.2 % on the 8th day ($P=0.0294$), as shown in Fig. 3. Figure 4 illustrates the ratio of small, medium and large-sized aggregates during the 8 days, which indicates that the size of platelet aggregates didn't change once the platelets

Table 1 ADP (4μM)-induced platelet aggregation rate (%) after aspirin ingestion.

	ADP 4μM (%)
pre	89.0 ± 10.0
1st day	69.0 ± 13.0
2nd day	71.0 ± 7.0
4th day	77.0 ± 7.0
6th day	80.0 ± 2.0
8th day	82.0 ± 2.0

Values are expressed as mean ± SD
ADP (4μM) induced platelet aggregation rate (%) by the conventional method.

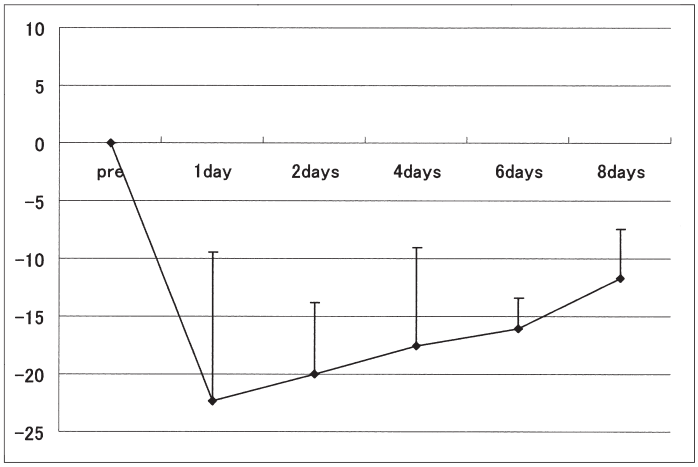

Fig. 3 Inhibition rate (%)of platelet aggregation.
The inhibition rate of platelet aggregation (%) was analyzed by the conventional light transmission method, on each day after ingestion of a single low-dose aspirin up to the 8th day.

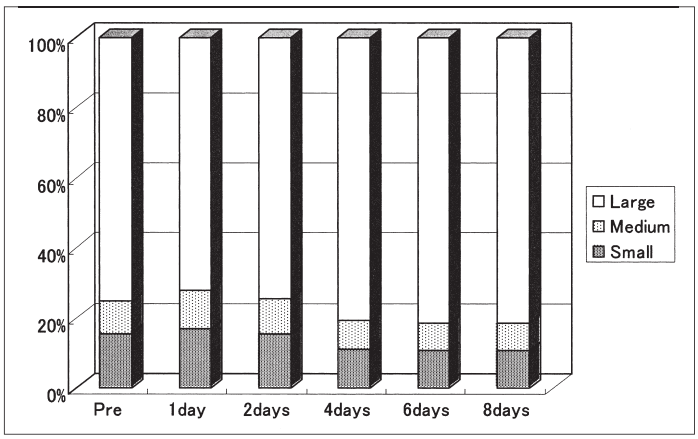

Fig. 4 Profiles (%) of aggregates of three sizes during platelet aggregation, on each day after ingestion of a single low-dose aspirin up to the 8th day, as established by laser light scattering aggregometry.

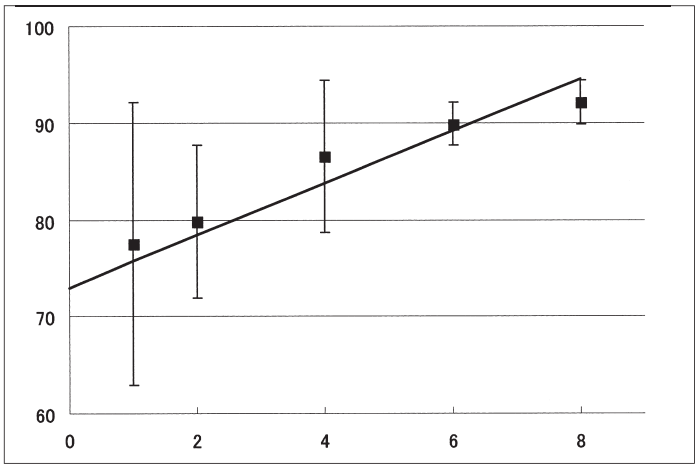

Fig. 5 Estimated life span of platelet.
Linear regression analysis was applied for the estimation of the life span of platelets, which is approximately 10.5 days.

aggregated.

DISCUSSION

As LDA, 81 mg of buffered aspirin per day is often administered for the treatment of recurrent abortions due to APS, from early to 35 weeks of gestation. This administration method is based on traditional practice and clinical experience. This LDA is evaluated as a high-safety medicine with few risks of gastrointestinal trouble, bleeding tendency, etc., presumably due to minimal inhibition of COX [16]. If an intermittent administration (1-2 a week) of 81 mg of aspirin is sufficient to induce the anti-platelet effect, it should be recommended. In this study, we investigated the duration of anti-platelet effect of 81 mg of aspirin by measuring platelet aggregation in healthy volunteers. As a result, anti-platelet effect was confirmed for 8 days. The mean inhibition rate of platelet aggregation on the 1st day was the largest, 22.3 ± 12.9 %. The mean inhibition rate of platelet aggregation gradually decreased (20.1 ± 6.1 % on the 2nd day, 17.6 ± 8.5 % on the 4th day, 16.1 ± 2.7 % on the 6th day, and 11.7 ± 4.2 % on the 8th day), but the value even on the 8th day was more than a half of anti-platelet effect of 81 mg of aspirin on the 1st day. Therefore, we speculate that anti-platelet effect of 81 mg of aspirin might endure for 8 days. It had also been reported previously that the inhibitory effect of aspirin on platelet aggregation persisted for five to seven days, and that aspirin maximally inhibited the formation of large-sized platelet aggregates within 30 min of ingestion [17]. This report and our results suggested that 81 mg of aspirin, which exhibits the maximal inhibitory effect on platelet aggregation within 30 min of ingestion, might also continue to inhibit platelet aggregation for 8 days. Since the previous studies including large scale of clinical trials considered only daily dose of aspirin [18, 19], we speculate that an intermittent administration (1-2 times a week) might be another candidate regimen to induce the anti-platelet effect for prevention purposes.

In our previous study [20], we demonstrated a significant difference in male and female platelet aggregation through the newly developed aggregometry with the LS method. In the present experiment, we utilized this new method in order to evaluate the inhibition of platelet aggregation by a single dose of aspirin, expecting to uncover the precise underlying process of platelet aggregation. However, the ratio of small, medium and large-sized aggregates hardly changed for 8 days after the aspirin ingestion. For additional information, we tried to analyze the effect of venipuncture needle size on platelet aggregation. While a 21 G needle is often used for drawing blood based on clinical experience, this is not the case with needles of other gauges. In our study, even with the newly developed aggregometry with laser, we could not detect the different profiles

of aggregation for different sizes of needle. However, compared with a 21 G needle, an 18 G needle causes greater pain to patients. And drawing blood with a 25 G needle takes longer. Therefore, it was suggested that a 21 G needle is acceptable for blood drawing and for measuring platelet aggregation. Hence, our main experiment with 21 G needles is confirmed to be properly designed for evaluating the effective duration of LDA on platelet inhibition.

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