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Ethyl Acetate Extract from Tissue-Cultured Mountain Ginseng Adventitious Roots Inhibits *In Vitro* Platelet Aggregation in Whole Human Blood and Augments Peripheral Blood Flow in Mice

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We previously reported that *in vitro* anti-platelet activity of tissue-cultured mountain ginseng (TCMG) ethanol extracts show improved efficacy when compared with commercial ginseng products such as Korean red ginseng and *Panax ginseng*. However, information on the anti-platelet activity of the ethyl acetate fraction from TCMG adventitious roots is limited. Therefore, in this study, we further investigated the effects of an ethyl acetate extract of TCMG (EA-TCMG) adventitious roots on *in vitro* antiplatelet activity in whole human blood and its effect on peripheral blood flow in mice. We found that EA-TCMG inhibited platelet aggregation with IC_{50} values of 271, 180, and 147 μg/mL induced by collagen, adenosine-5´-diphosphate, and arachidonic acid, respectively. Among the three agonists used, thromboxane A_2 formation induced by arachidonic acid was markedly suppressed. Furthermore, EA-TCMG improved the peripheral circulatory disturbance by improving vascular blood flow. In conclusion, these results suggest that ethyl acetate extracts from TCMG adventitious roots might inhibit vascular platelet aggregation and thrombus formation.

Keywords: *Panax ginseng*, Platelet aggregation, Human blood, Tissue-cultured mountain ginseng

INTRODUCTION

Certain herbal medicines and natural products show promise as agents for improving blood viscosity and microcirculation, as well as preventing atherosclerotic diseases, such as myocardial and cerebral infarction [1]. Many studies have reported that modulation of platelet activity using specific pharmacological agents is a successful strategy for preventing thrombotic disorders [2]. Ginseng radix, the root of *Panax ginseng* Meyer, is one of the world's best known medicinal plants and has long been studied for its medical efficacy [3]. In Korea, mountain ginseng has traditionally been considered more effective than cultivated ginseng products [4]. Although mountain ginseng is well known in pharmaceutics, much

remains to be understood to establish a scientific foundation for traditional use [5,6]. Mountain ginseng has become extremely scarce; thus, the commercial ginseng supply depends almost exclusively on field cultivation, which is a time-consuming and labor-intensive process. However, tissue culture techniques for mountain *P. ginseng* have made bioreactor technology a useful tool for large-scale production of root biomass [6,7]. Tissue culture techniques have allowed us to easily obtain a mass of adventitious roots, which can then be studied for the various biological effects of tissue-cultured mountain ginseng (TCMG). Recent findings have demonstrated that TCMG aids in the prevention or treatment of sper-

matogenic disorders [8,9], erectile dysfunction [10], symptoms of hyperlipidemia [11], and has anti-fibrotic and antioxidant activity [12,13]. In addition, TCMG stimulates immune cells and inhibits growth of cancer cells [14]. We recently reported that TCMG has more effective anti-platelet activity in 70% ethanol extracts than that of ginseng commercial products [15]. Platelets have multiple activation signaling mechanisms, so agents with anti-platelet activity may have wide therapeutic potential for blood flow disorders [16-18]. In this study, we found that ethyl acetate extract of TCMG (EA-TCMG) was a candidate for further study through confirmation of its *in vitro* effects on anti-platelet activity using whole human blood and *in vivo* peripheral blood circulation in a mouse model. Based on the results, we suggest that EA-TCMG shows higher anti-platelet potency than that of other TCMG solvent fractions, and that it also improves peripheral blood circulation.

