Effects of medicinal plants on the metabolism of platelet arachidonic acid

—— Studies on "oketsu" syndrome, platelet aggregation and changes in malondialdehyde values ——

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Abstract

The "oketsu" syndrome, i.e., blood stasis syndrome, was analyzed with respect to platelet functions and platelet metabolism of arachidonic acid. A significant difference was not recognized in platelet aggregation between patients and non-patients of the "oketsu" syndrome. However a significant difference was found in malondialdehyde (MDA) production of platelets, which was shown to accelerate in the "oketsu" state. The results of treatment with anti-"oketsu" drug given to 16 patients showed that the "oketsu" syndrome improved at the same time as the accelerated state of MDA production became suppressed. Also, from the results of the load test with the anti-"oketsu" drug, Keisi-bukuryô-gan (Gui-Zhi-Fu-Ling-Wan), administered to 13 healthy subjects as controls, it was shown that the anti-"oketsu" drug had the ability to suppress MDA production. Thus, these findings suggest that, at least in part, the "oketsu" syndrome is probably related to the metabolism of arachidonic acid in platelets.

Key words platelet aggregation, malondialdehyde, arachidonic acid, oketsu syndrome, blood stasis, Keisi-bukuryô-gan, anti-oketsu drug

Abbreviations MDA ; malondialdehyde, PPP ; platelet poor plasma, PRP ; platelet rich plasma, Keisi-bukuryô-gan (Gui-Zhi-Fu-Ling-Wan) ; 桂枝茯苓丸

Introduction

"Oketsu," blood stasis or stagnant syndrome, is one of the pathological physiological concepts unique to Chinese medicine. "Ketsu," blood, means human red body fluid, which circulates inside the body and maintains life. A pathological state induced by stagnation of this "ketsu" is called "oketsu." Although definition of "oketsu" in terms of Western medicine is difficult as it is a general name given to a wide range of diseases, it is recognized clinically as blood stagnation or disorders in the peripheral microcirculation. In the present report, the authors paid attention to disorders in the peripheral microcirculation and investigated platelet functions, one of the significant factors in thrombus formation. Thus, with respect to the relation between "oketsu" and platelet functions, we obtained interesting results

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by comparing platelet aggregation and activities of the enzymatic system in prostaglandin synthesis inside platelets.

Materials and Methods

Substances : The decoctions of Keisi-bukuryô-gan and its variants given to patients were prepared according to Table I. As raw materials, those in the Japanese market were obtained from Tochimoto Tenkaidô Co. (Osaka) and Uchida Wakanyaku Co. (Tokyo). A granulated preparation of Keisi-bukuryô gan extract (Tsumura Co., Tokyo, lot 3013621) was used for the load test.

Reagents : Epinephrine from Daiichi Pharmaceutical Co. (Tokyo, Japan) was used for epinephrine, collagen reagent Horm from Hormon-Chemie Co., Ltd. (Munich, West Germany) for collagen, and sodium salt of ADP from Sigma Co., Ltd. (St. Louis, Mo., U.S.A) for ADP. Special grade N ethyl maleimide and 2 thiobarbituric acid of Nakarai Chemicals Co. (Osaka, Japan) were employed in the determination of malondialdehyde (MDA). Standard material for the preparation of the MDA calibration curve was made by hydrolysis of MDA-di-(bis-methyl)-acetal (Sigma Co., Ltd.).[1] For the other reagents, special grade materials were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan).

Clinical study of "oketsu" syndrome : Out of the patients who were examined in our clinic of Sino Japanese medicine during the study period, those given anti-inflammatory drugs or analge-

Table I The composition of Keisi-bukuryô-gan.

medical plant	place of product	daily dosage	original plant
Cinnamoni Cortex	China	4.0 gram	*Cinnamomum cassia*
Hoelen	South Korea	4.0	*Poria cocos*
Moutan Cortex	Japan (Nara)	4.0	*Paeonia moutan*
Persicae Semen	North Korea	4.0	*Prunus persica*
Paeonia Radix	Japan (Nara)	4.0	*Paeonia albiflora*

Mixed all drugs and boiled with 600 ml of water down to 300 ml.
The decoction was adiministered 3 times daily before meals.

