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Anti-Thrombotic Effect of Potato in Animal Experiments

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ABSTRACT

Prevention of atherothrombotic diseases is an important and urgent social task in the developed world. Inappropriate diet plays a causative role in the development and clinical outcome of thrombotic diseases. We have been testing fruits and vegetables and found that some varieties have a significant anti-thrombotic effect. The present study aimed to test twenty potato varieties using *in vitro* test (Global Thrombosis Test-GTT) and animal models of thrombosis (laser-induced thrombosis in the carotid artery of mice). Potato varieties Touya (yellow), Hokkaikogane (yellow) and Star ruby (yellow) showed heat-resistant anti-thrombotic effects. Including these varieties in daily diet may be beneficial in thrombosis prevention.

Keywords: anti-thrombotic food, platelet aggregation, thrombolysis, vegetable

INTRODUCTION

Prevention of "lifestyle-related atherothrombotic diseases" such as myocardial infarction and stroke is an important task in developed countries. Studies have provided clear evidence for the causative role of an inappropriate diet in the pathogenesis and clinical outcome of thrombotic diseases (Lichtenstein *et al*. 2006). There is reason to believe that a diet containing fruits and vegetable with experimentally proven anti-thrombotic effect could be beneficial in prevention of arterial thrombotic events (Yamamoto *et al*. 2003; Yamada *et al*. 2004; Naemura *et al*. 2005, 2006; Yamamoto *et al*. 2006). In the present study, we screened 20 potato varieties for anti-thrombotic effect using an *in vitro* test (GTT-Global Thrombosis Test). Varieties, which were effective *in vitro* were tested further by the laser-induced animal thrombosis model after long term intake of the specific potatoes.

MATERIALS AND METHODS

Potato varieties

Twenty potato varieties (Yukirasha, Inca red, Sayaka, Hokkaikogane, Touya, Beni-akari, Inca purple, Andes red, Kita-akari, Danshaku-imo, Tokachikogane, Red moon, Kitamurasaki, Inca-no-hitomi, Inca-no-mezame, Star ruby, Northern ruby, May queen, Haruka, Konafubuki) were planted in April, 2002 in the same field of the National Agriculture and Food Research Organization (NARO) and National Agricultural Research Center for Hokkaido Region (NARCH), harvested in September and supplied in November after storage at 4°C and used in acute experiments. The color of the tuber flesh of each variety is shown in **Table 1**.

Ten potato varieties (Yukirasha, Inca red, Sayaka, Hokkaikogane, Touya, Beni-akari, Inca purple, Andes red, Kita-akari and Danshaku-imo) were found to be active in the first in vitro screen and were tested *in vivo* for anti-thrombotic effect after long-term intake. The flour prepared from these potatoes by freeze-drying was used for long-term intake experiments.

Table 1 Color of potato varieties.

Varieties: Japanese name (English name)	Flesh color	Varieties	Flesh color
Yukirasha	White	Tokachikogane	Light yellow
Inca red	Red	Red moon	Yellow
Sayaka	White	Kitamurasaki	Purple
Hokkaikogane	Yellow	Inca-no-hitomi	Deep yellow
Touya	Yellow	Inca-no-mezame	Deep yellow
Beni-akari	White	Star ruby	Yellow
Inca purple	Purple	Northern ruby	Red
Andes red	Yellow	May queen	White
Kita-akari	Yellow	Haruka	White
Danshaku-imo (Irish Cobbler)	White	Konafubuk	White

Preparation of potato filtrates for acute (*in vitro*) and flour for long-term intake (*in vivo*) experiments

The flour was prepared by freeze-drying. Having been washed, potatoes were stored at -30℃ until use by Ikeda Tohka Industries Company Ltd., Fukuyama, Japan. To prepare filtrate several potatoes per variety (to avoid inter-individual variation) were grated with a plastic grater at room temperature and the juice was squeezed and filtrated (pore size 0.5 μm; Schleicher & Schuell GmbH., Dassel, Germany). Filtrates were stored at –30℃ until use and diluted with saline before the experiment.

