

Korea red ginseng restores impaired endothelial  
function in aged mice through inhibition of  
arginase activity

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# Korea red ginseng restores impaired endothelial function in aged mice through inhibition of arginase activity

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## ABSTRACT

### **Korea red ginseng restores impaired endothelial function in aged mice through inhibition of arginase activity**

Korean red ginseng water extract (KRGE) has reported beneficial effects on the cardiovascular system. Cardiovascular disease is the leading cause of morbidity and mortality and the incidence of cardiovascular disease is predicted to increase as the population ages. There is accumulating evidence that arginase upregulation is associated with impaired endothelial function in aged vasculature. Here, we demonstrate that oral administration of KRGE to aged mice inhibits increased arginase activity, restores NO generation, and reduces ROS production via enhancement of endothelial nitric oxide synthase coupling. In vascular tension assay, attenuated vasorelaxation responses to acetylcholine and reduced vasoconstriction responses to phenylephrine in aged vessels were significantly improved following administering KRGE. Furthermore, KRGE showed a preventative effect on formation of peroxynitrite in plasma of aged mice. Taken together, these results suggest that KRGE may exert vasoprotective effects through augmentation of NO signaling by inhibiting arginase activity. Therefore, KRGE may be useful in the treatment of vascular diseases associated with aging.

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Keywords: *Panax ginseng*, Korean red ginseng extract, Aging, Arginase, Endothelial Nitric Oxide Synthase, Nitric Oxide, Vasorelaxation

# Korea red ginseng restores impaired endothelial function in aged mice through inhibition of arginase activity

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## I. INTRODUCTION

Cardiovascular diseases are the leading cause of morbidity and mortality in both industrialized and developing countries. The occurrence of cardiovascular disease is predicted to increase as population ages, although effective treatments for several established cardiovascular risk factors, such as hypertension and hypercholesterolemia. The hallmark of the aging cardiovascular system is decrease in nitric oxide (NO) bioavailability<sup>1, 2</sup> and increase in reactive oxygen species (ROS) production<sup>3, 4</sup>. Superoxide ( $O_2^-$ ) is a free radical that rapidly scavenges NO, thereby decreasing NO bioavailability. NO and  $O_2^-$  may react to produce peroxynitrite ( $ONOO^-$ ), a highly damaging ROS molecule. Thus, simultaneous generation of NO and  $O_2^-$  can raise  $ONOO^-$  to levels potentially detrimental to vascular cell function and viability<sup>5</sup>. This nitroso-redox imbalance contributes to age-related endothelial dysfunction and vascular stiffness<sup>6</sup>.

Korean red ginseng water extract (KRGE) has been extensively studied and its consumption progressively increased. Ginseng has shown beneficial effects to various diseases, including thrombosis, hyperlipidemia, cancer, and atherosclerosis <sup>7-11</sup>. In vasculature, it is well documented that KRGE has vasoprotective effects by eliminating superoxide derived from NADPH oxidase <sup>12</sup>, promoting endothelial cells proliferation and protecting from H<sub>2</sub>O<sub>2</sub>-dependent cell death <sup>8, 13</sup>, and inducing heme oxygenase-1 expression <sup>14</sup>. Ginseng extract exerts a direct vasodilatory effect by releasing NO in endothelium-dependent manner <sup>15</sup>. Furthermore, the beneficial effect of ginseng on vascular system may be dependent on activation of phosphoinositide 3-kinase (PI3K)/Akt signal transduction in endothelial cells <sup>16</sup>.

Vascular changes associated with aging have been investigated in humans and a number of other species <sup>17</sup>. However, the relative contributions of dysregulated mechanisms to age-related vascular pathology remain to be elucidated, because the contribution of vascular control mechanisms in health, aging, and disease conditions is influenced by vessel type and size <sup>17</sup>. In previous study, we demonstrated that KRGE inhibited arginase activity and reciprocally regulates NO production and enhanced NO-dependent vasorelaxation in wild type young mice <sup>18</sup>. Therefore, we, here, investigated which mechanisms contribute to age-related endothelial dysfunction in mice and determined whether orally administered-KRGE improves impaired endothelial function in aged mice.



## II. MATERIALS AND METHODS

### 1. Materials

KRGE (solid extract 64%, ginsenoside Rg1+Rb1 4 mg/g) was obtained from Korea Ginseng Corporation (chuncheon, Korea) and directly dissolved in distilled water. MnTBAP(Mn(III) Tetra(4-benzoic acid) porphyrin chloride) and L-NAME(N<sup>G</sup>-nitro-L-arginine methyl ester) were obtained from Calbiochem. All reagents were purchased from Sigma unless otherwise stated.