MATERIALS AND METHODS

Reagents and materials

Catechin was purchased from Wako Pure Chemical Co. (Osaka, Japan). Collagen, adenosine-5´-diphosphate (ADP) and arachidonic acid (AA) were purchased from (Chrono-Log Co., Havertown, PA, USA). A thromboxane B_2 (TXB_2) ELISA kit was obtained from (R&D Systems Inc., Minneapolis, MN, USA). The isofluran/foran solution (Choongwae Pharmaceutical Co., Seoul, Korea) was prepared by mixing oxygen (Shinheung Oxygen, Daejeon, Korea) and nitrogen (Shinheung Oxygen). TCMG was supplied by CBN Biotech in Ochang, Korea in 2008 and deposited at the TKM Converging Research Division, Korea Institute of Oriental Medicine. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction

Dried roots of TCMG (400 g) were extracted with 70% ethanol and then concentrated under vacuum using a rotary evaporator. All extracts were freeze-dried to yield extract powders (121.1 g; yield, 30%). The residue from the 70% ethanol extract was then extracted with n-hexane, followed by trichloromethane, ethyl acetate, n-butanol, and H_2O. Their yields were 3.38, 2.78, 2.35, 28.69, and 62.82%, respectively.

Volunteers

Seven (1 female and 6 males; age range, 20 to 38 years) healthy volunteers entered the study. None of the subjects had a history of bleeding disorders or cardiovascular disease and refrained from any pharmacological therapy for at least 2 wk prior to enrollment. None of the subjects smoked, nor did they exhibit hypertension, diabetes, or an abnormal hematocrit. All subjects gave written informed consent prior to participation. This study was approved by the Institutional Review Board of the Oriental Hospital of Daejeon University, Korea (approval no., djomc-24).

Whole blood platelet aggregation assay

Aggregation studies were performed with a 500VS Chrono-log aggregometer (Chrono-Log Co.), using the impedance method [19]. Intravenous blood for whole blood studies was drawn from healthy volunteers into Vacutainer tubes (Becton Dickinson, Franklin Park, NJ, USA) containing 3.8% sodium citrate, quickly diluted with one volume of 0.9% saline solution, and used directly. Whole blood was incubated at 37°C in 450 μL aliquots until the time of use. Samples were maintained at 37°C and stirred at 200 ×g during aggregation testing. Following determination of the amount of agonist, such as collagen, AA, and ADP, necessary to cause a suitable impedance (W) full-scale deflection, varied sample doses in 50 μL aliquots were added to 1 mL of diluted whole blood in a polystyrene cuvette (Chrono-Log Co.), 5 min prior to adding the agonist. The deflection observed at 8 min after adding the agonist was used for the calculation. Aggregation was quantified as the change in impedance. Several concentrations of EA-TCMG were used so that an IC_{50} value could be determined from a plot of concentration vs. percent inhibition.

Thromboxane B_2 measurement

Following the AA-induced aggregation assay, whole blood samples were centrifuged at 2,000 ×g for 15 min for the TXB_2 immunoassay. Supernatant aliquots were immediately stored at -20°C until assay. The concentration of TXB_2, a stable metabolite of thromboxane A_2 (TXA_2), was quantified using a TXB_2 ELISA kit (R&D Systems), according to the manufacturer's instructions. Absorbance was measured at 405 nm using a SpectaMAX 340 reader (Molecular Devices, Sunnyvale, CA, USA). TXB_2 concentration was calculated from a standard curve.

Animals and peripheral blood circulation disturbance model

Male ICR mice (Orient Bio Inc., Seongnam, Korea; weight, 25±3 g) were maintained in cages under light-

controlled conditions (12 h light/dark cycle) with *ad libitum* access to food and water. All experimental procedures were approved by the Animal Care and Use Committee of the Korea Institute of Oriental Medicine (approval no. 09-115). EA-TCMG or catechin was suspended in saline and administered orally to ICR mice at 500 mg/kg once daily for 3 d and once before initiating the experiment. All experiments were performed 1-h after the final administration. We previously developed the peripheral circulatory disturbance *in vivo* model [20]. Briefly, after induction with 2.5% isoflurane, animals were anesthetized with 1.5% isoflurane in a mixture of oxygen (0.1 L/min) and nitrogen (0.2 L/min). Peripheral blood flow in the mouse foot region was measured by laser Doppler flowmetry (LDF; BFL21, Transonic Instrument, Ithaca, NY, USA) and recorded using Chart4 for Windows software (ADI Instruments, New South Wales, Australia). Peripheral blood circulatory disturbance was induced in mice under cooling conditions using a temperature-controlled chamber (Chamlide IV; Live Cell Instruments, Seoul, Korea). The tissue perfusion unit value of stabilized peripheral blood flow was measured for 10 min, and this value was set as the baseline using LDF. In addition, rectal temperature was measured at 5 min intervals. Peripheral blood flow was expressed as a peripheral blood flow ratio (peripheral blood flow value/ baseline value). Each mouse was observed for 100 min to determine inhibition or recovery from the lower blood flow caused by the peripheral circulatory disturbance.