Animals

Double-homozygous apolipoprotein E and low-density lipoprotein receptor deficient mice (DK mice, 129×C57BL/6J background) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and bred in the Animal Units of Kobe Gakuin University. Mice were kept in air-conditioned rooms (22.5 ± 0.5℃ and humidity 50 ± 5%) with 12-h light and dark cycle. These spontaneous atherogenic male mice were used for long-term experiments. For acute experiments, male Wistar/ST rats (at least 14 weeks old) and C57BL/6 mice (10-11 weeks old) were purchased from SLC Co.

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Table 2 Composition of the potato diets.

Ingredient	High-fat diet (g)	Potato diet (g)
Potato flour	0.0	39.7
Casein	23.2	20.3
Cystine	0.3	0.3
Corn starch	36.2	0.0
Sucrose	9.95	9.95
Soy oil	3.0	2.6
Butter	7.5	7.5
Beef tallow	10.0	10.0
Cellulose	5.0	4.8
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
Choline bitartrate	0.25	0.25
tert-Butylhydroquinone	0.0041	0.0041
Cholesterol	0.05	0.05
Total	100.0	100.0

Ltd. (Hamamatsu, Japan). Animals had free access to diet, drinking water *ad libitum*, and they were fasted overnight before the test. Animals were kept in compliance with the "*Guiding Principles for the Care and Use of Animals in the field of Physiological Sciences*," published by the Physiological Society of Japan and the experiments were approved by the Animal Experiment Committee of Kobe Gakuin University.

Diets

A Western-style high-fat model diet was prepared from the freeze-dried potato flours or purified corn starch (control diet) and from purified materials as described in **Table 2** (Ijiri *et al.* 2002). Freeze-dried flours obtained from different potato varieties were used as carbohydrate source and the contents of protein, oil and dietary fiber in the diet were adjusted using the standard tables of food composition in Japan (published by the Japanese Ministry of Education, Culture, Sports, Science and Technology). Diets were stored at –30°C until use. DK mice were conditioned with a commercial solid diet (CE-2; Clea Japan Inc., Tokyo, Japan) and kept on the diets for three months from the age of 6 weeks. Wistar/ST rats and C57BL/6 mice were kept on standard MF diet (Oriental Yeast Co. Ltd., Tokyo, Japan).

Measurement of platelet reactivity and spontaneous thrombolytic activity *in vitro*

The Global Thrombosis Test (GTT) has been described in detail elsewhere (Ikarugi *et al*. 2003; Yamamoto *et al*. 2003; Yamamoto and Kovacs 2003; Yamashita *et al*. 2005; Kovacs and Yamamoto 2006; Gorog *et al*. 2008). The instrument was purchased from Montrose Diagnostics Ltd., London, UK (www.globalthrombosis.com). **Figs. 1** and **2** show the principle and the practical embodiment of the technique.

The GTT test-tube is a conical plastic tube with two steel balls (**Fig. 1**). There are four longitudinal flat segments in the inner surface of the conical part of the tube forming very narrow gaps between the inserted balls and the inner plastic surface of the tube. When blood flows through the four gaps by the upper ball, platelets are activated by the high shear stress (175 dyne/cm^2). Distal to this place, between the two balls, low shear and turbulence favor platelet aggregate formation. The activated and aggregated platelets generate thrombin, which stabilizes the loose aggregates. When these thrombi are carried by the flow into the gaps by the lower ball, they cause occlusion and arrest the flow. In essence, the instrument detects the time interval (d, sec) between consecutive blood drops. At the start, blood flow is rapid and hence (d) is small. Subsequently, as the gaps become occluded and the flow rate decreases, (d) gradually increases. When the actual (d) exceeds 15 seconds (arbitrarily set "occlusion-time-limit"), the instrument displays "Occlusion Time (OT)", which is the time elapsed from the detection of the first drop of blood until incomplete occlusion (d>15 sec). Later, as the gaps occluded, flow is arrested. Eventually, due to thrombolysis, flow is restored as indicated by the detection of blood drop(s). There is also an arbitrarily pre-set "lysis-time-limit" (lysis d >200 sec) for lysis time measurement. When (d) between the last drop before and the first drop after occlusion exceeds this (lysis-d), the instrument displays "Lysis Time (LT)". Lysis time is calculated as follows: LT= (time of first drop with d > (lysis-d)) – (time of last drop with d < (lysis-d)).