### 2. Animal protocol

Young (10±3 weeks) and aged (55±5 weeks) mice (C57BL/6J) were used for all experiments. Mice were housed at 23°C under a 12-h light/12-h dark cycle. The dark period was from 10:00 to 22:00. All animals had access *ad libitum* to water and food (Nara Biotech.). The study protocols were in accordance with the Guide for the Care and Use of Laboratory Animals (Institutional Review Board, Kangwon National University). KRGE was orally administered for 4 weeks. Given that each mouse consumed approximately 10 ml water/day, this represented a daily dose of ~20 mg/mouse/day of KRGE.

### 3. Arginase activity assay

Tissue lysates were prepared using lysis buffer (50 mM Tris-HCl, pH7.5, 0.1 mM EDTA and protease inhibitors) by homogenization at 4°C followed by centrifugation for 20 min at 14,000 x g at 4°C. The supernatants were used to assay for arginase activity as previously described<sup>19</sup>.

### 4. Western blotting analysis

Aortic vessels from C57BL/6 mice were homogenized in the buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 µg/ml of leupeptin, 1 µg/ml of pepstatin, 1 µg/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 mM NaF) and centrifuged for 30 min at 14,000 x g. The protein

amount of the supernatant was analyzed by the Bradford method. Protein (100 µg) were separated in a 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). The blots were incubated with a polyclonal anti-arginase II (Santa Cruz), anti-endothelial nitric oxide synthase (eNOS, BD Bioscience), or anti-actin (Santa Cruz) antibodies followed by the secondary antibody (Amersham). The signals were detected using an enhanced chemiluminescence detection reagent with X-ray films.

### **5. Determination of eNOS dimerization**

Dimers and monomers of eNOS were separated using low-temperature SDS-PAGE as previously described<sup>20</sup>. Band intensities were analyzed using NIH ImageJ Software.

### **6. Estimation of NO or ROS generation in isolated mice aorta using 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM) or dihydroethidine (DHE)**

NO and ROS production were estimated using microscope by measuring change of fluorescence intensity at different time intervals as described previously<sup>21</sup>.

### **7. Aortic vascular tension assay**

The study was approved in accordance with Guide for the Care and Use of Laboratory Animals (Institutional Review Board, Kangwon national University). Male mice C57BL/6J were anesthetized using isoflurane and the thoracic aorta was rapidly removed. The aorta were placed on ice-cold oxygenated Krebs-Ringer bicarbonate solution (NaCl 118.3, KCl 4.7, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 1.6, NaHCO<sub>3</sub> 25, glucose 11.1 (in mM)) and cleared off adherent connective tissues. The mouse aorta was cut into 1.5-mm rings and suspended between two wire stirrups (150 µm) in a myograph (Multi myograph system DMT-620) in 10 ml Krebs-ringer (95% O<sub>2</sub>-5%CO<sub>2</sub>, pH7.4, 37 °C). One stirrup was connected to a three-dimensional micromanipulator, and the other to a force transducer. The rings were passively stretched at 10-minutes intervals in increments of 100 mg to reach optimal tone (600 mg). After the arterial rings had been stretched to their optimal resting tone, the contractile response to 100 mM KCl was determined. For constriction assay, phenylephrine (PE) was applied at different concentrations (10<sup>-9</sup>-10<sup>-5</sup>)

and cumulative responses were recorded. For NO-dependent relaxation responses, vessels were pre-constricted with PE ( $10^{-6}$ ) for 15 minutes and acetylcholine (Ach,  $10^{-9}$ - $10^{-5}$ ) was then added to determine relaxation activities. To further confirm the vasorelaxation activity in a NO-dependent manner, the inhibitor of guanylate cyclase (1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one, ODQ, 1  $\mu$ M) was added at the end of experiments.

#### **8. Determination of thiobarbituric acid-reactive substances (TBARS)**

Plasma samples were mixed with trichloroacetic acid (20%) and the precipitate was dispersed in  $H_2SO_4$  (0.05 M). TBA (0.2% in 2 M sodium sulfate) was added and heated for 30 min in boiling water bath. TBARS adducts were extracted by *n*-butanol and absorbance was measured at 532 nm<sup>22</sup>. Malondialdehyde (MDA) was used as a standard.