Table II Diagnostic criteria for "oketsu" syndrome.[3]

symptoms	score male	score female		score male	score female
dark shade around the eyes	10	10	tenderness of left navel region	5	5
pigmentation over the face	2	2			
rough skin	2	5	tenderness of right navel region	10	10
purple discoloration of lips	2	2	tenderness under the navel region	5	5
purple discoloration of gums	10	5			
purple discoloration of tongue	10	10	tenderness of iliocoecal region	5	2
telangiectasis, vascular spider	5	5			
susceptibility to subcutaneous bleeding	2	10	tenderness of hypochondrial region	5	5
redness of palms, palmar erythema	2	5	tenderness of sigmoid region	5	5
			hemorrhoids	10	5
			dysmenorrhea		10

Evaluation : 20 points and less : non-"oketsu" state.
21 points and above : "oketsu" state.
40 points and above : severely affected "oketsu" state.

sics, which may affect platelet functions, were excluded. Then, the indication of Keisi-bukuryô-gan[2] was studied. Sixteen patients consisting of 8 men (27-58 y.o.) and 8 women (25-74 y.o.) were considered as appropriate subjects. The degree of the "oketsu" state was standardized according to the diagnostic criteria established by Terasawa *et al.* (Table II)[3] Changes in "oketsu" scores, platelet aggregability and MDA production in these patients at the start of treatment and after 2-3 months of drug administration were determined. Daily dose of the drug was listed in Table I.

Healthy volunteer study : 13 healthy volunteers (7 male and 6 female, aged 19-30) made up of staff members and students of this college were investigated as control. Their "oketsu" scores were under 20. During the test period, they were prohibited from taking alcoholic beverages and drugs which may affect platelet functions. Changes in platelet aggregations and MDA production were investigated after administrating 7.5g/day of a granulated preparation of Keisi-bukuryô-gan extract from Tsumura Co., Ltd. for one week.

Determination of platelet aggregation : Blood was taken with 3.8% (w / v) sodium citrate in the morning after overnight fasting. After centrifugation at 1000 rpm (139 × g) for 10 minutes, the supernatant was collected ; this fluid was called platelet rich plasma (PRP). The precipitate was further centrifuged at 3000 rpm (1249 × g) for 20 minutes, yielding another supernatant, platelet poor plasma (PPP). The number of platelets in PRP was determined using an automatic platelet counting apparatus (Toa Medical Electronics, Co., Ltd., PL-100) and, by adding autologus PPP, the platelet count was adjusted to 3×10^5 platelets per μl : 200μl of PRP were placed in a cuvette (7 mm diameter and 50 mm length) in an automatic platelet aggregometer, Haemameter Model PAT-4A (Nikô Bioscience Co., Tokyo, Japan), and the platelet aggregation was measured and recorded by adding 20μl of an aggregating agent, epinephrine (final concentrations : 2μM, 10μM), collagen (final concentration : 3μg/ml) and ADP (final concentrations : 2μM, 10μM). Aggregabilities were indicated with the maximum aggregation as 100 %.

Measurement of MDA production : MDA production was determined in order to estimate the activity of enzymes related to prostaglandin production in platelets. The method of measurement was based on that of Smith *et al.*[1] and Stuart *et al.*[4] One hundred μl of platelet aggregating agent (N-ethylmaleimide ; 1 mM) were added to 1 ml of PRP and shaken at 37℃ for 5 minutes (pH 7.4). Then an equal volume of 20 % (w/v) trichloroacetic acid in 0.6 M hydrochloride acid solution was added to the mixture and stirred so as to stop the reaction and extract MDA.