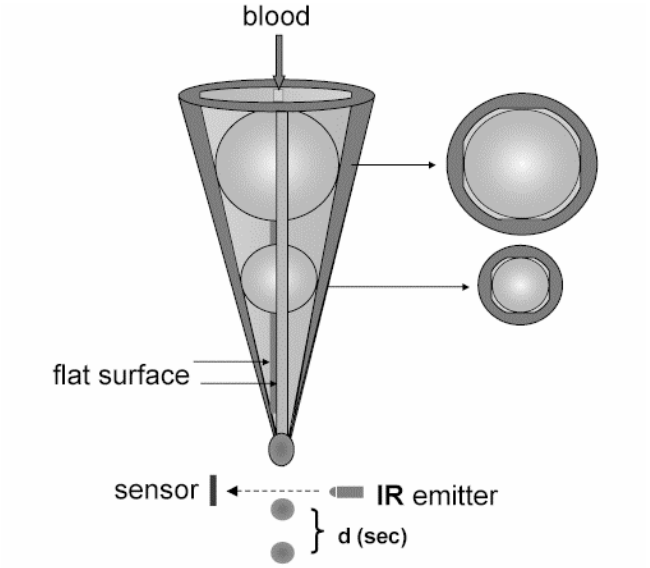

Fig. 1 Practical embodiment of the principle.

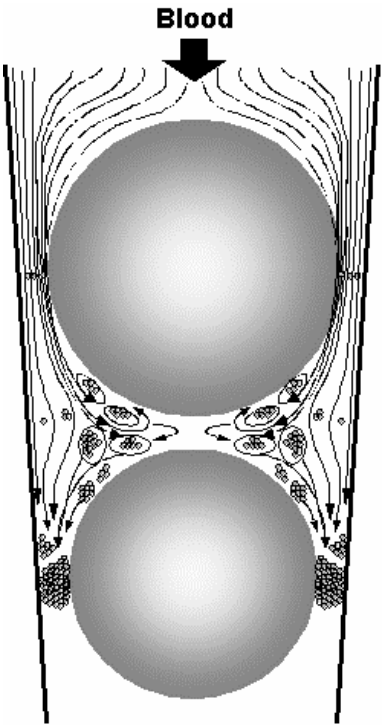

Fig. 2 Schematic diagram showing the principle of Global Thrombosis Test.

An increase or decrease in OT indicates inhibited or enhanced platelet reactivity, respectively while an increase or decrease of LT indicates inhibition or enhancement of spontaneous thrombolysis, respectively. From each sample six parallel measurements were made. Coefficients of variation (CV) for OT and LTs obtained by two-fold diluted rat blood were 6.07% and 33.67%, respectively (n = 15 each).

Effect of potato filtrates on platelet reactivity and spontaneous thrombolysis *in vitro*

Rats were anaesthetized with Nembutal (60 mg/kg, intramuscularly) and the blood was withdrawn from the abdominal aorta. The non-anticoagulated blood was mixed with saline (1:1) and 3.6 ml of such diluted blood and 0.4 ml of potato filtrate or saline (con-

trol) (blood: filtrate = 9:1) were mixed in a syringe by three inversions. Four ml of this mixture was tested by GTT.

Measurement of the anti-thrombotic effect *in vivo*

The helium-neon laser-induced thrombosis method has been described in detail (Kovacs *et al*. 1975; Ijiri *et al*. 2002; Yamamoto *et al*. 2006). Mice were anaesthetized, a polyethylene tube was placed into the left femoral artery to inject the dye and the carotid artery was exposed by incision. The animal was then placed on a special microscope stage and Evans blue dye was injected intra-arterially. Subsequent to the administration of dye, the center of the exposed carotid artery was irradiated with laser. Thrombus formation at the site of irradiation was monitored under epi-illumination and simultaneously recorded on videotape using CCD camera.

