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# Myricetin and Hesperidin Inhibit Cerebral Thrombogenesis and Atherogenesis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ Mice

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## Abstract

Flavonoids have been reported to possess strong antioxidant activities that moderate endothelial dysfunction and demonstrate protective effects on cardiovascular disease. Our previous studies confirmed that flavonoids, including hesperidin, naringin and nobiletin, inhibited thrombogenesis and hypertension in stroke prone spontaneously hypertensive rats (SHRSP) by protecting the endothelium from the adverse effects of free radical formation. We have now further investigated the protective effects of myricetin and hesperidin on cerebral thrombosis and atherogenesis in apolipoprotein E (apoE) and low-density lipoprotein receptor (LDLR) deficient (*Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout) mice. Three groups of mice were fed high fat diet alone and high fat diet mixed with myricetin (100 mg/kg/day and 200 mg/kg/day) or glucosyl hesperidin (G-hesperidin; 250 mg/kg/day and 500 mg/kg/day) for 8 weeks. There were no differences in body weight related to administration of the flavonoids. Thrombotic tendency was assessed using a He-Ne laser technique in the murine cerebral pial vessels. In addition, atherogenesis was quantified histologically after dissection of the aorta from each mouse and staining with Oil Red O solution. The percentages of stained area to whole area of dissected aorta were calculated as indices of anti-atherogenic activity. Both myricetin and G-hesperidin significantly inhibited thrombogenesis *in vivo* and significantly inhibited atherogenesis compared to control mice ($p < 0.001$). These findings demonstrated that daily intake of myricetin and hesperidin suppressed the development of atherogenesis and thrombogenesis, possibly associated with the potent antioxidant effects of the flavonoids.

**Keywords**

Myricetin, Hesperidin, Atherosclerosis, *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ Mice, Thrombosis

## 1. Introduction

Flavonoids are polyphenolic compounds present in substantial amounts in plants. They exert multiple biological effects, including antioxidant activity [1] [2], anti-inflammatory [3] [4], anti-cancer [5] [6] and neuroprotective properties [7]. Lipid oxidation has been recognized as the major source of deteriorative changes in the nutritional properties of fats and oils, and the anti-oxidative effects of flavonoids improve the stability of lipid containing foods. In addition, lipid oxidation has been shown to be associated with disturbances in the structure and functional loss of cell membranes, and oxidative modification appears to be an important initial event for the pathogenesis of atherosclerosis [8]. The potential beneficial effects of flavonoids on inhibiting the oxidation of low-density lipoproteins (LDL) and promoting anti-atherogenesis is believed to be beneficial for human health [9] [10].

Myricetin (**Figure 1**) is a well-defined flavonol with hydroxyl substitutions at 3, 5, 7, 3', 4' and 5' molecular sites, and occurs naturally in tea, fruits, berries, vegetables and medicinal herbs [11]. The role of myricetin as an antioxidant appears to be especially distinguished as an anti-carcinogen and in the treatment of type 2 diabetes mellitus [12]. In addition, myricetin has been found to be effective in scavenging free radicals [11] [13] and inhibiting the formation of malondialdehyde (MDA) from arachidonic acid when lipids undergo peroxidation [14].

Hesperidin, a citrus flavonoid also exerts numerous pharmacological activities including strong antioxidant effects [15], and our previous studies have shown that compounds of this nature, such as glucosyl hesperidin (G-hesperidin; **Figure 1**), naringin and nobiletin, improved endothelial function, and suppressed hypertension and thrombotic tendency possibly through antioxidant effects in stroke prone spontaneously hypertensive rats (SHRSP) [16] [17]. The evidence suggested that free radicals generated in the vicinity of the endothelium reduced endothelial function, caused vasoconstriction and led to increases in blood pressure and an enhanced thrombotic tendency.