To one volume of the supernatant (acid extract) obtained by centrifuging the above mixture at 10000 rpm (10733 × g) for 10 minutes, 0.2 volume of 0.12 M thiobarbituric acid in 0.26 M Tris-hydrochloric acid buffer (pH 7.0) was added, and the mixture was heated in a boiling bath for 15 minutes. After cooling to room temperature, absorbance of the solution at 532 nm was measured by UV spectrometer (Shimadzu, UV-120-01). The amount of MDA (n mol/10^9 platelets) produced in platelets was determined from the calibration curve prepared with known amounts of MDA. The molar absorbance of MDA was 1.37×10^5. As a control, a mixture of 1 ml PPP with 100 μl of platelet aggregating agent was treated in the same manner.

Results

"Oketsu" syndrome and platelet functions

Results obtained by comparing "oketsu" score with platelet aggregability (Table III) and MDA production are shown in Fig. 1 (a,b). With respect to platelet aggregability, no significant difference between "oketsu" and non-"oketsu" was observed. On the other hand, a significant difference was recognized in MDA production between the patients of the "oketsu" and non-"oketsu" states, with MDA production being accelerated in the "oketsu" state.

Effects of anti-"oketsu" drugs

Results are shown in Fig. 2. The first values are those obtained at the beginning of the treat-

Table III Platelet aggregation in "oketsu" and non-"oketsu" state.

Reagents	Concentration	non-"oketsu" state	"oketsu" state
Epinephrine	2 μM	64.6±8.3 SE%	60.9±7.2 SE%
	10 μM	74.6±7.6	82.1±5.6
Collagen	3 μg/ml	82.4±4.4	84.5±2.4
ADP	2 μM	34.4±6.5	29.5±4.5
	10 μM	82.9±3.5	70.1±5.8

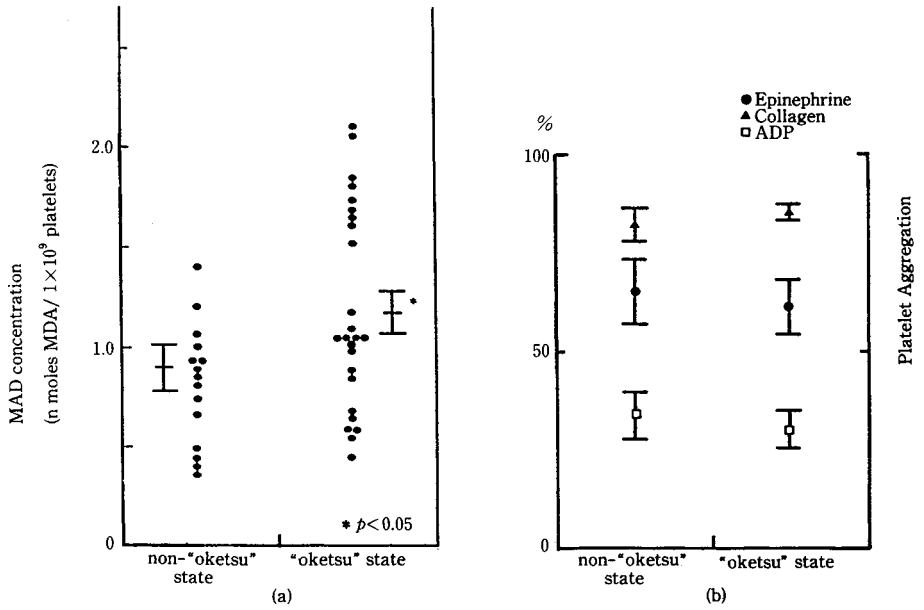

Fig. 1 Relation between platelet functions and "oketsu" score.
a) [a]MDA production in PRP, b) platelet aggregation.