Oral administration of potato filtrate to mice

Potato filtrate was diluted three-fold with distilled water (controls received distilled water) and administered through a gastric tube in a volume of 11.55 ml/kg. The same volume of filtrate or water was given again 30 min after the first treatment (Yamamoto *et al*. 2003). The mouse was anaesthetized and the thrombosis experiment started 90 min after the second oral administration. The effect on thrombus formation was assessed by the calculated total thrombus size. Reduced thrombus size indicated anti-thrombotic activity.

Calculation of thrombus size

Details of this technique have been described elsewhere (Ijiri *et al*. 2002). An image of thrombus was computer-analyzed in every 10 s. Area of the thrombus was outlined and the thrombus mass was calculated. Image analysis was performed using Image J software (Image Processing and Analysis Java version 1.30, National Institutes of Health, Maryland, USA). Thrombotic status was expressed by the total sum of mass measurements in the first 10 min after irradiation.

Measurement of antioxidant activity (superoxide anion activity)

Antioxidant activity was measured by chemiluminometry (Luminometer AB-2200; ATTO Co. Ltd., Tokyo, Japan). The following reaction mixtures were used: (A) positive control; (B) negative control; (C) sample mixture. For (A): 150 µl of 100 mM phosphate buffer and 60 µl of xanthine oxidase solution (XOD; 0.1 U/ml, Sigma-Aldrich Co, Ltd., St Louis, USA) were kept at 37°C for 1 min. 10 µl of MPEC (ATTO Co, Ltd., Tokyo, Japan), 30 µl of saline and 50 µl of 3.6 mM hypoxanthine were added and mixed for 1 min. Measurements were performed over 45 s at 37°C. For (B): XOD and saline were replaced with phosphate buffer and the test sample, respectively. For (C): 10 µl of saline in (A) was replaced with the test sample. Antioxidant activity was expressed as units of superoxide dismutase (SOD, units/ml filtrate) calculated from a standard curve. Five parallel measurements were made from each sample.

Statistical analysis

Means of different groups were analysed by ANOVA, followed by the *post hoc* test of Fisher's PLSD using commercially available statistical package Stat View (v. 5.0; SAS Institute Inc., North Carolina, USA). Raw OT data and logarithmic LT data were used for the analysis. Correlation was analysed by a Pearson test. Results were expressed as mean ± SEM. $P<0.05$ was considered to be statistically significant.

RESULTS

Body weight

Potato diets significantly suppressed body weights as compared with the control diet. No difference was observed between the varieties.

Effects of potato varieties on thrombotic tendency

(1) Long-term intake of diet
Results are shown in **Fig. 3**. Variety Touya had a significant anti-thrombotic effect when compared with the control and with other potato varieties.

(2) Acute experiments
Results are shown in **Table 3**. Variety Yukirasha had no effect on OT and LT in any dilutions. Undiluted Inca red had no effect on OT but significantly increased LT, indicating inhibition of thrombolysis. Variety Sayaka decreased OT at 10X dilution, indicating enhancement of platelet reactivity, but did not affect LT. Varieties Hokkaikogane, Inca purple and Star ruby increased OT in a 3X dilution, indicating inhibition of platelet reactivity, but they had no effect on LT. Variety Beni-akari increased OT (inhibited platelets) and LT (inhibited thrombolysis) in a 3X dilution. Anti-thrombotic activity of this variety was further assessed *in vivo* by the He-Ne laser-induced thrombosis test. In acute experiments, four out of 20 varieties showed anti-thrombotic activities. Heat stability of these four varieties was tested as shown below.

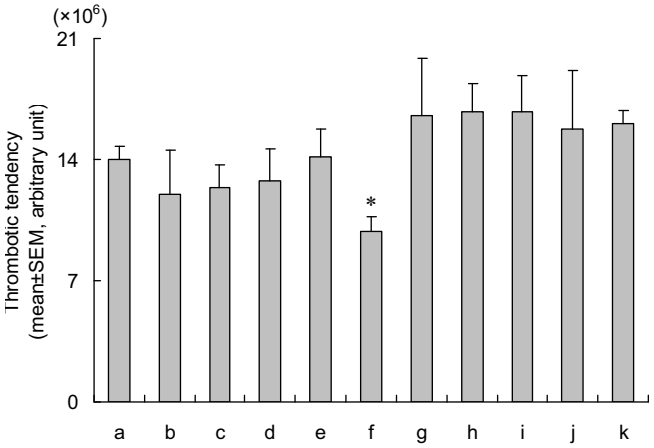

Fig. 3 Anti-thrombotic activity of potato varieties after long-term intake. a: control, b: Yukirasha, c: Inca red, d: Sayaka, e: Hokkaikogane, f: Touya, g: Beni-akari, h: Inca purple, i: Andes red, j: Kita-akari, k: Danshaku-imo; n=5-14; *: $P<0.05$ vs control.