#### **9. Statistics**

All data are represented as mean  $\pm$  S.D. of at least four independent experiments. An unpaired Student's *t*-test or 1-way ANOVA was used to assess significant differences. A value of  $p < 0.05$  was accepted as significant.

### **III. RESULTS**

#### **1. Effect of KRGE on arginase activity in aortas of aged mice**

With the previous report that KRGE inhibits arginase activity<sup>18</sup>, mice were orally administered KRGE at 10 or 20 mg/mouse/day for 4 weeks. At first, we measured arginase activity in isolated aortic vessels. Arginase activity in aorta of aged mice was significantly increased (\* vs. young control,  $140.0 \pm 13.5$  vs.  $100 \pm 6.9\%$ ,  $p < 0.01$ ) that was blocked by KRGE (\*\* vs. aged,  $117.1 \pm 10.2$  (10 mg/mouse/day) and  $110.0 \pm 9.2$  (20 mg/mouse/day) vs.  $140.0 \pm 13.5\%$ ,  $p < 0.05$ ).

#### **2. KRGE enhances NO production and decreases ROS generation in endothelium of aged mice**

Given that arginase competes with nitric oxide synthase (NOS) for the common substrate, L-arginine, and negatively regulates NO production, we measured whether KRGE-dependent inhibition of arginase activity was associated with increase in NO production. As demonstrated in Fig. 2A, NO production was significantly attenuated in aged mice (\* vs. young,  $0.58 \pm 0.16$  vs.  $0.90 \pm 0.06$  change of DAF fluorescence/second,  $p < 0.01$ ). It was restored in groups of KRGE administration (\*\* vs. aged,  $0.87 \pm 0.11$  (10 mg/mouse/day) or  $0.91 \pm 0.14$  (20 mg/mouse/day) vs.  $0.58 \pm 0.16$  change of DAF fluorescence/second,  $p < 0.01$ ).

The effect of KRGE on NO production was also observed in young mice (# vs. young,  $1.13 \pm 0.09$  vs.  $0.90 \pm 0.06$  change of DAF fluorescence/second,  $p < 0.01$ ). On the other hand, ROS generation in aged mice was markedly increased (\* vs. young,  $0.52 \pm 0.13$  vs.  $0.31 \pm 0.04$  change of DHE fluorescence/second,  $p < 0.01$ ). This was significantly inhibited with KRGE administration (\*\* vs. aged,  $0.37 \pm 0.13$  or  $0.28 \pm 0.06$  change of DHE fluorescence/second,  $p < 0.01$ ). In young mice, KRGE also attenuated ROS generation (# vs. young,  $0.24 \pm 0.02$  vs.  $0.31 \pm 0.04$  change of DHE fluorescence/second,  $p < 0.01$ ). Interestingly, treatment of NOS inhibitor, L-NAME, reduced ROS generation in aged

mice (## vs. aged,  $0.29\pm 0.11$  vs.  $0.52\pm 0.13$  change of DHE fluorescence/second,  $p<0.01$ ).

### **3. KRGE administration enhanced eNOS coupling**

Based on the data of NO production and ROS generation, we next assessed proteins expression to find underlying mechanism. Interestingly, eNOS protein expression in aged mice was significantly increased (young vs. aged,  $1.0\pm 0.03$  vs.  $1.23\pm 0.04$ ,  $p<0.01$ ) that was not affected by KRGE administration. On the other hand, arginase II protein expression was not changed in both groups (Fig. 3A). This result is not consistent with the above observation that NO production was decreased and ROS generation was increased in aged mice. Therefore, we tested whether eNOS coupling was reduced in aorta of aged mice. As shown in Fig. 3B, eNOS coupling was attenuated in aged mice, which was restored with KRGE administration (Fig. 3C). These results imply that endothelial function is impaired in aged mice through attenuated eNOS coupling despite increased eNOS expression and KRGE administration can restore augmented eNOS uncoupling in aorta of aged mice.