Statistical analysis

All analyses were performed with the SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). All results are presented as means±standard deviation. The IC_{50} values were calculated using the Regression Wizard from Sigma Plot. The inhibition of catechin or EA-TCMG on human platelet aggregation induced by collagen was calculated using the paired Student's t-test. Differences in TXB_2 production were determined by one-way analysis of the variance (ANOVA) followed by Tukey's post-hoc test. The peripheral blood flow ratio differences between the two experimental groups were calculated by repeated measure ANOVA followed by Tukey's post-hoc test. Differences were statistically significant at *p*-values <0.05.

RESULTS

Anti-platelet activity of ethyl acetate extract of tissue culture mountain ginseng in human whole blood

EA-TCMG showed the greatest inhibition of human

Table 1. Inhibitory effect of the solvent fractions from a TCMG ethanol extract on human platelet aggregation induced by collagen

Samples	Solvent fractions (500 μg/mL)	Inhibition (%)
Catechin		63.98±10.54
TCMG	*n*-hexane	44.74±7.44
	Trichloromethane	24.75±3.57
	Ethyl acetate	93.06±1.84 (*p*=0.004)[1]
	n-butanol	17.42±1.07
	H_2O	22.25±3.57

Data are mean±SD.
TCMG, tissue-cultured mountain ginseng.
[1]*p*-value in comparison to catechin as a reference by the paired Student's *t*-test.

whole blood platelet aggregation in a preliminary screening of solvent fractions. Using the impedance method of aggregometry, we tested the *in vitro* effect of solvent fractions from the TCMG ethanol extract on platelet aggregation induced by collagen in whole human blood. Table 1 lists the inhibitory effects of the solvent fractions on human platelet aggregation. Inhibition percentages for EA-TCMG were 93.06±1.84%, whereas values for *n*-hexane, trichloromethane, *n*-butanol and H_2O extracts, all at a 500 μg/mL, were 44.74±7.44%, 24.75±3.57%, 17.42±1.07%, and 22.25±3.57%, respectively. Among them, EA-TCMG (93.06±1.84%, *p*=0.004) was selected as an effective fraction in comparison with catechin as a control agent (63.98±10.54%). Fig. 1 shows the inhibitory activity of EA-TCMG on human platelet aggregation induced by agonists such as AA, ADP, and collagen in a dose-dependent manner and the pattern for the typical curves. Table 2 shows the IC_{50} values for EA-TCMG calculated from the concentration-inhibition curves as 271, 180, and 147 μg/mL for collagen, ADP, and AA-induced platelet aggregation, respectively.

