<Original Article>

Water soluble tomato concentrate regulates platelet function via the mitogen-activated protein kinase pathway

Dahye Jeong¹, Muhammad Irfan¹, Evelyn Saba¹, Sung-Dae Kim², Seung-Hyung Kim³, Man Hee Rhee¹,*

¹Department of Veterinary Medicine, College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Korea
²Research Center, Dongnam Institute of Radiological and Medical Sciences, Busan 46033, Korea
³Institute of Traditional Medicine & Bioscience, Daejeon University, Daejeon 34520, Korea

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Abstract: Tomato extract has been shown to exert antplatelet activity in vitro and to change platelet function ex vivo, but with limitations. In this study, antiplatelet activity of water soluble tomato concentrate (Fruitflow I) and dry water soluble tomato concentrate (Fruitflow II) was investigated using rat platelets. Aggregation was induced by collagen and adenosine diphosphate and granule-secretion, [Ca²⁺], thromboxane B2, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels were examined. The activation of integrin αIIbβ3 and phosphorylation of signaling molecules, including mitogen-activated protein kinase (MAPK) and PI3K/Akt, were investigated by flow cytometry and immunoblotting, respectively. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were examined. Moreover, in vivo thrombus weight was tested by an arteriovenous shunt model. Fruitflow I and Fruitflow II significantly inhibited agonist induced platelet aggregation, adenosine triphosphate and serotonin release, [Ca²⁺], and thromboxane B2 concentration, while having no effect on cAMP and cGMP levels. Integrin αIIbβ3 activation was also significantly decreased. Moreover, both concentrates reduced phosphorylation of MAPK pathway factors such as ERK, JNK, P38, and PI3K/Akt. In vivo thrombus formation was also inhibited. Taken together, these concentrates have the potential for ethnomedicinal applications to prevent cardiovascular ailments and can be used as functional foods.

Keywords: blood platelet, cardiovascular disease, natural cardioprotective agent, thrombosis, tomato

Introduction

The prevalence of cardiovascular diseases in the developed countries is increasing at a high pace [7]. Platelet hyperactivation is the causal factor for the growth and advancement of atherosclerosis and an important contributor in cardiovascular pathology [14]. Rupture of atherosclerotic plaque can lead to death by development of cardiovascular disease (CVD) [4]. Therapeutic antiplatelet agents have been proved to reduce the incidence CVD [15]. There is need to slow down the progression of these diseases along with focused attention on the influence of dietary compounds on the cardiovascular system [6]. Recently, increase in the popularity of ethnomedicine and natural products has strengthened interest in traditional remedies for CVD [10]. During the last few decades, consumption and popularity of tomatoes have been increased and previous literature suggests the overall health benefits of tomatoes and evidence to be considered as cardiovascular protective food [1, 5]. Nutrients available in the tomato are accompanying theoretical or proven effects on reduced risk of degenerative diseases [28] and cardiovascular system like cardioprotective effects of lycopene [1]. Previous studies have put forward a link between lower incidence of CVD and consumption of tomato in Mediterranean countries [5, 23]. Dutta-Roy et al. [2] and O’Kennedy et al. [16] proved that platelet activity can be influenced by water tomato concentrates (Fruitflow I and Fruitflow II) in vitro and ex vivo. But the available data is insufficient to recognize the underlying mechanism of action. In this study, we explored the effects of tomato ex vivo as well as in vivo in more detail using a rat model.

We hypothesize that observed cardioprotective benefits accredited to the tomato could be linked to reduce hyperactivity of platelets and the suppression of platelet function in vivo. This type of natural antithrombotic agent could have an application in the new era of ethnomedicine and prevention of CVD like atherosclerosis, myocardial infarction and coronary artery disease.

Materials and Methods

Chemicals preparation

Water soluble tomato concentrate (WSTC; Fruitflow I) and
dry water soluble tomato concentrate (DWSTC; Fruitflow II) were obtained from DSM Nutritional product (Switzerland). The Fruitflow II was diluted to the appropriate concentration immediately before all experiments were performed. Collagen and adenosine diphosphate (ADP) were purchased from Chrono-log Corporation (USA). Fura-2/AM was obtained from Sigma Chemical (USA). ATP Assay kit was obtained from Biomedical Research Service & Clinical Application (USA). TXB2 EIA kit was purchased from Enzo Life Sciences (USA). Serotonin EIA kit was purchased from LDN Labor Diagnostika Nord (Germany). Fibrinogen Alexa Fluor 488 conjugate was obtained from Molecular Probes (USA). Antibodies against phospho-p44/42, p44/42, phospho-p38, p38, phospho-SAPK/JNK, SAPK/JNK, phospho-PI3K (p85), PI3K (p85), phospho-Akt and Akt were acquired from Cell Signaling Technology (USA). All chemicals were of reagent grade.