### **4. NO-dependent relaxation of aortic vessels was augmented with KRGE administration**

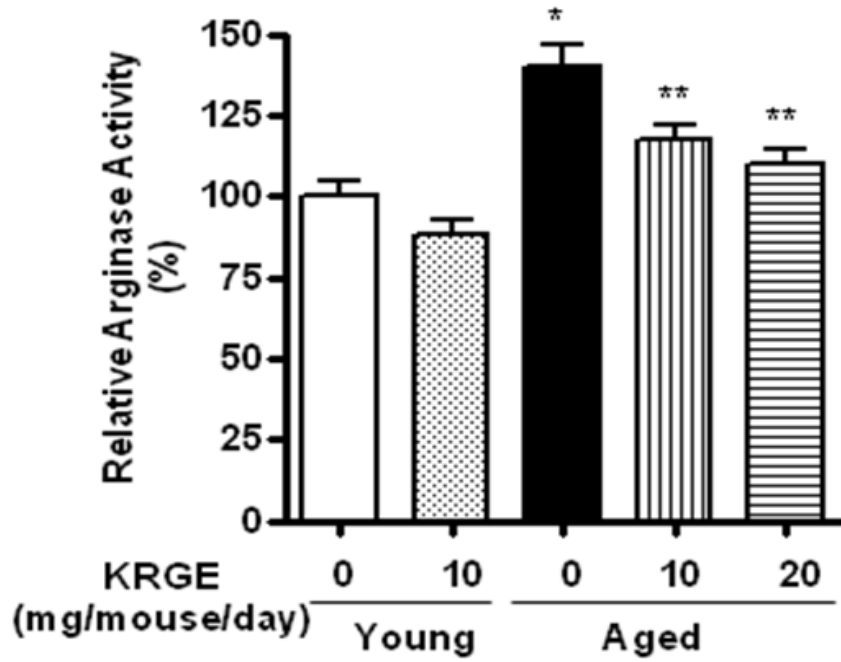
With the above data that KGWE administration induced eNOS coupling and NO production, we measured vascular tension to determine whether increased NO production by KGWE contributes to vasorelaxation in aged mice. Mouse aorta were precontracted with PE ( $10^{-6}$  M), and dose-response curves to endothelium-dependent vasodilator acetylcholine (Ach) were constructed. The vasorelaxation responses in aged mice were significantly attenuated compared with those from young (Fig. 4A, \*, young vs. aged,  $97.16\pm 4.08$  vs.  $73.30\pm 2.50\%$  ( $E_{max}$ ),  $p<0.01$ ;  $7.23\pm 0.13$  M vs.  $6.81\pm 0.09$  M ( $-\log EC_{50}$ ),  $p<0.01$ ). KRGE administration to aged mice enhanced vasorelaxation responses. Next, vasoconstrictor responses to the agonist phenylephrine (PE) were measured. As shown in Fig. 4B, the responses to PE were markedly attenuated in aorta from aged mice (\*, young vs. aged,  $59.92\pm 1.83\%$  vs.  $20.93\pm 1.68\%$  ( $E_{max}$ ),  $p<0.01$ ;  $8.21\pm 0.08$  M vs.  $7.72\pm 0.20$  M (-

logEC<sub>50</sub>),  $p < 0.01$ ). However, KRGE administration to aged mice enhanced vasoconstrictor responses in  $E_{max}$  (Fig. 4B, \*\*, aged vs. aged+KRGE (10 and 20 mg/kg/day, respectively),  $20.93 \pm 1.68\%$  vs.  $40.06 \pm 3.32\%$  and  $55.84 \pm 1.54\%$  ( $E_{max}$ ),  $p < 0.01$ ), but not in  $-\log EC_{50}$  (\*\*, aged vs. aged+KRGE (10 and 20 mg/kg/day, respectively),  $7.72 \pm 0.20$  M vs.  $7.56 \pm 0.19$  M and  $7.83 \pm 0.07$  M, not significance). On the other hand, the responses to the endothelium-independent vasodilator sodium nitroprusside (SNP) were not significantly changed in any group (Fig. 4C)