Demonty, *et al.* [18] reported, however, that citrus flavonoids did not affect serum cholesterol in moderately hypercholesterolemic men and women. Nevertheless, increases in oxidized LDL-cholesterol and triglyceride are known to be risk factors for atherosclerosis [19] [20]. Apolipoprotein E (ApoE) and LDL receptor (LDLR) deficient (*Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout) mice have been used in studies of atherosclerosis [21] [22]. These double mutant mice demonstrate cholesterol and lipoprotein profiles similar to *Apoe*$^{-/-}$ single mutant mice but reportedly develop atherosclerotic plaques even more rapidly than the

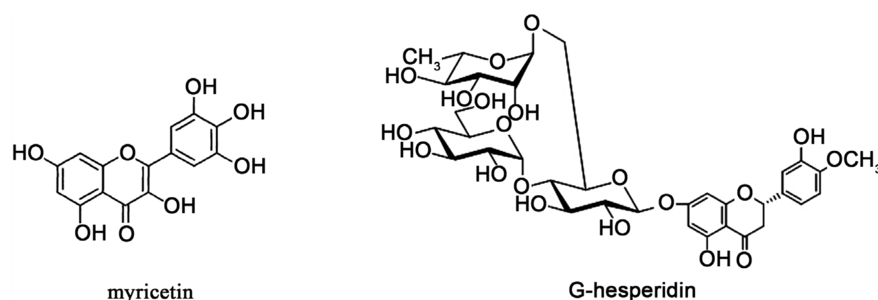


**Figure 1.** Chemical structure of myricetin and G-hesperidin.

*Apoe*$^{-/-}$ single knockout animals. Similarly, Ldlr deficient mice that were engineered to express only the ApoB-100 isoform, appeared to develop larger atherosclerotic plaques than age matched the *Apoe*$^{-/-}$ knockout mice, despite having similar total serum cholesterol levels [23]. The data suggested that the double-mutant mouse model offered benefits in studies of the effects of myricetin and G-hesperidin on atherosclerosis in a relatively short time-scale.

The present study was designed, therefore, to further clarify the anti-atherogenic and antithrombotic effects of myricetin and G-hesperidin utilizing these genetically sensitive *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout mice.

## 2. Materials and Methods

### 2.1. Animals

Apolipoprotein E (ApoE) and low-density lipoproteins receptor (LDLR) deficient mice (*Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout mice, 129xC57BL/6J background) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred in Kobe Gakuin University. All experiments were conducted in accordance with the ethical principles of animal care of Kobe Gakuin University and the guiding principles for the care and use of animals in the field of physiological sciences published by the Physiological Society of Japan [24]. Euthanasia was performed by $CO_2$ inhalation or cervical dislocation after experiments. Myricetin, purity more than 97% was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan and glucosyl hesperidin (G-hesperidin; purity of 78.8%) was kindly provided by Toyo Sugar Refining Co., Ltd, Tokyo, Japan. Control animals (N = 8) were fed high fat diet (F2HFD1), obtained from Oriental Yeast Co., LTD., Tokyo, Japan, to induce atherosclerosis. Myricetin or G-hesperidin were mixed with the high fat diet at concentrations of 0.06% and 0.12% and 0.3% and 0.6%, respectively. The animals (N = 8 in each group) were given myricetin diet or G-hesperidin diet and water ad libitum. Each animal was kept in an individual cage and water and food were changed at 9:00 am every day. Consumption was assessed by weighing the diet and water on each occasion. The daily average doses of myricetin and G-hesperidin, calculated from the food intake in each group were adjusted to approximately 100 and 200 mg/kg animal body weight/day [25] [26] and 250 mg/kg and 500 mg/kg animal body weight/day [16], respectively (**Table 1**).

**Table 1.** The experimental protocols.

| | Control (N = 8) | Myricetin (N = 8) | G-hesperidin (N = 8) |
|---|---|---|---|
| Diet | High Fat Diet (F2HFD1) | F2HFD1 + myricetin 100 mg/kg/day | F2HFD1 + G-hesperidin 250 mg/kg/day |
| | | F2HFD1 + myricetin 200 mg/kg/day | F2HFD1 + G-hesperidin 500 mg/kg/day |
| Period | for 8 weeks | for 8 weeks | for 8 weeks |
| Measurement | thrombotic tendency atherosclerosis | thrombotic tendency atherosclerosis | thrombotic tendency atherosclerosis |

### 2.2. Measurement of Thrombotic Tendency

Closed cranial windows were created as described by Morii *et al.* [27] and thrombotic tendency was measured as reported previously [16] [28].