**Animals and dosage**

Male Sprague Dawley rats (6 weeks old) weighing from 240 to 250 g were obtained from Orient Bio (Korea). The animals were acclimated for 1 week before the experiments and maintained in an air-conditioned animal room with a 12/12 h light/dark cycle at a temperature of 23 ± 2°C and humidity of 50 ± 10%. The rats were randomly divided into 3 groups; one normal group, one Fruitflow I treated group and one Fruitflow II treated group (n = 10 for each group). As based on tomato feeding of 3 g/day for human consumption [20], dose normalization for rats was done to 900 mg/kg dose with stirring and determined using the aggregometer. The Fruitflow II was diluted to the appropriate concentration immediately before all experiments were performed. Collagen and adenosine diphosphate (ADP) were purchased from Chrono-log Corporation (USA). Fura-2/AM was obtained from Molecular Probes (USA). Antibodies against phospho-p44/42, p44/42, phospho-p38, p38, phospho-SAPK/JNK, SAPK/JNK, phospho-PI3K (p85), PI3K (p85), phospho-Akt and Akt were acquired from Cell Signaling Technology (USA). All chemicals were of reagent grade.

**Platelet preparation**

Whole blood was obtained after 2 h of last oral injection by heart puncture and platelet preparation was conducted as previously described [18]. All experiments were carried out in accordance with the National Institutes of Health guidelines and approved by the Ethics Committee of the College of Veterinary Medicine, Kyungpook National University (2015-0094), Korea.

**Platelet aggregation assay**

Platelet aggregation was performed as previously described [11]. Aggregation was monitored by measuring light transmission using an aggregometer (Chrono-log Corporation, USA). Briefly, the washed platelets (3 × 10^9/mL) were pre-incubated at 37°C for 2 min in the presence of 1 mM CaCl_2 and stimulated with doses of collagen (1.25 µg/mL) and ADP (2.5 µM or 1.25 µM). The mixture was further incubated for 5 min with stirring and determined using the aggregometer.

\[ [Ca^{2+}] \text{ measurement} \]

The intracellular calcium ion concentration ([Ca^{2+}]) was mea-

\[ F \text{ (cAMP)} \text{ and cyclic adenosine monophosphate (cAMP)} \text{ levels} \]

After terminating the platelet aggregation reaction, cAMP or cGMP level was measured using a cAMP EIA kit or cGMP EIA kit according to the manufacturer’s protocol.

**Adenosine triphosphate (ATP) release assay**

ATP release was measured using a luminometer (GloMax 20/20; Promega, USA) using an ATP assay kit according to manufacturer’s instructions.

**Serotonin release assay**

After terminating the platelet aggregation reaction, the mixture was immediately centrifuged at 12,000 × g for 5 min at 4°C. The supernatant was collected and serotonin release was measured with a serotonin ELISA kit according to the manufacturer’s instructions.

**Evaluation of thromboxane B2 (TXB2) generation**

Platelet aggregation assay was performed and the reactions were terminated by adding ice-cold 2.5 mM EDTA and 100 µM indomethacin. After centrifugation at 12,000 × g for 5 min at 4°C, the supernatant was collected and the concentration of TXB2 was measured using a TXB2 EIA kit according to the manufacturer’s protocol.

**Measurement of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels**

After terminating the platelet aggregation reaction, cAMP or cGMP level was measured using a cAMP EIA kit or cGMP EIA kit according to the manufacturer’s protocol, respectively.

**Assessment of fibrinogen binding to integrin α_mβ_3**

Fibrinogen Alexa Fluor 488 conjugate binding to washed
Antiplatelet effects of tomato concentrate

Platelet aggregation was quantified by flow cytometry as previously described [8]. The fluorescence of each platelet sample was analyzed using a FACS Caliber cytomter (BD Biosciences, USA), and the data were analyzed using CellQuest software (BD Biosciences).