The inhibitory effects on thromboxane B_2 production

TXA_2 is rapidly hydrolyzed to TXB_2, a stable plasma product of the cyclooxygenase pathway. TXA_2 is a potent stimulator of platelet aggregation. Therefore, we examined whether EA-TCMG blocked the production of TXB_2 under AA exposure. The TXA_2 (determined as TXB_2) production percentages in the EA-TCMG and catechin treatments at 500 μg/mL were 68.64±8.57 and 56.42±9.44% when compared to the control group (saline+AA), respectively. As shown in Fig. 2, EA-TCMG mildly reduced TXB_2 production but significantly inhibited TXB_2 production at 500 μg/mL.
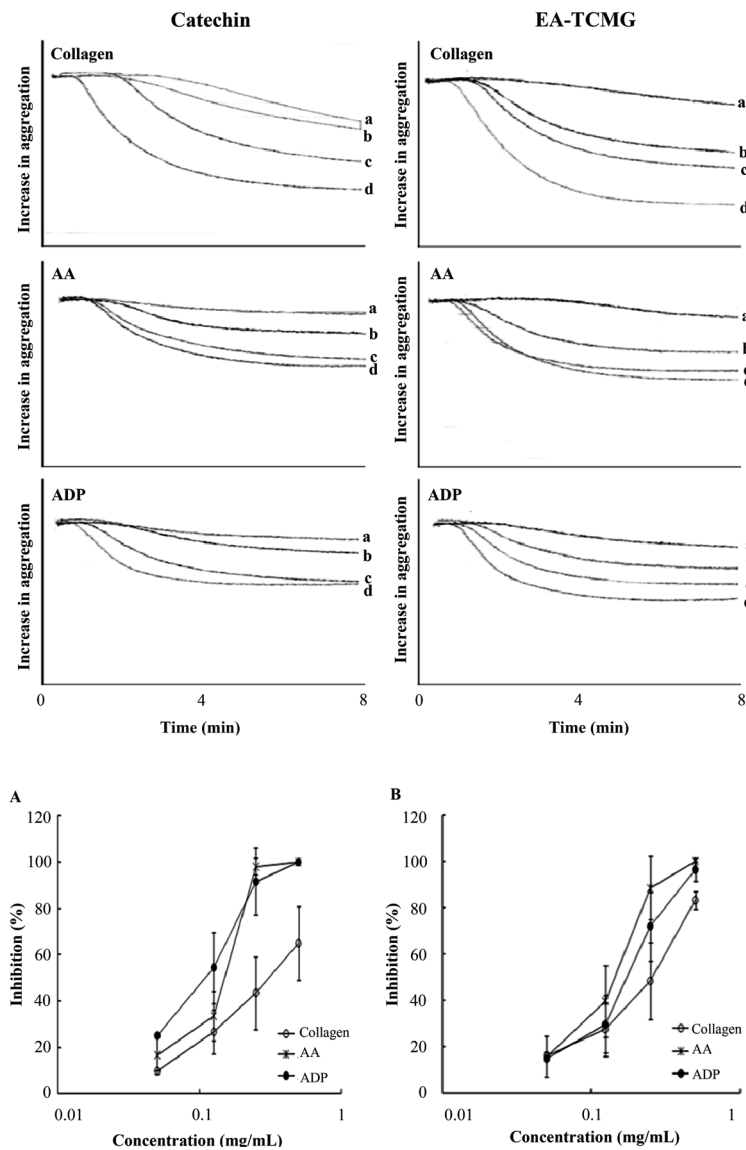
Fig. 1. Representative plots showing the pattern for typical curves of collagen-, arachidonic acid (AA)-, and adenosine-5′-diphosphate (ADP)-induced platelet aggregation obtained with dose-dependent samples of the ethyl acetate extract of tissue culture mountain ginseng (EA-TCMG) extract or catechin. a: 500 μg/mL, b: 250 μg/mL, c: 125 μg/mL, d: saline. Aggregation in the presence of the EA-TCMG extracts or catechin is expressed as a percentage of that in the absence of the extract. Results are mean±SD. (A) Inhibitory effects of catechin as a control agent. (B) Inhibitory effects of EA-TCMG extract.

Table 2. IC$_{50}$ values of EA-TCMG on human platelet aggregation induced by various agonists

Agonist	IC$_{50}$ value[1] (μg/mL)	
	Catechin	EA-TCMG
Collagen (2 μg/mL)	342	271
AA (0.5 mM)	143	147
ADP (15 μM)	120	180

IC$_{50}$, 50% inhibitory concentration; EA-TCMG, ethyl acetate extract of tissue culture mountain ginseng; AA, arachidonic acid; ADP, adenosine-5′-diphosphate.
[1]Calculated from a dose-dependent curve using the Regression Wizard in Sigma Plot.

