Development of an *in vivo* assay method for evaluation of “oketsu” using hen-egg white lysozyme (HEL)-induced blood flow decrease

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Abstract

“Oketsu”, or stagnant blood syndrome, is one of the important pathological concepts in therapy with Kampo formula and drugs. Here we show the effectiveness of our previously developed *in vivo* assay system for the evaluation of such drugs. The assay system measures blood flow in the microcirculation of the mouse tail after injection of hen-egg white lysozyme (HEL). The results showed the effectiveness of Kampo formula and crude drugs clinically used to treat “oketsu”, while little effect was found with drugs used for other purposes. These results indicated the effectiveness of the method for screening drugs aimed at “oketsu” treatment.

Key words  oketsu, blood flow decrease, Kampo formula, *in vivo* assay system.

Introduction

“Oketsu”, or “stagnant blood” in Kampo formula, is one of the leading causes menopausal symptoms, gynecopathy, cold constitution, and stiff shoulder. The development of novel drugs to treat “oketsu” should contribute to improving the quality of life for many suffering from these symptoms.

For the screening of anti-“oketsu” drugs, *in vitro* assays have been developed, such as those measuring blood viscosity, microcirculation in the conjunctiva, or the deformability of erythrocytes. However, the only *in vivo* method, to our knowledge, had been that in which steroid is repetitively administered to cause stagnant blood flow.

Here we examined the application of an *in vivo* bioassay method that we developed previously to search for novel anti-allergic drugs, and isolated the active compounds. This method monitors the decrease in blood flow in the tail vein of mouse subjected to sensitization with hen egg-white lysozyme (HEL). The blood flow of HEL-sensitized mice (control group) gradually and significantly decreases to 70~80% of the blood flow of normal mice at day 9, without changes in blood pressure. This blood flow decrease was improved by disodium cromoglycate, inhibitors of granulocytic elastase, endothelin-1, COX-1, COX-2, and thromboxane A2 synthase, and PGI2 agonist, while antagonists of 5HT2A, and histamine H1 were ineffective. We have also reported that inducible nitric oxide synthase (iNOS) and constitutive nitric oxide synthase (cNOS) exacerbated the stagnant blood condition. Further study of this phenomenon led to us to realize a similarity between the blood flow decrease in the model and the stagnant flow in the “oketsu” syndrome. We therefore tried assessing some clinically used anti-“oketsu” Kampo formula such as tokishakuyakusans, keishibukuryogan, kamishoyosan, and tokakujokito and crude drugs such as Moutan Cortex, Angelicae Radix, Persicae

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Semen, and Paeoniae Radix. They were compared with other Kampo formula such as shosaikoto and crude drugs such as Gardeniae Fructus, Bupleuri Radix, Poria, and Cinnamomi Cortex, which are rarely used for the “oketsu” syndrome. We here describe the results and the relationships of the drugs and their clinical response to “oketsu”.

Materials and Methods

Materials: Hen egg-white lysozyme (HEL), Complete Freund’s adjuvant (CFA), and crude drugs were purchased from Sigma Co., Ltd. (St. Louis, MO, U.S.A.), DIFCO (Michigan, USA), and Tochimoto Tenkaido Co., Ltd. (Osaka, Japan), respectively. All medicines used in this study such as kamishoyosan, tokakujikoto, tokishakuyakusan, keishibukuryogan and shosaikoto were purchased from Tochimoto Tenkaido Co., Ltd.

Animals: Male ddY mice (SPF grade), 5 weeks old, were obtained from Japan SLC, Inc. (Shizuoka, Japan) and housed at 24±2°C. Food and water were available ad libitum.

HEL sensitization: Immunization with HEL was performed as previously described with slight modifications. Male ddY mice of 5 weeks of age were sensitized subcutaneously with 50 μg of HEL in CFA on day 0.