Prothrombin time (PT)/activated partial thromboplastin time (aPTT)

aPTT and PT were measured using an Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory, Italy). Briefly, the platelet-poorn plasma (PPP) from rats treated Fruitflow I and Fruitflow II was incubated at 37°C for 7 min. 100 µL of the incubated PPP was mixed with 50 mL of cephalin in the process plate, and coagulation started with the addition of 1 mM CaCl₂ and 100 mL thromboplastin to the PPP for the aPTT and PT assays, respectively.

Ateriovenous shunt

The in vivo antithrombotic activity of Fruitflow I and Fruitflow II was evaluated in a rat extracorporeal shunt model by the method of Umetsu and Sanai [26], with a little modification. Briefly, the ateriovenous shunt model was used and blood circulation in the cannula was carried out for 15 min, and thrombus weight was determined immediately.

Immunoblotting

Immunoblots were performed as described previously [3]. Briefly, after terminating the aggregation reaction, lysates were then prepared by solubilizing and centrifuging the platelets in sample lysis buffer. Protein concentration was determined using a bicinchoninic acid assay. Total cell proteins (35 µg) from the platelet lysate were separated using 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes in transfer buffer. The membranes were blocked in TBS-T containing 5% dry skim milk and incubated with primary antibody diluted in 5% bovine serum albumin solution. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody that was visualized by binding an enhanced chemiluminescence (Advansta, USA).

Statistical analysis

Data were analyzed with a one-way analysis of variance followed by a post hoc Dunnett’s test in order to measure statistical significance of the differences observed (SAS Institute, USA). All data are presented as the mean ± SE. P values of 0.05 or less were considered to be statistically significant.

Results

Effects on agonist-induced platelet aggregation

The effect of WSTC (Fruitflow I) and DWSTC (Fruitflow II) on agonist-induced platelet aggregation was evaluated. Our result showed that Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) dramatically inhibited platelet aggregations induced with collagen (1.25 µg/mL) to 32 and 33% and with ADP 2.5
5% and 46 ± 6% respectively (Fig. 4) as well as serotonin release by 75 ± 10% and 71 ± 10% (Fig. 5).

**Effect on TXB2 production**

Thromboxane A2 amplifies platelet activation during hemostasis as a mediator. As it is physiologically unstable, so we investigated its metabolite, TXB2 production effect. The result showed that Fruitflow I and Fruitflow II markedly decreased both ADP and collagen-induced TXB2 production by 82 ± 7% and 85 ± 6% respectively (Fig. 6).

**Fruitflow I and Fruitflow II inhibit fibrinogen binding to integrin α_{IIb}β_{3}**

Binding of fibrinogen to integrin α_{IIb}β_{3} which take pivotal role in the platelet activation and aggregation, induce outside-in signaling leads to adhesion, spreading and complete aggregation [24]. So, we checked the effect of Fruitflow I

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**Fig. 4.** Inhibitory effects of Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) on granule secretion and ATP release. Washed platelets (3 × 10^8/mL) were pre-incubated for 2 min at 37°C in the presence of 1 mM CaCl_2 and then stimulated with collagen or ADP. After terminating the aggregation, ATP release assay performed. Bar graph shows the mean ± SE of at least four independent experiments. **p < 0.01 compared to the agonist control.

**Fig. 5.** Inhibitory effects of Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) on serotonin release from dense granules. After termination of aggregation reaction, serotonin release measured with ELISA kit. Bar graph shows the mean ± SE of at least four independent experiments. **p < 0.01 and ***p < 0.001 vs. control.

**Fig. 6.** Inhibitory effect of Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) on TXB2 production. After termination of aggregation reaction by adding ice-cold 2.5 mM EDTA and 100 µM indomethacin the mixture was centrifuged, the supernatant used and the concentration of thromboxane B2 (TXB2) measured using a TXB2 EIA kit. Bar graph shows the mean ± SE of at least four independent experiments. *p < 0.05 and **p < 0.01 vs. control.

**Fig. 7.** (A) The inhibitory effect of Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) on fibrinogen binding to integrin α_{IIb}β_{3}. Washed platelets (3 × 10^8/mL) were pre-incubated for 2 min at room temperature in the presence of 0.1 mM CaCl_2 and stimulated with collagen or ADP for 5 min and fibrinogen Alexa Fluor 488 (20 µg/mL), and then fixed with 0.5% paraformaldehyde at 4°C for 30 min. Representative FACS analysis results of four independent trials. (B) Bar graph summarizing the inhibitory effect of Fruitflow I and Fruitflow II on fibrinogen binding. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control.
and Fruitflow II on fibrinogen binding to integrin α_{IIb}β_{3} and the result showed that fibrinogen binding to integrin α_{IIb}β_{3} reduced by 45 ± 5% and 38 ± 2% respectively than the control group (Fig. 7).

