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Antiplatelet and Antithrombotic Activities of Korean Red Ginseng

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The antiplatelet and antithrombotic activities of Korean Red Ginseng (KRG) were examined on rat carotid artery thrombosis *in vivo*, and platelet aggregation *in vitro* and *ex vivo*. Administration of KRG to rats not only prevented carotid artery thrombosis *in vivo* in a dose-dependent manner, but also significantly inhibited ADP- and collagen-induced platelet aggregation *ex vivo*, while failed to prolong coagulation times such as activated partial thromboplastin time (APTT) and prothrombin time (PT), indicating the antithrombotic effect of KRG might be due to its antiplatelet aggregation rather than anticoagulation effect. In line with the above observations, KRG inhibited U46619-, arachidonic acid-, collagen- and thrombin-induced rabbit platelet aggregation *in vitro* in a concentration-dependent manner, with IC_{50} values of 620 ± 12, 823 ± 22, 722 ± 21 and 650 ± 14 μg/mL, respectively. Accordingly, KRG also inhibited various agonists-induced platelet serotonin secretions as it suppressed platelet aggregation. These results suggest that KRG has a potent antithrombotic effect *in vivo*, which may be due to antiplatelet rather than anticoagulation activity, and KRG intake may be beneficial to the individuals with high risks of thrombotic and cardiovascular diseases.

Key words: Korean Red Ginseng (KRG), Antithrombotic activity, Antiplatelet activity

INTRODUCTION

Platelets play an important role in both physiological haemostatic and pathological thrombotic processes. Once blood vessels become damaged, a diverse array of adhesive ligands (such as collagen and von Willebrand factor) and soluble agonists (such as ADP and thrombin) will be exposed or generated at the injury site, and stimulate platelets adhesion, activation and aggregation (Jackson *et al.*, 2003). Collagen supports the adhesion of platelets to the site of injury *via* membrane receptor glycoprotein (GP) VI and integrin α2β1 (Farndale *et al.*, 2004). The bindings of collagen to GPVI and α2β1 result in receptor clustering and thereby stimulate the downstream responses such as shape change, spreading, and the release or secretion of activating substances that activate and recruit platelets to the developing thrombus (Gruner *et al.*, 2004; Sarratt *et al.*, 2004, 2005).

Panax ginseng has been used for more than 2,000 years as a general tonic in traditional oriental medicine. White ginseng is air-dried, while red ginseng (RG) is produced by steaming and drying of raw ginseng. RG was reported to be pharmacologically more active than white ginseng. The different biological activities of red and white ginseng may result from production of different chemical constituents during steaming process. Ginseng saponins, referred to as ginsenosides, are believed to have a number of pharmacological roles. Several investigators have reported new ginsenosides from RG that are not usually found in raw ginseng (Baek *et al.*, 1996). In particular, Korean Red Ginseng (KRG, Ginseng Radix Rubra) has broad efficacious effects against hypertension, diabetes, nociception, and cancer, and it can improve weakness (Park *et al.*, 2005). With herb use increasing, interest in KRG's effect on cardiovascular risk factors has

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grown. It has been reported that KRG was able to normalize blood pressure, improve cholesterol, and lower blood glucose levels (Chen, 1982). Although inhibitory effects of several ginseng saponin fractions and ginsenosides (Rg1 and Rg3) on platelet aggregation have been reported (Cui *et al.*, 1999; Park *et al.*, 1995), the antithrombotic and antiplatelet activities of KRG remain unknown. In the present study, therefore, the inhibitory effects of KRG on artery thrombosis *in vivo* and platelet aggregation *in vitro* and *ex vivo* were investigated.

MATERIALS AND METHODS

Materials

Collagen, arachidonic acid, ADP and thrombin were purchased from Chrono-Log Co. (Havertown, PA, U.S.A.). U46619, bovine serum albumin (BSA), carboxymethyl cellulose (CMC), serotonin creatinine sulfate, *o*-phthalaldehyde (OPT) and imipramine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cephalin, thromboplastin, and bovine thrombin were purchased from Instrumentation Laboratory Co. (Milano, Italy).

