Research Article

DK-MGAR101, an extract of adventitious roots of mountain ginseng, improves blood circulation by inhibiting endothelial cell injury, platelet aggregation, and thrombus formation

Hye Rim Seonga, 1, Cuicui Wang a, 1, Muhammad Irfanc, Young Eun Kimc, Gooyoung Jungc, Sung Kyeong Parkd, Taemyoung Kima, Ehn-Kyoung Choia, Man Hee Rhee b, Yun-Bae Kim a, *

a College of Veterinary Medicine, Chungbuk National University, Cheongju, Republic of Korea
b College of Veterinary Medicine, Kyungpook National University, Daegu, Republic of Korea
c Pharmaceutical Technology Institute, Dongkook Pharmaceutical Co., Ltd., Jincheon, Republic of Korea
d Department of Beauty Care, Daejeon Health Institute of Technology, Daejeon, Republic of Korea

Abstract

Background: Since ginsenosides exert an anti-thrombotic activity, blood flow-improving effects of DK-MGAR101, an extract of mountain ginseng adventitious roots (MGAR) containing various ginsenosides, were investigated in comparison with an extract of Korean Red Ginseng (ERG).

Methods: In Sprague-Dawley rats orally administered with DK-MGAR101 or ERG, oxidative carotid arterial thrombosis was induced with FeCl3 (35%), and their blood flow and occlusion time were measured. To elucidate underlying mechanisms, the cytoprotective activities on rat aortic endothelial cells (RAOECs) exposed to hydrogen peroxide (H2O2) were confirmed. In addition, the inhibitory activities of DK-MGAR101 and ERG on agonist-induced platelet aggregation, thromboxane B2 production, and ATP granule release from stimulated platelets as well as blood coagulation were analyzed.

Results: DK-MGAR101 containing high concentrations of Rb1, Rg1, Rg3, Rg5, and Rk1 ginsenosides (55.07 mg/g) was more effective than ERG (ginsenosides 8.45 mg/g) in protecting RAOECs against H2O2 cytotoxicity. DK-MGAR101 was superior to ERG not only in suppressing platelet aggregation, thromboxane B2 production, and granule release, but also in delaying blood coagulation, FeCl3-induced arterial occlusion, and thrombus formation.

Conclusions: The results indicate that DK-MGAR101 prevents blood vessel occlusion by suppressing platelet aggregation, thrombosis, and blood coagulation, in addition to endothelial cell injury.

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1. Introduction

It is well known that vascular endothelial injury is one of the initial triggering factors of atherosclerosis and thrombosis, resulting in cardiovascular and cerebrovascular diseases (CVDs) [1]. Following arterial endothelial injury, blood-coagulating factors such as collagen, adenosine diphosphate (ADP), thrombin, and von Willebrand Factors are generated. These factors induce adhesion to the damaged intima, activation, and aggregation of platelets [2]. Eventually, aggregation of the stimulated platelets plays a central role for the thrombosis, blocking blood flow [3].

Activated platelets release ADP, thromboxane A2 (TXA2), and ATP from their alpha and dense granules for adherence to the injured arterial intima and for further autocrine activation [4]. As a strong inducer of platelet aggregation and vasoconstriction, TXA2 is produced during vascular oxidative stress process, but rapidly converted to thromboxane B2 (TXB2) [5–7].

Aspirin is widely prescribed for blood flow improvement to prevent CVDs, based on its anti-thrombotic activity [8]. However, aspirin can cause gastro-intestinal adverse effects, and exacerbate respiratory and cutaneous diseases such as asthma and urticarial [9,10]. Thrombolytic tissue-type plasminogen activator, prescribed
for the emergency treatment of acute stroke, has a potential hae-
morrhagic adverse effect, too [11].

Panax ginseng Meyer may be the most-widely used herbal
products for the improvement of immune function, cognitive
function, blood flow, tumour prevention, and stamina in the world
[12–14]. The effectiveness of ginsenosides covers improvement of
circulatory problems such as ischemia [15], hypertension [16], and
metabolic syndrome [17], based on their anti-oxidant and anti-
fibrotic activities [18].

Wild mountain ginseng, naturally growing in shady forests in
Asia continent, has been believed to be superior to cultivated
ginseng in the active ingredients and pharmacological activities
[19,20]. Notably, investigators have established tissue culture
techniques of mountain ginseng using bioreactors [21,22], obtain-
ing massive MGAR. Furthermore, high amounts of ginsenosides can be
obtained via such tissue culture process of mountain ginseng,
especially treating with methyl jasmonate [21,23]. Indeed, MGAR
effects of DK-MGAR101 and ERG against the cytotoxicity of H2O2
by collagen (0.625

m

104 cells/well) and then freeze-
/collagen (0.625

m

104 cells/well) or ADP (2.5

m

M) for 24 h. After
washing twice and adding 50 mL MTT (stock solution 5 mg/mL,
filtered), the cells were incubated for 4 h more [14]. The culture
medium was removed, and formazan blue produced by surviving
cells was extracted with 150 mL DMSO. The optical density of for-
mazan was read at 570 nm using an ELISA reader.