**Arteriovenous shunt thrombosis model**

It is well established that the arteriovenous shunt thrombosis models have been used to evaluate *in vivo* antithrombotic effects [3, 26]. We therefore, investigated the effect of Fruitflow I and Fruitflow II on extracorporeal shunts model thrombus formation. As shown in Figure 8, Fruitflow I and Fruitflow II potently reduced the thrombus weight by 58 and 61% respectively.

**Fruitflow I and Fruitflow II attenuates agonist induced mitogen-activated protein kinase (MAPK) and PI3K/Akt phosphorylation**

It is well-known that the phosphorylation of MAPKs (ERK, JNK and P38-MAPK) mediate platelet activation pathway and PI3K/Akt signaling pathway is another critical step for platelet activation. The above findings propose that impaired α_{IIb}β_{3} conformational changes may be induced by pretreatment with Fruitflow I and Fruitflow II on exposure to its high affinity fibrinogen binding site formerly called as inside-out signaling, followed by the platelet agonist interactions. Inversely, these findings also suggests that the outside-in signaling (the next step in fibrinogen binding followed by post-ligand occupancy proceedings) leading to platelet shape change and spreading may be weakened by given treatment. Number of evidences are available indicating that platelets are constantly exposed to a variety of activating factors, including collagen, fibrinogen, ADP, vWF, thrombin and thromboxane and inhibitory factors such as endothelial-derived NO, prostacyclin (PGI_{2}) and ADPase [21, 27]. Thrombotic or bleeding disorders can be developed by impairment of this equilibrium. Thus, a durable equilibrium between two opposing processes of platelet activation and inhibition is thought to be critical for normal hemostasis. Our study suggests that pretreatment of activated

**Discussion**

Previous studies proposed that prothrombotic suppression of platelet activation can prevent prothrombotic state [25]. It slows down the progression of atherosclerosis and minimizes the risk of stroke and myocardial infarction, and side effects of prothrombotic regimes outweigh their benefits [19]. A previous study demonstrated that Tomato extract inhibited collagen and ADP induced platelet aggregation *ex vivo* [16]. However, the underlying mechanism is not completely understood.
Fig. 9. Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) attenuated the phosphorylation of mitogen-activated protein kinase (i.e. ERK1/2, JNK and p38) (A) and PI3K/Akt (B). Cell proteins were extracted after aggregation termination and proteins separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes which then probed to antibodies against total and phospho ERK1/2, JNK and p38, and PI3K/Akt. All immunoblots were carried out in at least four independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control.
platelets with Fruitflow I and Fruitflow II may contribute to the maintenance of this balance.

In the present study, observations indicate that Fruitflow I and Fruitflow II inhibits collagen and ADP-induced ERK1/2, JNK and p38-MAPK and potent inhibitory effect on PI3K/Akt signaling, indicating modulation of both pathways may be involved in tomato’s anti-platelet activity. Our study indicate that tomato have GPVI and P2Y12 Suppression potential, thus antagonism of these receptors may represents a novel therapeutic regime. PT and aPTT results did not show difference between normal and treatment group suggesting that Fruitflow I and Fruitflow II do not induce changes to the integrity of extrinsic and intrinsic cascade of coagulation system (data not shown).

In accordance with previous findings on platelet hemostasis and pathophysiology of coagulation cascades [21, 27] we conclude the ex vivo capability of Fruitflow I and Fruitflow II to inhibit agonist induced aggregation, TXA2 production, granule secretion, αIIbβ3 activation, [Ca2+] mobilization, via MAPK and PI3K/Akt phosphorylations and in-vivo thrombus formation inhibition without affecting coagulation time, which illustrates the potential use of given compound as a nominee to be considered as an effective ethnomedicinal antithrombotic agent.

WSTC (Fruitflow I) and DWSTC (Fruitflow II) are potent inhibitors of agonist induced ex vivo platelet aggregation and granule secretion. In addition, it also significantly inhibited in vivo thrombus formation, while it had no effect on coagulation. Our findings indicate that Fruitflow I and Fruitflow II inhibit collagen and ADP-stimulated platelet function through modulation of signaling downstream via MAPK pathway.

Acknowledgments

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