In brief, animals were ventilated artificially after anesthetizing and Evans blue was administered through a femoral vein. Closed cranial windows were formed with 2 mm diameter in the right parietal bone and artificial cerebrospinal fluid was infused continuously within this window. The animals were placed in a stereotaxic frame on the stage of an optical microscope and the cerebral vessels on the surface of the brain were monitored and recorded on videotape with a CCD camera (Pulnix, Takenaka System, Kyoto). A He-Ne laser beam (15 μm in diameter) was focused on the center of selected blood vessels through the optical path of the microscope and thrombi were formed in venules (25 - 40 μm) by repeated irradiation for 10 s at 20 s intervals at a power of 13 mW. The number of laser pulses required to generate an occlusive thrombus was used as an index of thrombotic tendency.

### 2.3. Measurement of Atherosclerosis

Arteriosclerosis was assessed by estimating the development of atherosclerotic lesions as a percent of the entire surface area of the aorta [29] [30]. Briefly, hearts were exposed by abdominal incision and 50 mL of phosphate buffered saline (pH 7.4) infused through butterfly needle, followed by 10% neutral buffered formalin solution by reflex through a femoral artery. The aortic arch was carefully dissected from connective tissue and minor branching blood vessels. The extracted vessels were kept in 10% neutral buffered formalin solution until processed. The vessels were then incised along the longitudinal plane, inverted on a black rubber sheet and held in place using stainless steel pins. The tissue was washed with distilled water for 30 s, treated with 60% isopropyl alcohol for 1 min, and stained with Oil Red O stain solution at 37˚C for 15 min. Finally, the vessel was washed with 60% isopropyl alcohol and distilled water.

The Oil Red O stained specimens were photographed using a digital camera (Olympus Tokyo Japan). Images were transferred to a PC and analyzed using NIH Image. The entire area of the dissected blood vessels and the area of atherosclerotic lesions identified by Oil Red O staining were calculated. The percentages of Oil Red O staining area to entire area were used as an index of atheros-

clerotic development.

### 2.4. Statistical Analyses

The results illustrate the number of animals (N) and the mean and standard error (SEM) of each experiment. Statistical evaluation was performed by one-way analysis of variance (ANOVA), by the post hoc test of Dunnett and Mann-Whitney test using commercially available statistical packages (Prism 5.0; GraphPad Software, Inc., San Diego, CA, USA). A value of $p < 0.05$ was considered to be statistically significant.

## 3. Results and Discussion

### 3.1. Changes in Body Weight by Myricetin and G-Hesperidin in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ Double Knockout Mice

The doses of myricetin (100 and 200 mg/kg/day) and G-hesperidin (250 and 500 mg/kg/day) were adjusted every day for 8 weeks, respectively by weighing the consumption of high fat diet (F2HFD1) mixed with myricetin or G-hesperidin. Changes in body weight over time after administration of myricetin and G-hesperidin from 8 weeks of age up to 16 weeks of age are shown in Figure 2. The body weights increased with age, and there were no statistically significant differences among these groups.