2.3. Animals

Sprague-Dawley rats (male, 240–260 g) were purchased from
Onti-Stio (Seongnam, Korea). The rats were housed in a specific
pathogen-free room with controlled environment [temperature: 22 ± 2
°C, relative humidity: 40–70%, 12-h light-dark cycle (150–300 lux), ventilation: 12 times/h]. Filtered clean water and
pellet chow were available ad libitum. The animal experiment
protocols were approved by the CBNU Institutional Animal Care
and Use Committee (Document No, CBNUA-1096-18-02).

2.4. Measurement of platelet aggregation

2.4.1. In vitro platelet aggregation assay

Blood sample was obtained from the rats into a syringe con-
taining citrate coagulant. Platelet-rich plasma (PRP) was prepared
through centrifugation-washing procedures, and washed platelets
were adjusted to 3 × 108 platelets/mL [25–27].

Aggregation of platelets was assessed with a turbidimetric
method [28]. Platelet suspension with 1 mM CaCl2 was pre-
incubated with the test materials (DK-MGAR101, ERG or aspirin;
125–500 μg/mL) in an aggregometer (Chrono-Log, Harbertown,
USA) under stirring at 37 °C. Following pre-incubation for 3 min,
aggregation of the platelets was measured for 8 min after addition
of collagen (0.625 μg/mL) or ADP (2.5 μM).

2.4.2. Ex vivo platelet aggregation assay

Rats (n = 6/group) were treated with DK-MGAR101, ERG (250
or 500 mg/kg) or aspirin (30 mg/kg) for 7 d. Forty min after the last
day treatment, blood sample was collected. After preparation of
PRP, aggregation of platelets was measured by inducing with collagen
(0.625 μg/mL) or ADP (2.5 μM) as described above.

2.5. Measurement of TXB2 and ATP release

2.5.1. In vitro TXB2 release assay

TXB2 released from stimulated platelets was measured using an
ELISA kit (Dupont, Hayward, USA). Platelets suspension (4 × 108
cells/mL) was pre-incubated with DK-MGAR101 or ERG (125–500 μg/mL) for 3 min, and aggregation was induced by
collagen (0.625 μg/mL) or ADP (2.5 μM) described above. The re-
action was stopped with ice-cold 2.5 mM EDTA and 2.5 mM indo-
methacin. After centrifugation at 1,200 rpm for 5 min, TXB2 was
analyzed [27–29].

2.5.2. Ex vivo TXB2 and ATP release assays

Rats (n = 6/group) were administered DK-MGAR101, ERG (250
or 500 mg/kg) or aspirin (30 mg/kg) for 7 d. Forty min after the last
administration, blood was withdrawn. After collection of PRP, TXB2
was analyzed via ELISA after induction with collagen (0.625 μg/mL)
or ADP (2.5 μM) as described above.

The supernatant after centrifugation of the platelets aggregated
by collagen (0.625 μg/mL) or ADP (2.5 μM) was obtained. ATP in
dense granules released from platelets was quantified with a
commercial kit (Biomedical Research & Service Center, Redding,
USA) using GloMax 20/20 luminometer (Promega, Madison, USA)
[29].
2.6. Measurement of blood flow

Rats (n = 8/group) were orally given DK-MGAR101, ERG (125–500 mg/kg) or aspirin (30 mg/kg) for 3 wk. Forty min after the last administration, they were anaesthetized with urethane, and body temperature (36–37 °C) was maintained on a heating pad. After exposure of the right carotid artery, blood flow was recorded using a laser Doppler probe (AD Instruments, Colorado Springs, USA). One h after the last treatment, the artery was wrapped with a FeCl3 (35%)-immersed filter paper (3 mm wide), near the laser probe, for 5 min to induce thrombosis [27–29]. During monitoring the blood stream for 40 min, the occlusion time was recorded. After sacrifice, and the arteries including FeCl3-exposed area were cut for examination of thrombus in the artery.

2.7. Measurement of blood coagulation

After 40-min monitoring of blood flow, blood was collected into a tube containing citrate anti-coagulant. After centrifugation (2500 rpm, 15 min), the supernatant (platelet-poor plasma) was measured for coagulation times [prothrombin time (PT) and activated partial thromboplastin time (aPTT)] with Vetscan VSPRO (Abaxis Inc, Union City, USA) [30].