Preparation of Korean Red Ginseng powder

KRG powder (Cheong-Kwan-Jang) for the present experiments was provided from Korea Ginseng Corporation (Daejeon, Korea). For animal experiment, KRG powder was directly dissolved in 1% carboxymethyl cellulose solution. However, for *in vitro* platelet aggregation assay, KRG powder was extracted three times with 10 volumes of distilled water at 85°C for 8 h. The aqueous extracts were combined, concentrated under vacuum at 40°C and lyophilized to a brownish powder. The content of ginsenosides in the KRG powder was determined as follows. In brief, KRG powder was extracted with 10 volumes of 80% methanol at 85°C for 1 h. The alcoholic extract was concentrated under vacuum at 40°C, dissolved in water, and then passed through SepPak C18 cartridge. Saponins were eluted with 90% methanol, and then HPLC analysis was performed. The total content of ginsenosides of KRG powder was 1.64%, and its compositions were 3.04 mg/g for Rg1, 2.03 mg/g for Re, 0.79 mg/g for Rf, 5.73 mg/g for Rb1, 1.79 mg/g for Rb2, 1.95 mg/g for Rc, 0.72 mg/g for Rd, and 0.34 mg/g for Rg3, respectively.

Animals

Male Sprague-Dawley rats were purchased from Dae-Han Biolink Co. (Eumsung, Korea), and acclimatized for 1 week at a temperature of 24 ± 1°C and humidity of 55 ± 5%. New Zealand white rabbits were purchased from Sam-Tako Animal Co. (Osan, Korea) and acclimatized for 1 week at 24°C and 55% humidity. The animals had free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Korea) and drinking water before experiments. The animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, Chungbuk National University, Korea.

Washed rabbit platelet preparation and platelet aggregation assay

Blood was withdrawn from the ear artery of male New Zealand white rabbits and collected directly into 0.15 (v/v) of anticoagulant citrate dextrose (ACD) solution that contained 0.8% citric acid, 2.2% trisodium citrate and 2% dextrose (w/v). Washed platelet was prepared as previously described (Jin *et al.*, 2005). Briefly, platelet rich plasma (PRP) was obtained by centrifugation of rabbit blood at 230 × *g* for 10 min. Platelets were sedimented by centrifugation of the PRP at 800 × *g* for 15 min and washed with Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, and 3.8 mM Hepes, pH 6.5) containing 0.35% BSA and 0.4 mM EGTA (ethylene glycol bis (β-aminoethyl ether) N,N,N'N'-tetraacetic acid). The washed platelets were resuspended in Hepes buffer (pH 7.4) and adjusted to 4×10^8 cells/mL.

Platelet aggregation was measured by using an aggregometer (Chrono-Log Co., Havertown, PA, U.S.A.) according to the turbidimetry method of Born (Born & Cross, 1963) as previously described (Jin *et al.*, 2005). Briefly, washed platelet suspension was incubated at 37°C in the aggregometer with stirring at 1,000 rpm, and then distilled water or various concentrations of KRG were added. After 3 min preincubation, platelet aggregation was induced by addition of collagen (5 μg/mL), arachidonic acid (100 μM), U46619 (1 μM), or thrombin (0.05 U/mL), respectively. The resulting aggregation measured as the change in light transmission was recorded for 8 min. The results were expressed as percentage of control.

In vitro serotonin secretion assay

Serotonin secretion was determined by the fluorimetric method of Holmsen and Dangelmaier (1989), with a little modification. In brief, to prevent the reuptake of secreted serotonin, imipramine (a serotonin re-uptake inhibitor, 5 μM) was added to platelet suspension. Washed rabbit platelets were treated with various concentrations of KRG or saline at 37°C for 3 min prior to addition of agonists (collagen 5 μg/mL, arachidonic acid 100 μM, U46619 1 μM, thrombin 0.05 U/mL) for 5 min. An aliquot (0.35 mL) of the washed rabbit platelet was mixed with 5 mM EDTA in ice, and centrifuged at 12,000 × *g* for 2 min. The supernatant was mixed with 6 M trichloroacetic acid (TCA) and centrifuged at 12,000 × *g* for 2 min. An aliquot (0.3 mL) of TCA supernatant was mixed with 1.2 mL of the solution (0.5% *o*-phthalaldehyde in ethanol diluted 1:10 with 8 N HCl), placed in a boiling water bath for 10 min, and then

cooled in ice. The excess TCA was extracted with chloroform and fluorophore was measured at the wavelength of excitation (360 nm) and emission (475 nm). Serotonin creatinine sulfate was used as a standard solution to calculate the extent of serotonin release.