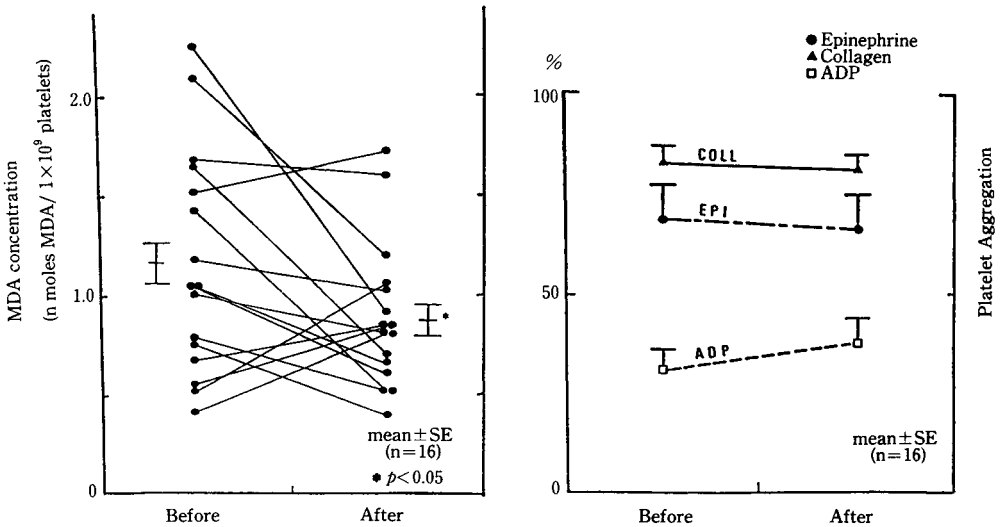

Fig. 2 Effects of anti-"oketsu" drugs.
Former values are those obtained at the beginning of the treatment and the latter are those obtained after 2-3 months of treatment with anti-"oketsu" drugs.

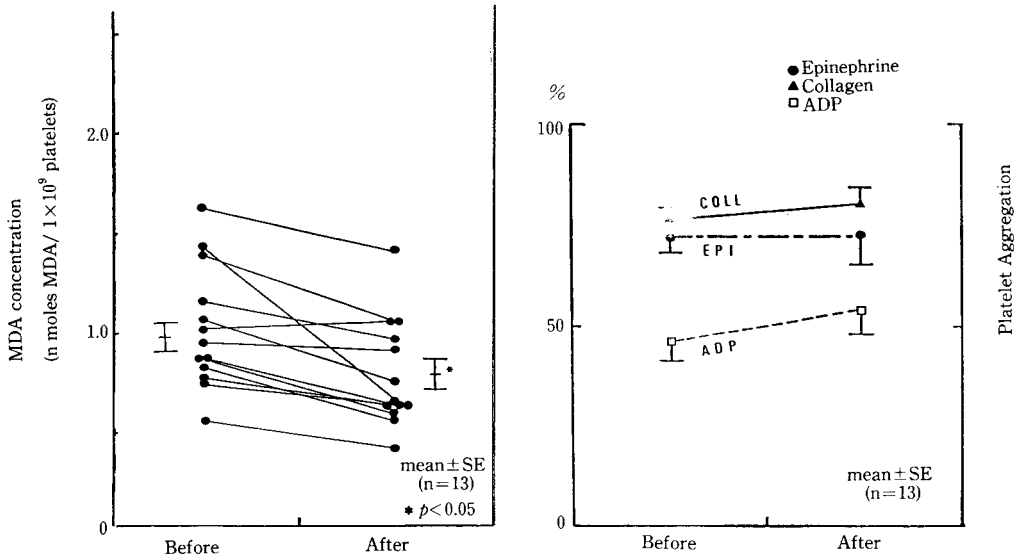

Fig. 3 Effects of Keisi-bukuryô gan extract on MDA production in PRP and platelet aggregation in normal subjects.

ment and the latter after 2-3 months of treatment with the anti-"oketsu" drugs. Significant decrease of MDA production was observed after the treatment ; however, there was no significant difference in platelet aggregation.

With respect to the "oketsu" scores, they were high at the beginning (33.1±4.5 SE), and reduced significantly ($p<0.05$) after treatment (19.7±2.3).