2.8. Thrombus weight and histopathology

After blood collection, thrombus regions of the arteries were cut at 1-mm intervals, and analyzed for entire length and weight of the thrombi.

For microscopic observation, the damaged arteries were fixed in a buffered-formalin. Paraffin-embedded slides were prepared, and stained with hematoxylin and eosin [27,28]. The proportions of platelet plug and blood clot in cross-sectioned vessels were analyzed using digital Image Inside analyzer (Focus, Seoul, Korea).

2.9. Statistical analysis

Data are presented as mean ± SE. Statistical analysis was performed with SPSS version 18.0 program (SPSS Inc, Chicago, USA). Differences among groups were analyzed with one-way ANOVA, followed by Dunnett’s multiple-range test and Fisher’s LSD test, at a level of P-value < 0.05.

3. Results

The total concentration of ginsenosides including Rb1, Rg1, Rg3, Rg5, and Rk1 was much higher in DK-MGAR101 (55.07 mg/g) than in ERG (8.45 mg/g) [Supplementary Fig. 1; Supplementary Table 1].

Exposure to H2O2 (60 μM) for 24 h decreased the survival rate of RAOECs to 43.2%. However, DK-MGAR101 protected against the H2O2 cytotoxicity: i.e., it significantly recovered the survival rate to 76.1–96.2% at 50–200 μg/mL (Fig. 1). By comparison, ERG exerted significant cytoprotective activity at only 100 and 200 μg/mL, increasing the survival rate to 70.2 and 74.8%, respectively.

DK-MGAR101 and ERG inhibited in vitro all the collagen (0.625 μg/mL)- and ADP (2.5 μM)-induced aggregation of platelets (Fig. 2A). Notably, DK-MGAR101 with higher concentration of ginsenosides was superior to ERG at all concentrations tested (125–500 μg/mL).

Such platelet aggregation-inhibitory effect was also achieved ex vivo: i.e., 7-d oral administration of DK-MGAR101 and ERG (250–500 mg/kg) strongly inhibited the platelet aggregation (Fig. 2B). DK-MGAR101 was more effective than ERG, showing a comparable activity at 500 mg/kg with aspirin (30 mg/kg).

DK-MGAR101 and ERG inhibited in vitro both the collagen- and ADP-induced thromboxane production, in which DK-MGAR101 was
superior to ERG (Fig. 3A). Also, DK-MGAR101, ERG, and aspirin decreased the thromboxane production ex vivo (n = 6/group) (Fig. 3B).

In addition, ATP release from the stimulated platelets was inhibited by the 3 compounds (DK-MGAR101, ERG, and aspirin), indicative of the role of granular secretion for further activation and aggregation of platelets (Fig. 3C).

Following exposure to 35% FeCl₃ of the surface of arterial wall, the blood flow gradually decreased and near-fully ceased in 30 min (Fig. 4A). However, 3-wk oral treatment with DK-MGAR101 or ERG preserved the blood flow: i.e., the blood flow was preserved over 40 min in the animals given DK-MGAR101 or ERG (≥250 mg/kg), as observed in aspirin-treated rats (30 mg/kg). The arterial occlusion time in control animals was 26.0 min, calculated from the practical cessation time point when the blood steam velocity decreased to 10% of initial flow rate (Fig. 4B). DK-MGAR101 and ERG markedly delayed the occlusion time to 30.3±6.2 min and 29.6±4.9 min at 125–500 mg/kg, respectively. Expectedly, aspirin (30 mg/kg) also exhibited blood flow-improving activity, delaying mean occlusion time to 76.6 min.

Three-wk oral administration of DK-MGAR101 or ERG (125–500 mg/kg) did not remarkably delayed PT in the blood exposed to FeCl₃. However, both DK-MGAR101 (≥250 mg/kg) and ERG (375 mg/kg) significantly prolonged aPTT as shown in aspirin (30 mg/kg) (Supplementary Fig. 2).

Forty min after FeCl₃ application, the mean weight of thrombi produced was 0.99 ± 0.08 mg/mm in control group (Table 1). Treatment with DK-MGAR101 (375–500 mg/kg) significantly decreased the thrombus weight to 0.65±0.77 mg/mm. Such decreased thrombus formation was also observed in the rats treated with ERG (≥250 mg/kg) or aspirin (30 mg/kg).