Ex vivo platelet aggregation assay

Ex vivo antiplatelet aggregation activity was investigated as previously described (Jin *et al.*, 2004). Male Sprague-Dawley rats weighing 200-250 *g* were orally administered with KRG powder solution (1% CMC solution *via* gastric tube at doses of 500 and 1000 mg/kg body weight) for one week. Blood was collected 60 min after sample treatment. PRP was obtained by centrifuging the blood sample at 180 × g for 10 min, and platelet poor plasma (PPP) was obtained by centrifuging at 2,100 × g for 10 min continuously. PRP was adjusted to 3×10^8 platelets/mL with PPP. Platelet aggregation was induced by addition of ADP (final concentration, 5 μM) or collagen (final concentration, 3 μg/mL).

Ex vivo coagulation assay

The activated partial thromboplastin time (APTT) and prothrombin time (PT) were automatically measured by using Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory Co., Milano, Italy) as previously described (Jin *et al.*, 2004). Briefly, the PPP from KRG-administered rats was incubated at 37°C for 7 min. 100 μL of the incubated plasma was mixed with 50 μL of cephalin in the process plate, and the coagulation was started by addition of $CaCl_2$ (1 mM), 100 μL of thromboplastin and 100 μL of bovine thrombin into 100 μL of incubated plasma for APTT and PT assay, respectively.

In vivo antithrombotic activity assay

Male Sprague-Dawley rats (200-250 g) were orally administered with KRG powder solution (1% CMC solution *via* gastric tube at doses of 500 and 1000 mg/kg body weight) for one week. After rats were anaesthetized with pentobarbital sodium salt (60 mg/kg, i. p.), a segment of the right carotid artery was exposed and dissected free of the vagus nerve and surrounding tissues, and fitted on the Doppler flow probe (1 mm diameter). Blood flow was measured with a Doppler velocimeter (Crystal Biotech, U.S.A.). After 60 min of administration of KRG, thrombosis was induced by placing a 2 mm^2 Whatman No. 1 filter paper saturated with 50% $FeCl_3$ on the carotid artery near the probe for 10 min. The time needed for occlusion to occur was measured for up to 60 min, and occlusion time was assigned a value of 60 min for vessels that did not occlude with in 60 min.

Statistical analysis

The experimental results were expressed as the mean ± S.E.M. A one-way analysis of variance (ANOVA) was used for multiple comparison followed by Dunnett's test. The data were considered a significant difference with probability less than 0.05.

RESULTS

Effect of KRG on washed rabbit platelet aggregation *in vitro*

Platelet aggregation plays an important role in thrombus formation. Therefore, inhibition of platelet aggregation is an effective treatment for patients with cardiovascular diseases. As shown in Fig. 1, KRG inhibited U46619 (1 μM)-, arachidonic acid (100 μM)-, collagen (5 μg/mL)- and thrombin (0.05 U/mL)-induced washed rabbit platelet aggregation in a concentration-dependent manner, with IC_{50} values of 620 ± 12, 823 ± 22, 722 ± 21 and 650 ± 14 μg/mL, respectively. Considering that inhibitions of KRG against various agonists-induced platelet aggregations were almost in the same range, it seems that the antiplatelet mechanism of KRG may be resulted from interfering with a common pathway rather than inhibition of any receptor specifically.

Effect of KRG on serotonin secretion

Granule secretion occurs during platelet aggregation, and plays an important role in recruitment and amplification of platelet thrombus formation. Here we determined various agonists-induced serotonin secretion as a marker for platelet granule secretion. In line with the inhibition of platelet aggregation, KRG inhibited collagen-, arachidonic