The load test with Keisi-bukuryô-gan extract

Fig. 3 shows the results obtained in the administration of granulated Keisi-bukuryô-gan extract to 13 volunteers for one week. It can be seen that MDA production was significantly suppressed after the administration. Significant change in platelet aggregability was not observed.

Discussion

When the "oketsu" syndrome is thought to be a kind of peripheral circulatory disorder, the following causes can be considered : deterioration of blood fluidity, thrombus formation inside blood vessels, as well as their contraction and narrowing. Arichi *et al.*[5] and the authors[6] have reported that hyperviscosity was seen in the "oketsu" state. However, there has been no investigation on the platelet functions in this condition. In the present study, we have investigated the relationship between the "oketsu" state and platelet functions, and further examined the effects of anti-"oketsu" drugs on the platelets.

The results indicate that acceleration of MDA production is present in "oketsu," but there is no significant change in platelet aggregability.

Thiobarbituric acid reaction for MDA is not only specific for cyclo-oxygenase pathways but also for lipoxigenase. However, under the conditions employed in this study (pH 7.4, 37℃, 5 minutes), MDA values correlated with the activity of cyclo-oxygenase pathways in human platelets.[1,4,7] Therefore, our data suggest that in cases of "oketsu" the cyclo-oxygenase pathways are accelerated in the platelets.

With respect to platelet aggregability, however, significant differences were not found in this study. The discrepancies between accelerated MDA production and unchanged platelet aggregability might be due to the fact that platelet aggregation is caused not only by thromboxane B_2 but by many other factors as well.

In order to elucidate the effects of anti-"oketsu" drugs, the clinical course of the MDA values and platelet aggregability were estimated following Keisi-bukuryô-gan adiministration. The patients were selected by their "oketsu" score

(above 21), so that the MDA value before treatment (1.17±0.13 SE) is similar to that of the "oketsu" state (1.17 ± 0.10 SE) in Fig. 1 (a). Following the treatment of Keisi-bukuryô-gan, the MDA values in the platelets have become significantly decreased (0.89±0.09 SE), corresponding to the value of the non-"oketsu" state (0.90±0.11 SE) in Fig. 1. Results obtained by clinical investigation, however, are the possible effects of other factors, i.e., diet, restriction of alcoholic beverages, change of life style, *etc.* In order to eliminate such contamination factors during the investigation, a load test using volunteers was performed in this study. The volunteers were selected by their "oketsu" score (under 20), with the MDA values before treatment (0.96± 0.07 SE) corresponding roughly to that of the non-"oketsu" state in the patients. As shown in Fig. 3, this MDA value decreased further following Keisi-bukuryô-gan administration.

According to the present studies, the effects of Keishi-bukuryô-gan on MDA productivity in human platelets were determined. It was shown that the drug inhibits cyclo-oxygenase pathways in human platelets, and corrects the accelerated activity of these pathways in the "oketsu" state. Kiuchi *et al.*[8] have reported that medical plants containing Keisi-bukuryô-gan inhibit prostaglandin biosynthesis *in vitro*. The present studies may support their data *in vivo*.

Hirai *et al.*[9] revealed that *Moutan Cortex* and its main component, paeonol, inhibits platelet aggregation *in vivo*. In the present study, however, any significant changes in platelet aggregation could not be found. These discrepancies might be due to differences of concentration of the reagent adopted.

Improvement of the "oketsu" scores was also observed in this study. In consideration of the unchanged platelet aggregability, improvement of the score may be due not to any platelet factors but rather to other pharmacological effects of the drug, i.e., anti-inflammatory effects,[10] correction of blood viscosity,[11] *etc.*[12]

As discussed above, the "oketsu" state, when related to platelet functions, could be considered as the condition in which the activities of cyclo-oxygenase pathways are elevated, but still not high enough to induce changes in platelet aggregability. Further, the anti-"oketsu" drug (Keisi-bukuryô-gan) may then correct the accelerated state of the pathways.

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