As observed 40 min after application of FeCl₃, the arteries were near-fully plugged with thrombi in control animals (Fig. 5A),
consisting of 79.1% blood clot and 20.9% platelet plug (Fig. 5B). However, following administration of DK-MGAR101, ERG or aspirin, the thrombi were relatively loose and showed larger portions of permeable platelet plugs compared to the flow-blocking blood clots. Aspirin (30 mg/kg) enhanced the portion of platelet plugs to 48.5%. In comparison, DK-MGAR101 (125–500 mg/kg) increased the platelet plug ratio to 29.6–48.2%, and ERG (125–500 mg/kg) expanded to 22.3–41.0%, too. Notably, DK-MGAR101 was superior to ERG at all dose ranges.

4. Discussion

We investigated whether DK-MGAR101 containing ginsenosides Rb1, Rg1, Rg3, Rg5, and Rk1 higher than ERG has per-oral anti-thrombotic activity, since ERG is well-known blood flow enhancer [30]. Indeed, it was demonstrated that ginsenosides Rg3 and Rk1 inhibited platelet aggregation by reducing ATP-containing granule release, thromboxane production, and integrin αIIbβ3 activation [30,31]. In addition, geometric isomers Rk1/Rg5 were found to exert diverse inhibitory activities against platelet aggregation, oxidative response, and inflammation [32]. Especially, they increased the expression of vascular endothelial nitric oxide synthase (eNOS) related to vasodilatation [33]. Therefore, we

| Treatment     | Dose (mg/kg) | Thrombus (mg/mm) |
|---------------|--------------|------------------|
| FeCl3 alone   | 0            | 0.99 ± 0.08      |
| DK-MGAR101    | 125          | 0.87 ± 0.06      |
|               | 250          | 0.94 ± 0.04      |
|               | 375          | 0.65 ± 0.10*     |
|               | 500          | 0.77 ± 0.05*     |
| ERG           | 125          | 0.83 ± 0.08      |
|               | 250          | 0.74 ± 0.05*     |
|               | 375          | 0.67 ± 0.04*     |
|               | 500          | 0.73 ± 0.11*     |
| Aspirin       | 30           | 0.78 ± 0.06*     |

DK-MGAR101, an extract of Korean Red Ginseng (ERG) or aspirin (30 mg/kg) were orally administered for 3 weeks prior to FeCl3 exposure. *P < 0.05 from vehicle (FeCl3 alone) control.

Table 1

Thrombus weight in the carotid arteries after application of FeCl3 outside the arterial wall.

Fig. 5. Representative findings of arterial thrombi (A) and the ratios of platelet plug (gray) to blood clot (black) in the thrombi (B) produced by FeCl3 application. DK-MGAR101, an extract of Korean Red Ginseng (ERG) or aspirin (30 mg/kg) was orally treated for 3 wk prior to FeCl3 challenge. ★: blood clot (reddish), ★&: platelet plug (gray).
attempted to find out that DK-MGAR101 might be effective for improvement of blood circulation via in vitro and in vivo assays: i.e., oxidative endothelial cell injury, platelet aggregation, and FeCl3-induced arterial thrombosis.

Vascular endothelial injury is one of the major triggering factors of atherosclerosis and CVDs [1]. In our study, DK-MGAR101 exerted a substantial protective activity against H2O2-induced oxidative cytotoxicity in RAOECs, in which DK-MGAR101 containing higher RB1 (18.15 mg/g) was more effective than ERG (2.01 mg/g), as inferred from that ginsenoside RB1 reduced H2O2-induced dysfunction and aging of human umbilical vein endothelial cells (HUVECs) [34].

In addition, DK-MGAR101 and ERG delayed both the collagen- and ADP-induced platelet activation and thromboxane production, wherein DK-MGAR101 was more potent than ERG. Interestingly, ADP-induced platelet aggregation and related TXA2 production in vitro were more sensitive to DK-MGAR101 and ERG than collagen-triggered aggregation. However, DK-MGAR101, ERG as well as aspirin were more effective on platelet responses to collagen than ADP ex vivo. The in vivo platelet aggregation is a complex process taking place mainly through 3 steps [35]. Base on the complex cascades of platelet aggregation, it is believed that the high sensitivity to ADP may be due to an inhibition by ginsenosides on the direct interaction of soluble factors with surface receptor in vitro. By comparison, the ginsenosides and aspirin may predominantly control the early stage of collagen binding to platelets, their activation, and ATP release in vivo, in addition to their anti-inflammatory and anti-oxidant activities on the damaged endothelial cells [32,34].