Heat stability of Hokkaikogane, Beni-akari, Inca purple and Star ruby

Results are shown in **Table 4**. Anti-platelet activity of Hokkaikogane was preserved after heat treatment. Both the anti-platelet activity and inhibition of thrombolysis by Beni-akari was lost after heating. Heat treatment of Inca purple facilitated blood coagulation. Anti-platelet activity of Star ruby enhanced after heat treatment but the inhibitory effect on thrombolytic activity remained unchanged. It was decided to subject Hokkaikogane and Star ruby varieties for further test by the He-Ne laser-induced thrombosis model.

Anti-thrombotic activity of Hokkaikogane and Star ruby varieties *in vivo* as assessed by the He-Ne laser-induced thrombosis test

Results are shown in **Fig. 4**. Filtrates prepared from the specific potato varieties were heated at 100°C for 10 min and were administrated orally. Filtrates from both varieties showed anti-thrombotic activity in this *in vivo* test.

Relationship between antioxidant activity and anti-platelet/spontaneous thrombolytic activities (GTT) of raw potato filtrates

All filtrates showed oxidant rather than anti-oxidant activity and significant correlations were not demonstrated between

Table 3 Effect of potato variety filtrates on platelet reactivity (occlusion time) and spontaneous thrombolysis (lysis time)