Blood flow measurement: Subcutaneous blood flow in the mouse tail was monitored using a laser Doppler blood flow meter of the non-contact type (FLO-C1, Neuroscience, Tokyo, Japan) as previously reported. Each mouse was pre-warmed for 15 min at 36°C, prior to the experiment and was placed on a holder in a measuring chamber kept at 36°C throughout the measurement. The systolic blood flow of the venous microcirculation of tail hypodermic of the unanesthetized mouse was measured (n = 5). The normal blood flow was measured for 10 min at 1 day before the experiment. The blood flow of the sensitized mouse was measured for 10 min every day up to 9 days after the sensitization (0 day) without anesthesia. The results were expressed as a relative percent of normal blood flow of each mouse.

Assay of blood flow decrease in response to HEL sensitization: All of the crude drugs and Kampo formula were administered orally (200 mg/kg) 1 h prior to measurement at 0 (the start day), 3, 6 and 9 days after the sensitization using effective quantity and medication methods described in the literature. The control group was administered water in the same schedule. The statistical calculations were determined in comparison with control group. All experiments were performed in accordance with the Guidelines for Animal Experiments of Mukogawa Women’s University.

Statistical analysis: Statistical analysis was performed using Dunnett’s multiple range test coupled with Bonferroni inequality for significant differences between each test group and the control group. For the Bonferroni test, 5 points were used 4 days after the HEL sensitization, because a significance difference was observed between the blood flow of non-treated and sensitized mice after 4 days.

Preparation of Kampo formula and crude drugs

1) Kampo formula: Kampo formula used in this study were prepared according to literature. All extracts were dissolved in distilled water and used for the bioassays.

Kamishoyosan: A mixture of Bupleuri Radix (Saiko, 3.0 g), Paeoniae Radix (Shakuyaku, 3.0 g), Angelicae Radix (Toki, 3.0 g), Atractylodis Rhizoma (Byakujutsu, 3.0 g), Poria (Bukuryo, 3.0 g), Gardeniae Fructus (Sanshishi, 2.0 g), Moutan Cortex (Botanpi, 2.0 g), Glycyrrhizae Radix (Kanzo, 1.5 g), Zingiberis Rhizoma (Shoukyou, 1.0 g), Mentheae Herba (Hakka, 1.0 g) and water (400 mL) was heated at reflux for 30 min. After filtration, the resulting filtrate was evaporated in vacuo to afford kamishoyosan (7.6 g).

Tokakujikoto: A mixture of Persicae Semen (Tounin, 4.0 g), Glycyrrhizae Radix (2.0 g), Cinnamomi Cortex (Keihi, 2.0 g), Natrii Sulfas (Boushou, 2.0 g), Rhei Rhizoma (Daiou, 0.5 g) and water (400 mL) was heated at reflux for 30 min. After filtration, the resulting filtrate was evaporated in vacuo to give tokakujikoto (4.77 g).

Tokishakuyakusan: A mixture of Paeoniae Radix (6.0 g), Alismatis Rhizoma (Takusha, 4.0 g), Poria (4.0 g), Cnidii Rhizoma (Senkyu, 3.0 g), Angelicae Radix (3.0 g), Atractylodis Rhizoma (3.0 g) was heated at reflux for 30 min. After filtration, the resulting filtrate was
evaporated in vacuo to afford tokishakuyakusan (5.94 g).

Keishibukuryogan: A mixture of Cinnamomi Cortex (2.0 g), Paeoniae Radix (2.0 g), Persicae Semen (2.0 g), Poria (2.0 g), and Moutan Cortex (2.0 g) was heated at reflux for 30 min. After filtration, the resulting filtrate was evaporated in vacuo to afford keishibukuryogan (4.68 g).

Shosaikoto: A mixture of Bupleuri Radix (6.0 g), Pinelliae Tuber (Hange, 6.0 g), Scutellariae Radix (Ougon, 3.0 g), Zizyphi Fructus (Taisou, 3.0 g), Ginseng Radix (Ninjin, 3.0 g), Glycyrrhizae Radix (2.0 g) and Zingiberis Rhizoma (1.0 g) was heated at reflux for 30 min. After filtration, the resulting filtrate was evaporated in vacuo to give shosaikoto (5.14 g).