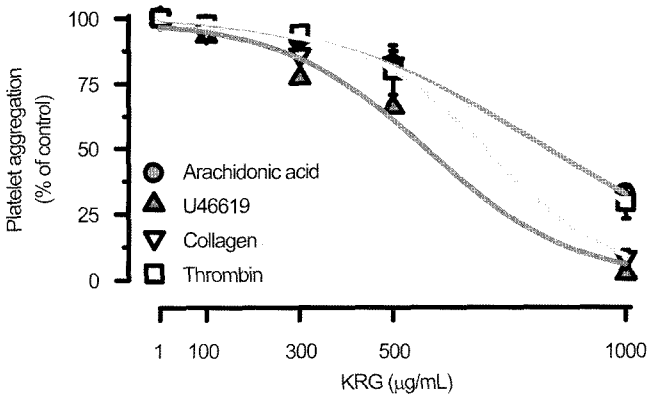

Fig. 1. Effect of KRG on washed rabbit platelet aggregation *in vitro*. Washed rabbit platelet suspension was incubated at 37°C in an aggregometer with stirring at 1,000 rpm, and then KRG was added. After preincubation for 3 min, the platelet aggregation was induced by addition of collagen (5 μg/mL), arachidonic acid (100 μM), U46619 (1 μM) and thrombin (0.05 U/mL), respectively. The aggregation percentages were expressed as percentage of maximum aggregation induced by respective inducers. Data are expressed as mean ± S.E.M. (n = 4).

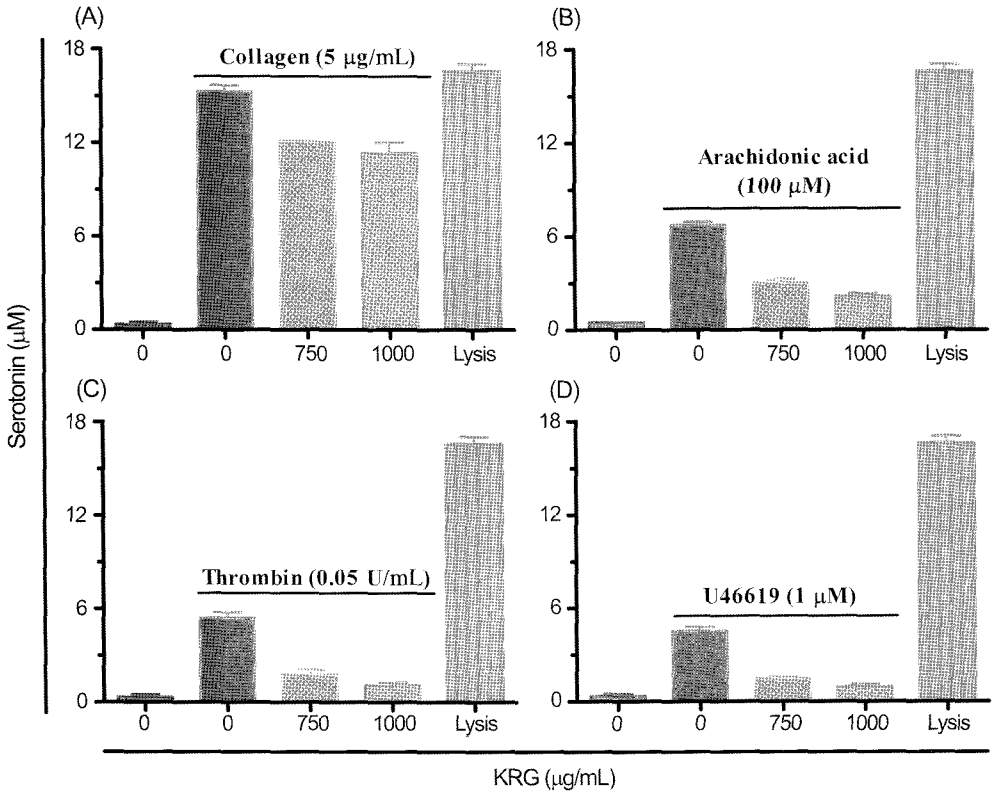

Fig. 2. Effect of KRG on serotonin secretion. Washed rabbit platelet suspension was incubated with imipramine (5 μM) and various concentrations of KRG at 37°C for 3 min prior to addition of collagen (A), arachidonic acid (B), thrombin (C) and U46619 (D). The reaction was incubated for 5 min and terminated by addition of 5 mM ice-cold EDTA. The reaction solution was obtained by centrifugation at 12,000 × g for 2 min. And then the serotonin concentration was determined by a fluorimetric method as described in Materials and Methods. Data are expressed as mean ± S.E.M. (n = 3).

acid-, U46619- and thrombin-induced serotonin secretions by 17, 83, 91 and 91% at the concentration of 750 μg/mL, and 18, 86, 92 and 93% at the concentration of 1000 μg/mL, respectively (Fig. 2).