Varieties	Dilution	Occlusion time	Lysis time
Yukirasha	control	306.7 ± 30.8	1634.3 ± 119.0
	X1	nd	nd
	X3	234.7 ± 27.5	2504.2 ± 517.2
	X10	260.1 ± 42.4	2322.8 ± 365.0
Inca red	control	293.3 ± 20.3	1456.8 ± 283.4
	X1	308.2 ± 56.8	5555 ± 0 b
	X3	361.9 ± 16.9	1353.3 ± 379.2
	X10	321.3 ± 10.0	1711.0 ± 179.1
Sayaka	control	310.9 ± 21.7	1325.2 ± 84.1
	X1	nd	nd
	X3	303.4 ± 50.8	2271.8 ± 231.3
	X10	230.6 ± 46.5 a	2262.3 ± 536.2
Hokkaikogane	control	306.8 ± 19.3	1262.2 ± 114.0
	X1	nd	nd
	X3	357.9 ± 20.2 a	1574.8 ± 201.1
	X10	321.1 ± 34.0	1393.7 ± 209.9
Touya	control	259.0 ± 18.7	967.5 ± 139.2
	X1	nd	nd
	X3	274.2 ± 49.5	1114.7 ± 116.5
	X10	330.9 ± 35.2	1418.7 ± 394.0
Beni-akari	control	268.2 ± 14.1	1171.3 ± 57.7
	X1	nd	nd
	X3	395.5 ± 15.2 b	2394.7 ± 168.1 b
	X10	276.5 ± 17.4	1632.3 ± 371.8
Inca purple	control	294.8 ± 20.7	1429.3 ± 217.3
	X1	nd	nd
	X3	380.6 ± 31.9 b	1329.5 ± 117.7
	X10	298.7 ± 19.7	1301.3 ± 134.9
Andes red	control	284.3 ± 29.8	1770.3 ± 237.8
	X1	nd	nd
	X3	340.5 ± 58.3	1889.3 ± 178.3
	X10	157.4 ± 30.6 b	1489.3 ± 156.0
Kita-akari	control	325.3 ± 27.7	1591.7 ± 257.1
	X1	nd	nd
	X3	406.0 ± 11.1	1659.7 ± 172.9
	X10	351.7 ± 13.4	1261.5 ± 161.7
Danshaku-imo	control	296.1 ± 36.9	1290.0 ± 77.5
	X1	nd	nd
	X3	341.6 ± 40.6	1279.8 ± 140.2
	X10	279.6 ± 24.4	2531.3 ± 410.2 b
Tokachikogane	control	318.5 ± 29.7	1066.5 ± 120.2
	X1	nd	nd
	X3	390.4 ± 13.8	868.3 ± 75.2
	X10	324.5 ± 31.8	1099.7 ± 117.2
Red moon	control	374.0 ± 79.7	908.7 ± 76.8
	X1	nd	nd
	X3	279.5 ± 55.0	968.8 ± 120.6
	X10	321.0 ± 51.7	1071.7 ± 282.3
Kitamurasaki	control	396.8 ± 37.3	1295.7 ± 256.6
	X1	nd	nd
	X3	355.5 ± 15.8	7708 ± 67.4
	X10	352.2 ± 27.0	895.5 ± 126.7
Inca-no-hitomi	control	350.7 ± 31.9	892.5 ± 75.5
	X1	nd	nd
	X3	245.2 ± 38.5	806.3 ± 95.8
	X10	305.1 ± 63.0	832.7 ± 103.6
Inca-no-mezame	control	367.2 ± 33.7	938.7 ± 83.1
	X1	nd	nd
	X3	301.2 ± 24.3	1380.5 ± 166.3
	X10	336.6 ± 20.6	944.7 ± 48.7
Star ruby	control	349.3 ± 32.7	1212.3 ± 129.3
	X1	nd	nd
	X3	494.1 ± 25.3 a	922.0 ± 90.5
	X10	288.7 ± 21.1	1172.5 ± 101.5
Northern ruby	control	366.1 ± 27.7	1043.8 ± 175.6
	X1	nd	nd
	X3	314.2 ± 27.1	1401.3 ± 413.6
	X10	368.5 ± 23.5	1319.8 ± 192.7
May queen	control	357.0 ± 40.9	1248.8 ± 324.8
	X1	nd	nd
	X3	356.2 ± 38.0	1633.0 ± 298.1
	X10	389.2 ± 32.4	1435.8 ± 246.1
Haruka	control	294.1 ± 25.7	996.5 ± 98.2
	X1	nd	nd
	X3	370.6 ± 43.8	1441.5 ± 321.2
	X10	350.9 ± 28.5	1240.7 ± 129.4
Konafubuki	control	301.1 ± 20.3	1093.3 ± 202.4
	X1	nd	nd
	X3	354.5 ± 23.4	1327.7 ± 216.3
	X10	322.8 ± 32.1	1171.0 ± 213.9

nd: not determined; a: P<0.05; b: P<0.01.

Table 4 Heat stability of four potato varieties

Varieties	Heat treatment	Occlusion time (OT)	Lysis time (LT)
Hokkaikogane	control	195.8 ± 20.4	961.8 ± 62.5
	before	268.7 ± 17.6 a	913.7 ± 42.4
	after	260.3 ± 18.4 a	980.0 ± 24.6
Beni-akari	control	323.2 ± 22.0	1488.2 ± 109.8
	before	441.6 ± 30.7 a	3108.5 ± 482.0 a
	after	350.6 ± 25.3	2733.7 ± 452.8
Inca purple	control	350.9 ± 27.4	1410.0 ± 230.5
	before	459.9 ± 25.8 b	1396.2 ± 210.0
	after	coagulation	coagulation
Star ruby	control	334.5 ± 21.9	1336.5 ± 100.8
	before	454.5 ± 34.1 a	1275.0 ± 94.2
	after	501.3 ± 54.8 b	2384.7 ± 264.3 b

a: P<0.05; b: P<0.01.