EA-TCMG alleviates the peripheral blood circulation disturbance in mice

A peripheral blood circulatory disturbance was induced in mice under cooling conditions using a temperature-controlled chamber [20]. To examine the effects of EA-TCMG on improving peripheral blood circulation in the *in vivo* model, peripheral blood flow was compared between the experimental group (*n*=8, EA-TCMG oral administration), the control group (*n*=8, saline oral administration), and the positive drug group (*n*=8, catechin oral administration). Rectal temperatures were similar
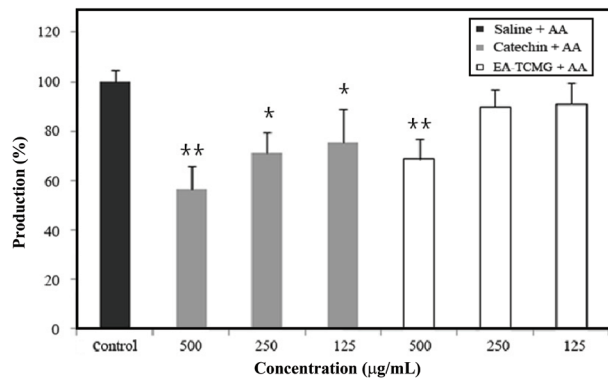
Fig. 2. Effects of an ethyl acetate extract of tissue culture mountain ginseng (EA-TCMG) on the production of thromboxane B_2 (TXB$_2$) induced by arachidonic acid (AA). Whole blood was preincubated with the EA-TCMG extract for 5 min and then stimulated with AA (0.5 mM). TXB$_2$ was investigated as described in Materials and Methods. The amount produced was measured using a TXB$_2$ EIA kit. Values are given as mean±SD. *$p<0.05$, **$p<0.01$ represents a significant difference between the experimental and control values.

among the EA-TCMG, catechin, and saline experimental groups. Peripheral blood flow was severely reduced after cooling in all mice. After 30 min, peripheral blood flow ratios in the EA-TCMG and saline groups decreased to 0.77±0.22 and 0.63±0.20, respectively. Note that the decrease in the saline group was induced by cooling. After 60 min, peripheral blood flow ratios in the EA-TCMG group and the saline group were 0.21±0.07 and 0.19±0.07, respectively. During 60 min after measurement, the improvement in peripheral blood flow was mild in the EA-TCMG (500 mg/kg, $p=0.041$) group compared with that in saline group but significant in the catechin group (500 mg/kg, $p=0.001$) (Fig. 3). These results indicate that the peripheral blood flow ratio decreased in both groups; however, the decrease in the blood flow ratio was lower in EA-TCMG group than that in the saline group. These findings indicate that the EA-TCMG treatment was effective for improving peripheral blood flow.
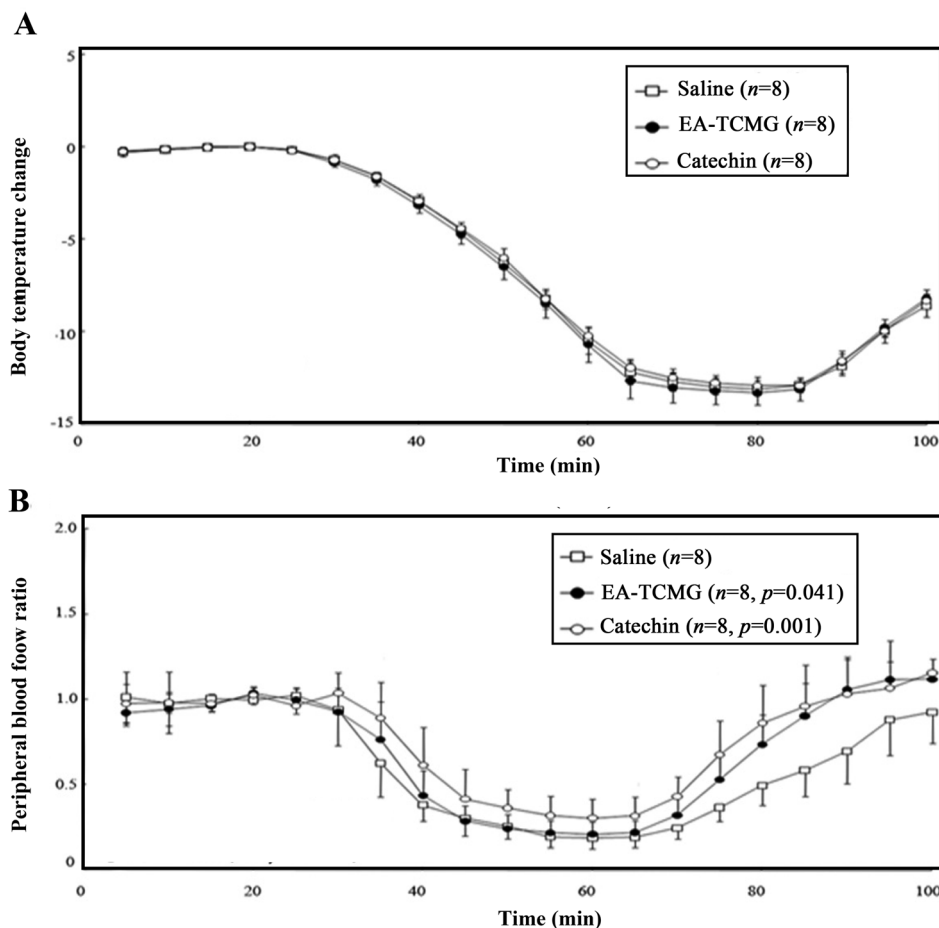