### **5. KRGE prevents peroxynitrite formation in aged mice**

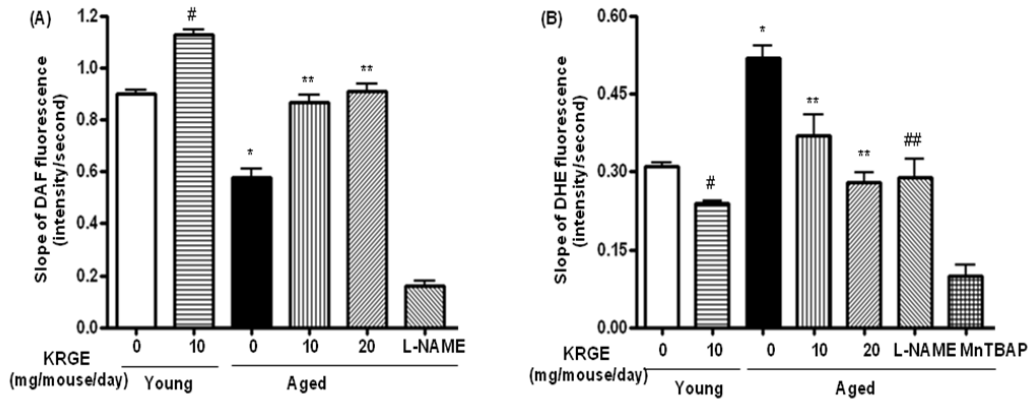
Given that enhanced oxidative species such as peroxynitrite formation increase arginase activity<sup>23</sup> and enhanced peroxynitrite formation is associated with vascular aging<sup>24</sup>, we performed TBARS assay to measure lipid peroxidation. In plasma of aged mice, lipid peroxidation was significantly increased that was reduced to those of young by administration of KRGE (Fig. 5, \*, young vs. aged,  $0.23 \pm 0.08$  vs.  $0.52 \pm 0.13$   $\mu$ M MDA/ml plasma,  $p < 0.01$ ; #, aged vs. aged+20 mg/mouse/day,  $0.52 \pm 0.13$  vs.  $0.29 \pm 0.09$   $\mu$ M MDA/ml plasma,  $p < 0.01$ ).

Figure 1



**Fig. 1. Increased arginase activity in aged mice aorta was inhibited by administration of KRGE.** Oral administration of KRGE for 4 weeks resulted in decrease in arginase activity. \* vs. Young,  $p < 0.01$ ; \*\* vs. Aged,  $p < 0.01$ .