2) Crude drugs: The crude drugs, Persicae Semen, Moutan Cortex, Angelicae Radix, Paeoniae Radix, Gardeniae Fructus, Poria, Cinnamomi Cortex and Bupleuri Radix (20 g each), were extracted overnight with 30% EtOH (400 ml) at room temperature, respectively. After filtration, the filtrate was evaporated in vacuo to yield each extract from the crude drugs, respectively (1.92 g, 4.64 g, 6.92 g, 5.88 g, 5.40 g, 360 mg, 1.32 g, or 2.76 g). These extracts were dissolved in distilled water and used for bioassays.

Results

Effect of Kampo extract on HEL-induced blood flow decrease: We evaluated clinically used Kampo extract for anti-“oketsu” treatment such as tokishakuyakusan, keishibukuryogan, kamishoyosan, and tokakujokito, and compared the results with that for shosaikoto which is rarely used for this purpose.

Tokishakuyakusan is clinically used for anemia and menopausal syndrome. This medicine reduced the significantly decrease of blood flow after 5 days, and clearly improved the blood flow after 6 days; that is, the flow was almost equal to that on the start day (0 day) while in control, the flow was reduced to less than 85% compared with 0 day (Fig. 1A).

Kamishoyosan, which is clinically used to treat menopausal syndrome and dysphoria, showed significantly effectiveness only after 9 days (Fig. 1A).

Keishibukuryogan, used to treat menstrual irregularity and neuralgia, showed a reductive effect after 6 days (Fig. 1A)

Tokakujokito is often used for treatment of menstrual irregularity and neuralgia. This medicine exhibited the most potent activity; that is, the flow was significantly improved after 5 days and was almost equal to that at 0

![Fig. 1](image-url)  
**Effect of extracts of Kampo formula prescriptions.**  
- ●: control (HEL-sensitized mice), (A); ◇: pretreatment with kamishoyosan; □: pretreatment with tokishakuyakusan; △: pretreatment with keishibukuryogan, (B); ▽: pretreatment with shosaikoto; ●: pretreatment with tokakujokito, at 1 h prior to measurement at 0 (the start day), 3, 6, and 9 days from the sensitization. Each value presents the mean ± S.E. (n = 5). * and †p<0.05 as compared with control group (Dunnett’s test with Bonferroni).
day while in control, the blood flow was reduced almost 80% compared with 0 day (Fig. 1B).

Shosaikoto is used for hepatic dysfunction and acute febrile disease. It is not usually used to treat “oketsu”. Shosaikoto showed only weak effectiveness only after 9 days with significant (Fig. 1B).

**Effect of crude drugs on HEL-induced blood flow decrease:** We evaluated Moutan Cortex, Angelicae Radix, Persicae Semen, and Paeoniae Radix, which have been clinically applied for the treatment of “oketsu”. Gardeniae Fructus, Bupleuri Radix, Poria, and Cinnamomi Cortex were also evaluated although these drugs have little effect on improvement of stagnant flow.

Moutan Cortex (Botanpi) has been reported to show anti-platelet, anti-inflammatory effects. Moutan Cortex demonstrated potent activities; that is, the flow was significantly improved after 5 days. The blood flow was maintained at the normal level while in control, the blood flow was reduced to less than 80% against 0 day (Fig. 2A).

Angelicae Radix (Tokig) has been reported to show anti-coagulation, anti-inflammatory, and anti-vascular hyperpermeability effects, and analgesic effect. This drug demonstrated potent activity, significantly improving the flow after 5 days, similar to Moutan Cortex. The blood flow was maintained at the normal level after 9 days while in control, the blood flow was reduced to less than 80% against 0 day (Fig. 2B).

Persicae Semen (Tounin) is used for its anti-“oketsu” effect and is known to show anti-inflammatory, analgesic, and thrombolytic effects. This medicine exhibited potent activity, significantly maintaining blood flow at the normal level after 5 days. The blood flow was

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**Fig. 2** Effect of crude drug components of Kampo formula prescriptions.