Ex vivo antiplatelet effect of KRG

To get a view whether KRG was effective in inhibition of platelet aggregation *in vivo*, *ex vivo* platelet aggregation was determined after oral administration of KRG at doses of 500 and 1000 mg/kg body weight. As shown in Fig. 3, administration of KRG to rat at doses of 500 and 1000 mg/kg body weight significantly inhibited ADP- and collagen-platelet aggregation. The inhibition percentages were 19.2 and 23.1% at dose of 500 mg/kg, and 20.5 and 25.5% at dose of 1000 mg/kg for ADP- and collagen-stimulation, respectively.

Effect of KRG on coagulation times *ex vivo*

To clarify whether KRG affected coagulation system, the coagulation time was also examined *ex vivo* after oral administration of KRG at doses of 500 and 1000 mg/kg body weight. The platelet poor plasma was separated from rat administered with KRG, and the coagulation

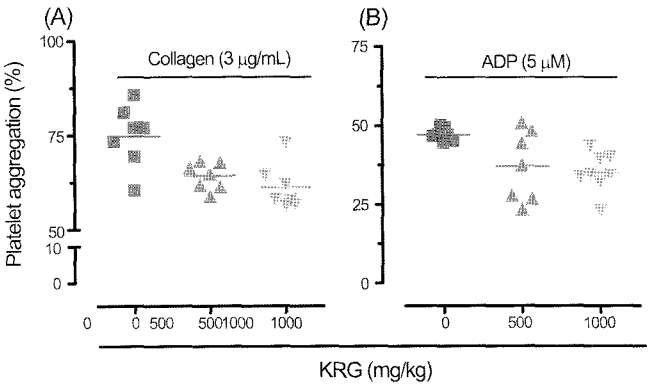

Fig. 3. *Ex vivo* antiplatelet effect of KRG. KRG was orally administered to rats at doses of 250 and 500 mg/kg body weight for 1 week. Blood was collected 60 min after last time sample treatment. Platelet rich plasma (PRP) was obtained by centrifuging the blood sample at 180 × g for 10 min and platelet poor plasma (PPP) was obtained by centrifuging the PRP at 2,100 × g for 10 min continuously. Platelet aggregation was induced by collagen (3 μg/mL) or ADP (5 μM). Data are expressed as mean values (n = 7-8).

times were assayed. As shown in Fig. 4, KRG has no effect on APTT and PT, indicating that KRG did not affect coagulation system.

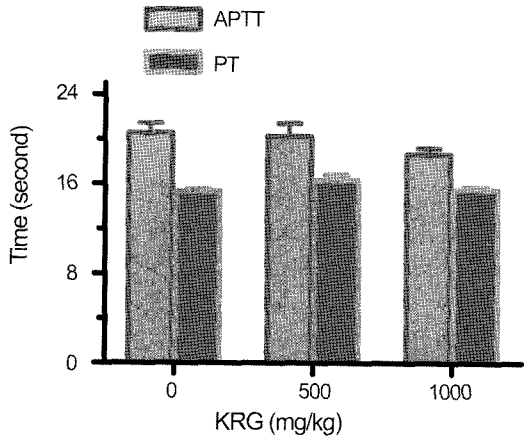

Fig. 4. Effect of KRG on coagulation times *ex vivo*. Blood was collected 60 min after last time sample treatment. Platelet poor plasma (PPP) was obtained by centrifuging the PRP at 2,100 × g for 10 min continuously. Activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured as described in Materials and methods. Data are expressed as mean ± S.E.M. (n = 5).

In vivo antithrombotic effect of KRG

To investigate the relevance of KRG to pathologic and occlusive thrombus formation *in vivo*, rat carotid artery injury was induced by $FeCl_3$. After $FeCl_3$ application, the injured vessel in control group was occluded within 15 min. After oral administration of KRG to rats, the occluded times in KRG-treated groups were significantly prolonged to 33 min ($p < 0.01$, n = 8) at the dose of 500 mg/kg body weight and 51 min ($p < 0.01$, n = 8) at the dose of 1000 mg/kg body weight (Fig. 5).