Fig. 3. Effects of an ethyl acetate extract of tissue culture mountain ginseng (EA-TCMG) on peripheral blood circulation disturbance in mice. Change in average peripheral blood flow ratio and temperature distribution during temperature-controlled chamber cooling and heating phases after oral administration of EA-TCMG (500 mg/kg). (A) Time course of change in rectal temperature. (B) Time course of change in peripheral blood flow ratio (peripheral blood flow value/baseline value). Data are mean±SD. A p-value in comparison to vehicle control (saline) by repeated-measures analysis of variance with a Tukey's post-hoc means comparison.

DISCUSSION

We have reported that TCMG adventitious root ethanol extract has anti-platelet activity in human whole blood, compared with ginseng commercial products [15]. We then selected the TCMG extract as a candidate for further anti-platelet activity study. In this study, we investigated the *in vitro* effect of EA-TCMG on platelet aggregation and then evaluated the *in vivo* effects on a peripheral circulatory disturbance in a mouse model. Our data on anti-platelet activity in human whole blood show that EA-TCMG could be protective against platelet aggregation (Fig. 1), and that it reduced TXB_2 production induced by various agonists (Fig. 2). In particular, EA-TCMG suppressed release of AA from platelet membrane phospholipids and significantly reduced formation of TXB_2, as a biomarker of platelet activation. The inhibitory effect of EA-TCMG on AA release and TXB_2 formation may contribute to reduced platelet aggregation. Irreversible platelet aggregation and vasoconstriction caused by TXA_2 formation has been reported [21]. Experimental animal models, such as the acute pulmonary thromboembolism [22], the arteriovenous shunt thrombosis [23] and the arterial thrombosis models have been developed to evaluate anti-platelet or anti-thrombotic agents [24]. However, peripheral circulatory disturbance in an *in vivo* model, which occurs as a result of changes in blood flow, has not been established. Several studies have reported on factors causing sensitive changes in blood flow [25,26]. Of the factors that affect blood flow, changes in external temperature trigger a change in skin temperature, which, in turn, causes changes in blood flow, because long-term exposure of the blood vessels to a cold-temperature environment and the resulting vasoconstriction cause abnormalities in blood flow, thereby inducing platelet aggregation [27]. Similarly, exposure of the whole body to a low temperature results in decreased blood flow [26]. Based on these studies, we previously developed an *in vivo* model of peripheral circulatory disturbance to confirm the efficiency of aspirin [20]. In this study, our results indicate that oral administration of EA-TCMG was effective for improving peripheral blood flow using the model of peripheral disturbance circulation. These results confirmed the efficacy, including improvement in peripheral blood circulation. In conclusion, EA-TCMG, as a natural product derived from tissue culture techniques, has a potential effect on blood circulation, as determined by an *in vitro* whole blood aggregation assay and an *in vivo* model of peripheral blood circulatory disturbance. These results suggest that EA-TCMG has potent anti-platelet activity

and that it improves peripheral blood circulation. Further elucidation of the active compounds in EA-TCMG could lead to the development of potent anti-platelet agents for preventing various vascular diseases; however mechanistic studies are necessary to define the effectiveness of EA-TCMG.

ACKNOWLEDGEMENTS

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