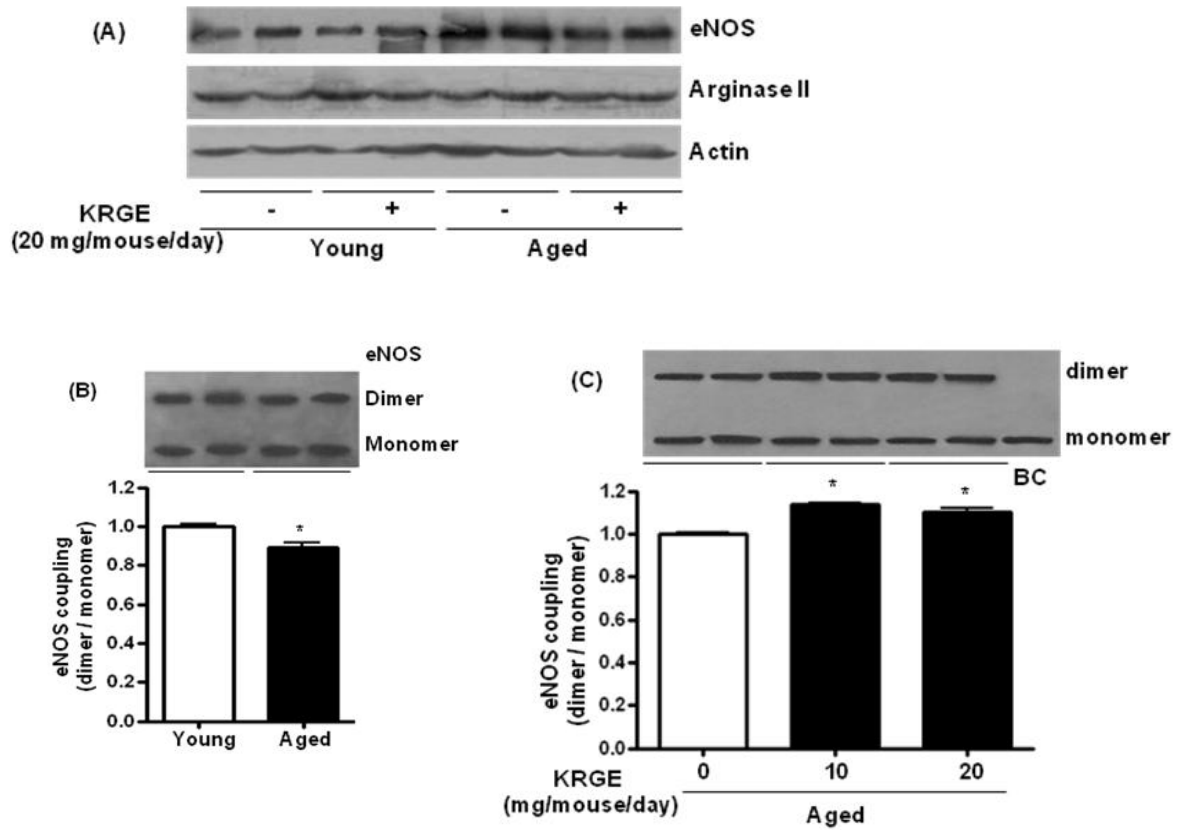
**Figure 2**



**Fig. 2. KRGE restored impaired endothelial function in aged mice.** Isolated aortic segments were incubated with DAF-AM (5  $\mu$ M) and fluorescence was measured in real-time (endothelium side up). The slope of DAF fluorescence was determined. (A) KRGE administration with young vessels increased the slope of DAF fluorescence (# vs. young,  $p < 0.01$ ). However, the decreased DAF fluorescence in aged mouse aorta was increased after KRGE ingestion (\* vs. young,  $p < 0.01$ ; \*\* vs. aged,  $p < 0.01$ ;  $n = 4$  mice). L-NAME was used as a control. (B) ROS production in aortic endothelium was measured with DHE (5  $\mu$ M), and the slope of DHE fluorescence was determined using cumulative data. KRGE intake reciprocally regulated ROS production (# vs. young,  $p < 0.01$ ; \* vs. young,  $p < 0.01$ ; \*\* vs. aged,  $p < 0.01$ ; ## vs. aged,  $p < 0.01$ ;  $n = 4$  mice). MnTBAP was used as a control.