- ●: control (HEL-sensitized mice), (A);
- ○: pretreatment with Moutan Cortex, (B);
- ◆: pretreatment with Angelicae Radix; △: pretreatment with Persicae Semen, (C);
- ▲: pretreatment with Paeoniae Radix; ▽: pretreatment with Gardeniae Fructus, (D);
- ▼: pretreatment with Bupleuri Radix, (E); □: pretreatment with Poria, (F);
- □: pretreatment with Cinnamomi Cortex, at 1 h prior to measurement at 0 (the start day), 3, 6, and 9 d from the sensitization. Each value presents the mean ± S.E. (n=5). * and ** p<0.05 as compared with the control group (Dunnett’s test with Bonferroni).
maintained at the normal level even after 9 days while in control, the blood flow was reduced to less than 80% against 0 day (Fig. 2B).

Paeoniae Radix (Shakuyaku) showed anti-inflammatory and anti-platelet effects; it is usually used for analgesic effect. This drug showed a weak anti-“oketsu” effect.\textsuperscript{19-21} Paeoniae Radix showed significant effects after 7 and 8 days, but its effectiveness was decreased after 9 days (Fig. 2C).

Gardeniae Fructus (Sanshishii) is known to have a pus discharge effect, and is clinically used for analgesic, anti-inflammatory, hemostatic, and anti-dysphoric effects.\textsuperscript{22-26} Gardeniae Fructus did not show significant effects (Fig. 2C).

Bupleuri Radix (Saikyo) is known to show anti-inflammatory, anti-hepatic dysfunction and chronic hepatitis effects.\textsuperscript{27} Bupleuri Radix showed a weak but significant effect only after 8 days (Fig. 2D).

Poria (Bukuryo) is known to have diuretic and antiallergy effects. It is widely used for diuresis and defervesence.\textsuperscript{28-29} Poria showed weak effects although a non-significant improvement effect was recognized (Fig. 2E).

Cinnamomi Cortex (Keihi) is known to show defervesence, sedation, spasmylosis, and coagulation. This drug is usually used as an aromatic stomachic and carminative.\textsuperscript{30-32} Cinnamomi Cortex showed little effect (Fig. 2F).

**Discussion**

We evaluated standard Kampo formula such as kamishoyosan, tokishakuyakusan, tokakujokito, keishibukuryogon, and shosaikoto. Among them, drugs that are clinically used for anti-“oketsu” treatment such as kamishoyosan, tokishakuyakusan, tokakujokito, and keishibukuryogon exhibited improvement effects in our model while shosaikoto, which is not usually used for this purpose, showed only little improvement effects. However, drugs bearing anti-inflammatory effects such as Gardeniae Fructus and Bupleuri Radix showed weak but significant improvement effects in our test system, which indicated that some inflammatory mechanism could be involved in the onset and/or maintenance of the “oketsu” syndrome.

Well-known anti-“oketsu” drugs such as Moutan Cortex, Persicae Semen, Angelicae Radix, and Paeoniae Radix exhibited improvement of the stagnant flow after injection of HEL. On the other hand, Gardeniae Fructus, Bupleuri Radix, Poria, and Cinnamomi Cortex, which are not usually used against the “oketsu” syndrome, showed only a weak effect in our model system.

These relationships between the effectiveness of drugs used in clinical treatment for “oketsu” and those in our model indicated that anti-“oketsu” effects could be evaluated by the method. To the best of our knowledge, the only in vivo method for evaluation of “oketsu” reported thus far is that in which repetitive administration of steroids is used to initiate a stagnant flow.\textsuperscript{4} However, using this method to screen for novel anti-“oketsu” drugs would be difficult as repetitive administration of steroid is a troublesome procedure. In contrast, our in vivo method requires only one injection of HEL.

We have shown that the amount of NO in blood flow increases significantly after the HEL injection. This coincides with a clinical report by Maruyama et al.\textsuperscript{33} that the amount of NOx in the blood of the “oketsu” patient is significantly increased.

Our model should be useful for identifying novel anti-“oketsu” drugs and elucidating the mechanism of drugs to be used for “oketsu” treatment.

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