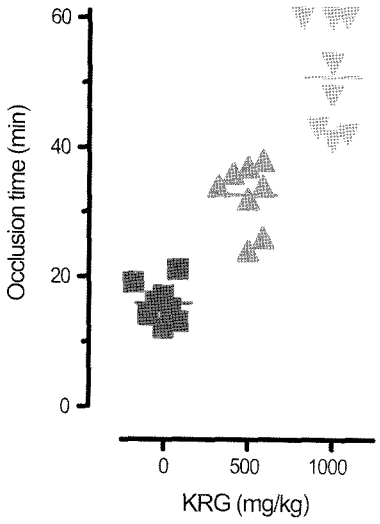

Fig. 5. *In vivo* antithrombotic effect of KRG. After oral administration of KRG for 1 week, the carotid artery was subjected to chemical injury by 50% $FeCl_3$. Blood flow was measured with a Doppler velocimeter. After 60 min of administration of KRG, thrombosis was induced by placing a 2 mm^2 Whatman No. 1 filter paper saturated with 50% $FeCl_3$ on the carotid artery near the probe for 10 min. The results are expressed as occlusion time (n = 8).

DISCUSSION

The results of the present study demonstrated that KRG had a potent protective effect on carotid artery thrombosis *in vivo* in rat and strong inhibition on platelet aggregation *in vitro* and *ex vivo*, whereas it had no effect on the coagulation times such as PT and APTT. These results indicate that KRG intake has a potential to prevent thrombotic and cardiovascular diseases, in which platelet aggregation is very important.

Platelet aggregation is a complex process; it is generally considered that platelet activation is mainly via three pathways (Blockmans *et al.*, 1995). The first is related to the release of ADP; the second involves the cyclooxygenase-mediated formation of thromboxane A_2; the last involves platelet activation factor. ADP and arachidonic acid can activate the first two pathways and then result in platelet aggregation, whereas collagen and thrombin activate all three pathways (Cowan, 1981; Cowan *et al.*, 1981; Woulfe, 2005). In the present in vitro platelet aggregation assay, KRG inhibited washed rabbit platelet aggregation induced by collagen, arachidonic acid, U46619 and thrombin in a concentration-dependent manner. Accordingly the serotonin secretion, which was a marker for platelet granule secretion, induced by various agonists was also inhibited in the same pattern. The antiplatelet mechanism of KRG remains unknown. However, judging from the aggregation results, the IC_{50} values of KRG against various agonist-induced platelet aggregations are in the same concentration range, indicating that KRG may interfere with a common step in signal transduction of platelet aggregation, which may be involved in the modulation of cytosolic calcium mobilization, second messenger transduction or thromboxane formation. Further study of the antiplatelet mechanism of KRG is in progress.

The antiplatelet effect of KRG was also confirmed by *ex vivo* platelet aggregation assay in rats. As shown in Fig. 3, after oral administration of KRG at dosages of 500 and 1000 mg/kg body weight for 1 week, a dose-dependent inhibition of the rat platelet aggregation induced by collagen and ADP were observed. On the other hand, administration of KRG had no effect on plasma coagulation times, indicating that KRG did not affect coagulation system.

The rat carotid artery thrombosis model was also used to examine the *in vivo* antithrombotic effect of KRG. In this model, ferric chloride induces an oxidative injury exposing the subendothelial matrix (Furie and Furie, 2005). Platelet interacts with the matrix via GPIb-V-IX and $\alpha IIb\beta 3$ on the platelet membrane, and collagen and von Willebrand factor in the matrix. Glycoprotein VI binding to collagen was required for platelet activation, and activated platelets underwent to calcium mobilization and the release of ADP and thromboxane A_2 to accelerate platelet recruitment

and activation, and thrombus formation (Furie & Furie, 2005). As shown in Fig. 5, administration of KRG increased the occlusion time in a dose-dependent manner, which indirectly indicated that KRG inhibited thrombus formation *in vivo* and was correlated well with the *in vitro* and *ex vivo* platelet aggregation results. The *in vivo* antithrombotic and *ex vivo* antiplatelet effects of KRG may be also related to the increase of nitric oxide level. It has been reported that saponin fraction of KRG enhances formation of citrulline from exogenously added arginine, which activates nitric oxide synthase, and ginsenosides purified from KRG enhanced nitric oxide release from endothelial cells of rat aorta (Gillis, 1997).

In conclusion, our present results indicate that KRG shows a potent protective effect on artery thrombosis *in vivo*, which may be due to antiplatelet rather than anti-coagulation activity, and KRG intake may be beneficial for the individuals with high risks of thrombotic and cardiovascular diseases.

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