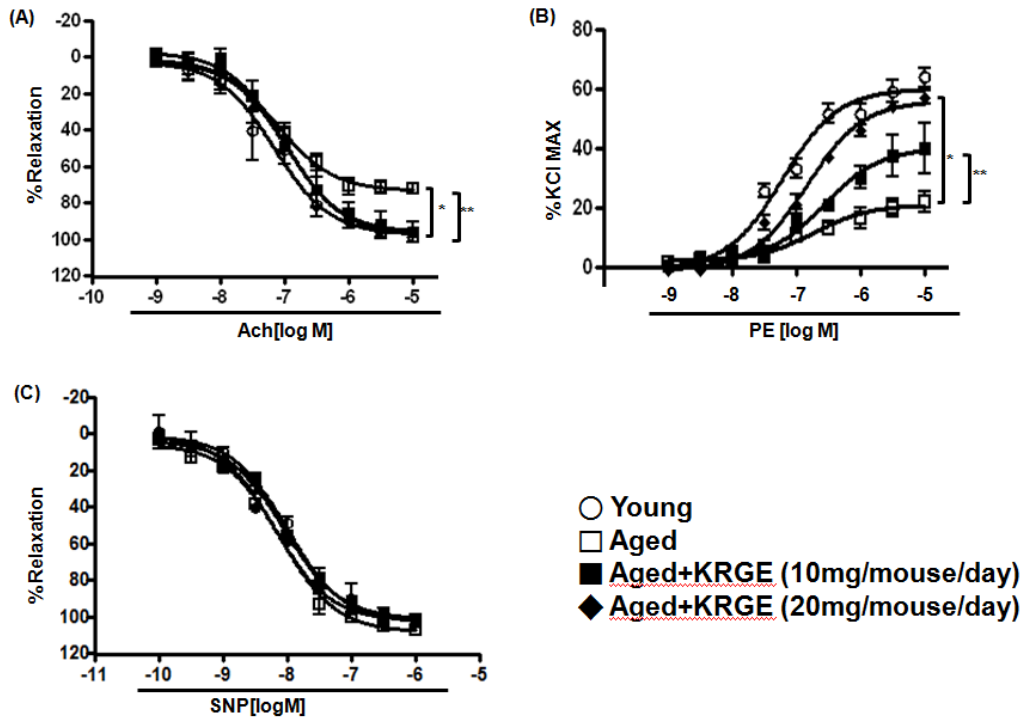


**Figure 3**



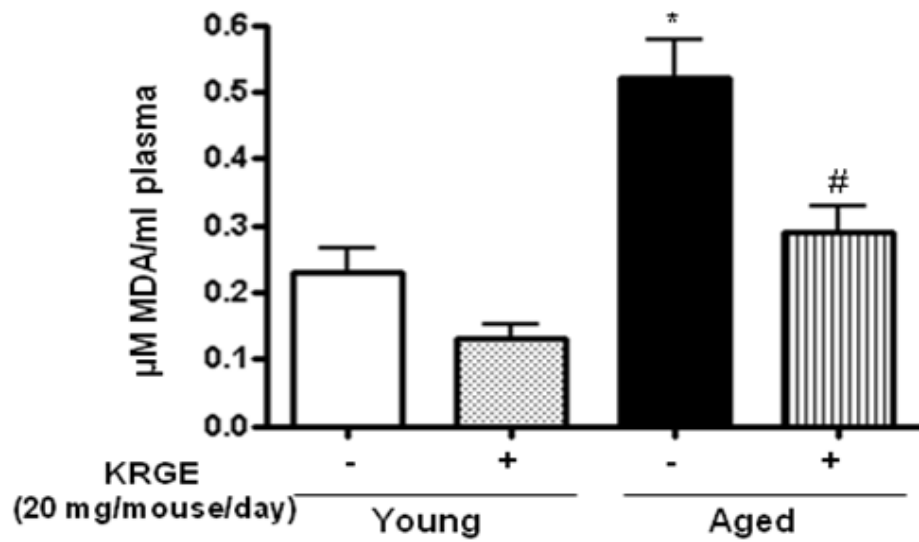
**Fig. 3. KRGE contributes to improvement of aging-dependent eNOS uncoupling.** (A) The expression levels of proteins was not changed by KRGE in aortas of young and aged mice. (B). eNOS dimerization was analyzed by low temperature SDS- PAGE and Western blot analysis and eNOS was uncoupled in aged mice aorta. \* vs. young,  $p < 0.05$ . (C) KRGE induced increase of eNOS coupling in aged mice aorta. \* vs. aged,  $p < 0.05$ .

**Figure 4**



**Fig. 4. KRGE improved impaired vascular reactivity in aged mice.** (A) Endothelium-dependent relaxation responses to Ach were impaired in aged aortas (\*, young vs. aged,  $p < 0.01$ ). Impaired relaxation was recovered by KRGE administration (\*\*, aged vs. aged+KRGE,  $p < 0.05$ ). (B) Aged aortic vessels had attenuated contractile responses to PE compared to young mice (\*, young vs. aged,  $p < 0.01$ ). KRGE restored the PE-mediated pressor responses of aged aortic vessels (\*\*, aged vs. aged+KRGE,  $p < 0.01$ ). (C) Relaxation responses to NO donor (sodium nitroprusside, SNP) are not significantly different in all groups.  $n = 4$  mice per each group.

Figure 5



**Fig. 5. KRGE administration prevented peroxynitrite formation.** Peroxynitrite content in plasma were measured by TBAR assay. Increased peroxynitrite content in aged mice was significantly inhibited with administration of KRGE. young vs. aged, \* $p < 0.01$ ,  $n = 6$ ; aged vs. aged+KRGE, # $p < 0.01$ ,  $n = 6$ .

## IV. DISCUSSION

Endothelial arginase can constrain the activity of eNOS by depleting the critical substrate, L-arginine. In turn, increased arginase activity reduces NO bioavailability and contributes to vascular diseases such as aging, hypertension, and atherosclerosis <sup>6, 25-27</sup>. Here, we show that oral administration of KRGE for 4 weeks enhances NO generation, reduces ROS production by inhibiting arginase activity, and induces the vasorelaxation in aortic vessels from aged mice. Furthermore, KRGE reduces the formation of peroxynitrite in plasma.

Ginseng has been shown to have beneficial effects in the treatment of various diseases. In the vasculature, it is well documented that KRGE eliminates superoxide <sup>12</sup>, promotes endothelial cell proliferation <sup>8</sup>, protects cell death by H<sub>2</sub>O<sub>2</sub> stimulation <sup>13</sup>, and induces heme oxygenase-1 expression <sup>14</sup>. Furthermore, the beneficial effects of ginseng on vascular system may be dependent on the activation of Akt/PI3K signal transduction <sup>16</sup>, inhibition of angiotensin converting enzyme <sup>28</sup>, and inhibition of calcium ion influx <sup>29</sup>. Here, we demonstrate that KRGE inhibits arginase activity and is associated with decreased peroxynitrite formation through increased NO production and decreased ROS generation in aged vasculature.

Aging is associated with changes in arterial wall structure and function. The most frequent modifications are luminal enlargement, vessel wall thickening due to intimal and medial expansion, elastin depletion and fragmentation, collagen and calcium deposition, glycation of proteins, and impaired vasomotor function associated with endothelial dysfunction <sup>30-32</sup>. These structural and functional alterations in aging contribute to increased vascular stiffness, which is an independent risk factor for cardiovascular morbidity and mortality <sup>33-35</sup>.