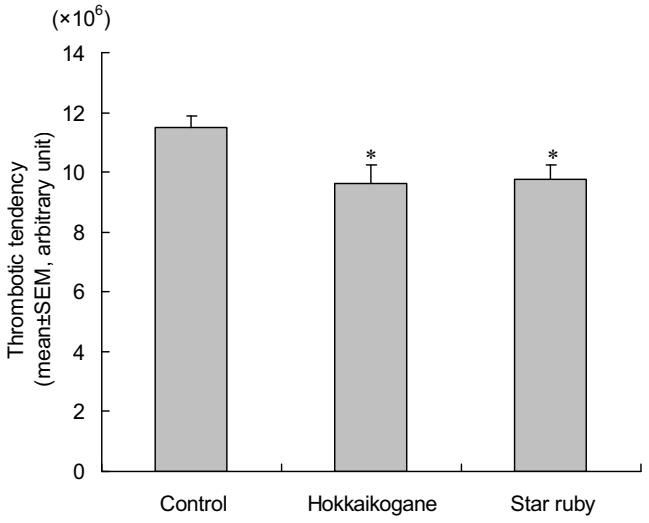

Fig. 4 Anti-thrombotic activity of heat treated filtrates from Hokkaikogane and Star ruby after oral intake. *n* = 6; *: P<0.05 vs control.

such activity and the anti-platelet effects (P = 0.831 for platelet reactivity and P = 0.862 for spontaneous thrombolytic activity).

DISCUSSION

Platelets play a pivotal role in the development of arterial thrombotic diseases such as myocardial infarction and stroke. A large variety of laboratory tests, both *in vitro* and *in vivo*, have been developed for the assessment of platelet function. The use of physiologically relevant technique to assess thrombotic status is of critical importance. We have chosen the Global Thrombosis Test to screen fruits and vegetables for anti-thrombotic effect *in vitro* and the He-Ne laser-induced thrombosis test in mice for further testing the *in vitro* active substances *in vivo*. In our opinion, a test which uses non-anticoagulated whole blood and shear forces as agonist is likely to be more relevant for the assessment of the *in vivo* thrombotic status than those using anticoagulated blood sample and different chemical agonists (Yamamoto and Kovacs 2003). We have also shown a good

correlation between *in vitro* platelet function tests using non-anticoagulated whole blood and shear forces as sole agonist and the *in vivo* test of laser-induced thrombosis (Yamamoto 2007).

In our earlier studies using shear-induced *in vitro* platelet function tests and laser-induced thrombosis test we have shown that fruits and vegetables could be classified into subclasses according to their anti-thrombotic activity (Yamamoto *et al*. 2003; Yamada *et al*. 2004; Naemura *et al*. 2005; Yamamoto *et al*. 2006). Some varieties did not affect platelets while others were either pro- or anti-thrombotic. Recently we have shown that an experimentally anti-thrombotic strawberry variety also exerted anti-platelet effect in human volunteers (Naemura *et al*. 2006).

In the present study, the effectiveness *in vitro* was not always confirmed by the further *in vivo* test after long-term intake. The freeze-dried and unheated flour from Touya variety and the heat-treated filtrates from Hokkaikogane and Star ruby varieties were anti-thrombotic both in acute experiment and after long term intake *in vivo*. In contrast, raw Beni-akari and Inca purple potatoes showed anti-platelet activity in acute experiments, but the heated filtrates of these did not have an anti-platelet effect. Touya variety showed anti-thrombotic activity in long-term experiment, but no such activity was observed in the acute experiment. The reason of such inconsistency between acute and long-term experiments is unclear, but one should consider the use of filtrates in acute experiments and whole material in long-term experiments.

Epidemiological studies have raised doubts about the effectiveness of antioxidants in the prevention of cardiovascular diseases (Brown *et al*. 2001). Earlier we found a significant correlation between antioxidant and anti-platelet activities of some strawberry varieties (Naemura *et al*. 2005) but not in mulberries (Yamamoto *et al*. 2006). In the present study, potato filtrates did not have antioxidant but rather oxidant activity and no correlation was found between the latter and the anti-platelet activities.

CONCLUSION

Our present findings showed that potato varieties could be classified into subclasses according to their anti-thrombotic activities shown *in vitro* and *in vivo*. Touya, Hokkaikogane and Star ruby varieties exerted experimental anti-thrombotic effect. Consumption of these potato varieties may have anti-thrombotic effect in humans and this could be exploited by including these potato varieties in an anti-thrombotic diet.

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