### 3.2. Suppression of Thrombogenesis by Myricetin and G-Hesperidin in the Pial Vessels of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ Double Knockout Mice

Thrombogenesis in cerebral vessels for each group of animals, as assessed by the He-Ne laser technique, is summarized in Figure 3. The number of laser pulses

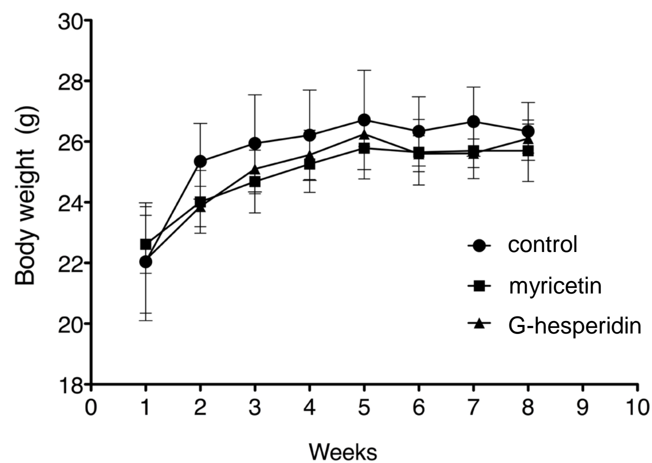


Figure 2. Changes in body weights after administration of myricetin and G-hesperidin ApoE and LDLR deficient mice were fed high fat diet (F2HFD1) alone (N = 8), F2HFD1 plus myricetin at 200 mg/kg/day (N = 8) or F2HFD1 plus G-hesperidin at 500 mg/kg/day (N = 8) for 8 weeks from the age of 8 weeks. Body weight increased in each group of mice, and there were no significant differences among each group at the same age. Statistical significance was assessed by one-way ANOVA and by the post hoc test of Dunnett.

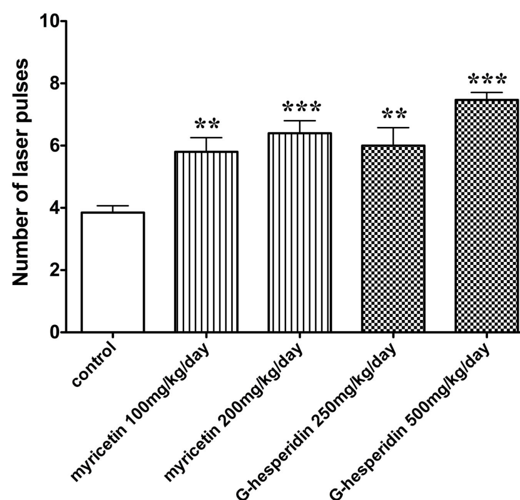


**Figure 3.** Effects of myricetin and G-hesperidin on cerebral thrombogenesis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout mice. *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout mice were fed F2HFD1 alone (N = 8), F2HFD1 plus myricetin at 100 and 200 mg/kg/day (N = 8) or F2HFD1 plus G-hesperidin at 250 and 500 mg/kg/day (N = 8) for 8 weeks from the age of 8 weeks. Occlusive thrombi were generated in pial arterioles by irradiation with a He-Ne laser at a power of 13 mW for 10 sec and 20 sec intervals. The number of laser irradiations (laser pulses) was used as an index of thrombotic tendency. Myricetin and G-hesperidin inhibited thrombogenesis significantly as compared to control. Statistical significance was assessed by one-way ANOVA and by the post hoc test of Dunnett. **: $p < 0.01$ vs control, ***: $p < 0.001$ vs control.

required to generate occlusive thrombi in pial vessels in control and myricetin-fed mice at 100 and 200 mg/kg/day were 3.9 ± 0.2 (N = 5), 5.8 ± 0.5 (N = 4), 6.4 ± 0.4 (N = 5), respectively and G-hesperidin-fed animals at 250 and 500 mg/kg/day were 6.0 ± 0.6 (N = 4) and 7.5 ± 0.2 (N = 8), respectively. Thrombus generation was significantly delayed at both concentrations of myricetin and G-hesperidin as shown in **Figure 3**. ($p < 0.01$ and $p < 0.001$). These results were consistent with our former studies demonstrating that flavonoids, including hesperidin, naringin and nobiletin, inhibited thrombus generation, possibly by scavenging free radicals and resulting in increased nitric oxide (NO) which inhibits platelet aggregation [16] [17]. Our new data provided further evidence that mechanisms underlying the effects of the flavonoids were related to strong antioxidative properties.