Accumulating evidence indicates that arginase contributes to age-associated endothelial dysfunction and arterial stiffening. The detrimental effect of arginase in vascular remodeling is attributable to its ability to stimulate vascular smooth muscle cell and endothelial cell proliferation, and collagen deposition by promoting the synthesis of polyamines and L-proline, respectively.

Vascular ROS production is enhanced in aged blood vessels<sup>3, 24, 36, 37</sup>. One of enzymatic systems that contribute to increased ROS production in pathophysiological states may be eNOS. Although eNOS normally produces vasoprotectant molecule NO, it can also produce  $O_2^-$  in the absence of either L-arginine or  $BH_4$  because electrons flow from the reductase domain in the heme to molecular oxygen rather than L-arginine in uncoupled eNOS. Actually, we showed that arginase activity and peroxynitrite formation was increased, and eNOS was uncoupled in aged vessels. Furthermore, increased ROS production in the endothelium of aged mice was prevented with preincubation of the eNOS inhibitor L-NAME that is consistent with previous observations<sup>38</sup>. Peroxynitrite may stimulate arginase II activation via RhoA-dependent ROCK activation without a change in mRNA and protein levels<sup>23</sup>. One study reported that peroxynitrite can react with the redox-active cysteine (Cys<sup>18</sup>) of RhoA, which enhances GDP release from RhoA and thus modulates their activity<sup>3</sup>. Indeed, we found peroxynitrite formation as measured by the thiobarbituric acid reactive substance assay, was significantly increased in plasma from aged mice, which is consistent with previous publication<sup>24</sup>.

Increased expression of eNOS may depend on shear stress and hemodynamic forces<sup>39, 40</sup> because of the presence of a shear stress-responsive element in the promoter region of the eNOS gene<sup>41</sup>. Consistent with the demonstration, eNOS expression was increased in aorta<sup>42</sup> and not changed in artery<sup>43</sup> and decreased in arteriole<sup>2</sup>. Furthermore, increased expression of eNOS protein in aged aorta may be one of the compensatory mechanisms to counterbalance endothelial dysfunction by increased arginase activity.

In summary, we demonstrate that KGRE, in aged aortic vessels, inhibits increased arginase activity that is associated with enhanced eNOS dimerization and increased NO production. Furthermore, KRGGE augments vasorelaxation in NO-dependent manner and attenuates peroxynitrite formation. These findings suggest that KGRE possesses therapeutic potential for cardiovascular diseases associated with aging.

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## Abstract

### 혈관기능이 약화된 노화생쥐에서 **arginase** 길항작용을 통한 홍삼의 효과

국내산 홍삼 추출액은 심혈관계에 도움 되는 효과가 있는 것으로 알려져 있다. 심혈관계 질환은 사망률과 이환율에 큰 부분을 차지하며 이런 심혈관계 질환의 발생률은 나이, 즉 노화와 밀접한 관계가 있다. 노화된 혈관에서 **argniase**의 발현 증가는 혈관 내피세포의 기능 부전을 일으킨다는 연구가 많이 진행되었다. 본 연구에서는 홍삼 추출액을 노화된 생쥐에 구강 투여 하였을 때 일산화질소 합성효소(nitric oxide synthase)의 결합을 통하여 **arginase**의 활성도를 낮추고, 일산화질소의 생성을 복구하고, 활성산소의 생성을 억제함을 보이고 있다. 홍삼 추출액의 구강 투여로 혈관 탄성 분석을 통해서도 나이든 혈관의 아세틸콜린에 의한 혈관이완능력과 페닐레프린에 의한 혈관수축능력이 비약적으로 향상되는 것을 알 수 있었으며, 세포 내에서 **peroxynitrite**의 생성이 저해되는 것을 발견하였다. 종합해보면 이런 결과들을 통해 홍삼 추출액이 일산화질소의 신호전달 체계를 통하여 **argniase**의 활성도를 저하시켜 혈관보호 효과를 나타낸다고 할 수 있다. 따라서 홍삼 추출액은 노화에 따른 혈관질환의 치료에 효과적일 수 있다.

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**Keywords:** 인삼, 홍삼추출액, 노화, **arginase**, 일산화질소, 내피세포 일산화질소 생성제, 혈관이완