Following vascular wall injury, collagen fibers exposed to circulating platelets play a crucial role in hemostasis by producing a physical barrier for blocking blood loss [35–37]. Collagen also activates platelets, and recruits more platelets to the lesions, and consolidates the thrombus [36]. Activated platelets change their shape, secrete granules, and finally, aggregate, which are triggered by collagen-induced an increase in [Ca2+]i [38]. As reported for ginsenosides [30,39], the inhibitory activity of DK-MGAR101 for the collagen-induced aggregation of platelets may be due to suppressing Ca2+ influx by decreasing thromboxane production. Actually, collagen-mediated increase in platelet [Ca2+]i was blocked by controlling thromboxane formation with cyclooxygenase inhibitors including aspirin [40–42]. Similarly, it was confirmed that the anti-platelet aggregation effect of ginsenosides including Rk1 was in part due to the reduced release of thromboxane [30,31].

In a previous report, a ginseng extract did not affect blood coagulation times such as PT and aPTT [30]. However, DK-MGAR101 and ERG delayed the aPTT, but not PT. Interestingly, aspirin prolonged only the aPTT, too. Such a discrepancy may be due to the exposure of the blood to FeCl3 in our study: i.e., after oxidative stress of blood cells, the aPTT might be changed and affected by anti-oxidant and anti-inflammatory ginsenosides and aspirin. It is believed that delayed blood coagulation should influence the blood (fibrin) clot formation, and eventually improvement of blood flow.

Here, we evaluated the anti-thrombotic potentials of DK-MGAR101 and ERG in a FeCl3-triggered arterial occlusion model, leading to oxidative vascular injury and occlusive thrombosis [28]. As shown in Fig. 4, oral treatment of DK-MGAR101 and ERG markedly delayed the occlusion time. Notably, anti-inflammatory and anti-oxidant activities of DK-MGAR101 rich in Rg1, Rk1, and Rg5 as well as aspirin might additionally contributed to the higher effectiveness than ERG [32,34].

In the FeCl3-mediated thrombus formation model, the thrombi composed of platelets, fibrin, and entrapped RBCs. In humans, such type of thrombus is observed in the coronary arteries after heart attack, leading to fatal myocardial infarction [43]. In our observation, the thrombi were divided into loose (gray) platelet plugs and compact (reddish) blood (fibrin) clots. Interestingly, DK-MGAR101, ERG, and aspirin lowered the ratio of impermeable blood clots, increasing the permeable platelet plugs, as implied from the delayed aPTT. Since fibrin polymers and clots are produced from fibrinogen by an enzyme thrombin, such effects of blood flow-improving compounds on the ratio of platelet plug/fibrin clot suggests that they have a potential to alter the composition and structure of fibrin meshwork. As demonstrated ex vivo platelet aggregation assay and in vivo thrombus analysis, ginsenosides and aspirin may interfere with the early step of collagen-induced hemostasis. Therefore, it is believed that through interruption of initial hemostasis, relatively-loose platelet plugs remained, so decreasing compact blood clot generation, and thereby letting the blood vessels obstruct slowly.

During processing of ginseng products, drying, steaming, puffing, and extraction conditions result in different amounts of ginsenosides [44]. It is of interest to note that addition of methyl jasmonate to tissue medium facilitate the accumulation of original and newly-generated ginsenosides [21]. Methyl jasmonate stimulated the ginsenoside biosynthetic pathway, and thereby enhanced the ginsenoside contents in adventitious roots [45,46]. Among ginsenosides, Rg3 is the most potent in blocking aggregation of platelets by decreasing Ca2+-mediated thromboxane formation and granule secretion [29,47]. Actually, Rg3 was rich in DK-MGAR101 (4.46 mg/g), by comparison in ERG (1.07 mg/g). More importantly, the contents of novel Rk1/Rg5, possessing anti-platelet aggregation, vaso-dilating, anti-oxidant, and anti-inflammatory activities [28–30], were much higher in DK-MGAR101 (31.24 mg/g) than in ERG (4.96 mg/g). It was expected that such high contents of Rg3, Rg5, and Rk1 in DK-MGAR101 would display a high blood flow-improving activity. Although there is a limitation to explain exact action mechanisms of the extracts DK-MGAR101 and ERG containing additional functional and non-functional ingredients.

Here, we investigated whether an extract of MGAR improves blood flow, since an extract of Korean Red Ginseng was approved as a functional food for blood flow enhancer by the Ministry of Food and Drug Safety, Korea. DK-MGAR101 containing high concentrations of Rb1, Rg1, Rg3, Rg5, and Rk1 improved blood circulation through inhibition of platelet aggregation triggered by innate inducers as well as thrombosis induced by oxidative endothelial damage. Therefore, DK-MGAR101 could be a promising natural candidate for the prevention and improvement of vascular circulation and CVDs.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2022.01.001.

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