Mechanisms of He-Ne laser induced thrombus generation in small blood vessels may depend on free radical and/or active oxygen produced in response to laser irradiation [31], and such reactive oxygen species (ROS) may be involved in vascular dysfunction. ROS reduce levels of bioactive NO by conversion into toxic peroxynitrite ($ONOO^-$) leading to a deterioration in protective endothelial function [32] [33]. Povlishock demonstrated that the endothelial dysfunction caused by He-Ne laser irradiation induced platelet aggregation without cell-surface denudation [34]. Furthermore, Wachowicz concluded that ROS are produced during platelet aggregation and play an important role in platelet function [35]. Other studies have shown, however, that flavonoids blocked

platelet aggregation by inhibiting thromboxane receptors [36] or thromboxane $A_2$ [37].

In alternative studies, Rong *et al.* reported that hesperidin pretreatment protected neonatal rats from brain injury by reducing free radicals [38], and Mastantuono *et al.* also reported that hesperidin, diosmin, and apigenin had dose-related protective effects on microvascular damage induced by hypoperfusion and reperfusion in the rat pial circulation, possibly related to a decrease in ROS production [39].

Our previous studies indicated that the antioxidant activity of G-hesperidin inhibited He-Ne laser induced thrombosis by scavenging ROS [16], and overall the present studies are consistent with the likelihood that the adverse effects of ROS produced by laser irradiation and platelet aggregation were moderated by the flavonoids

### 3.3. Effects of Myricetin and G-Hesperidin on Atherogenesis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ Double Knockout Mice

The entire area of the dissected blood vessels and the area of atherosclerotic lesions identified by Oil Red O staining in control, myricetin at 200 mg/kg/day and G-hesperidin at 500 mg/kg/day are shown in **Figure 4**. The lipid-stained areas in control were dominant and clear, as compared to myricetin and G-hesperidin treated aorta. The severity of atherogenesis, as expressed as percentages of lipid-stained areas to entire area of the aorta is shown in **Figure 5**. The percentages in control, myricetin and G-hesperidin treated mice were 33.4 ± 3.1% (N = 8), 14.2 ± 2.3% (N = 8; $p < 0.001$ vs. control) and 22.0 ± 1.4% (N = 8; $p < 0.001$ vs. control), respectively. The data clearly demonstrated that the control

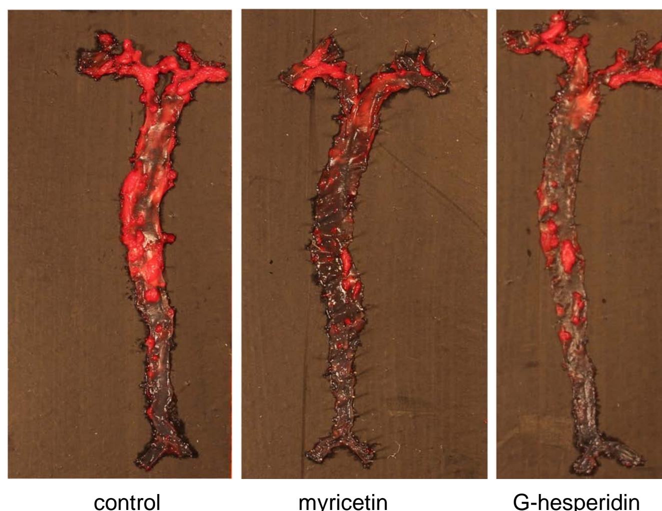


**Figure 4.** Effects of myricetin and G-hesperidin on atherogenesis in the aortas of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout mice treated for 8 weeks. *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout mice were fed F2HFD1 alone and together with myricetin at 200 mg/kg/day and G-hesperidin at 500 mg/kg/day. Dissected aortas were stained with Oil-Red-O as described in Materials and Methods. The Oil-Red-O stained area appeared more dominant in control mice as compared to the aortas of myricetin and G-hesperidin-treated animals.

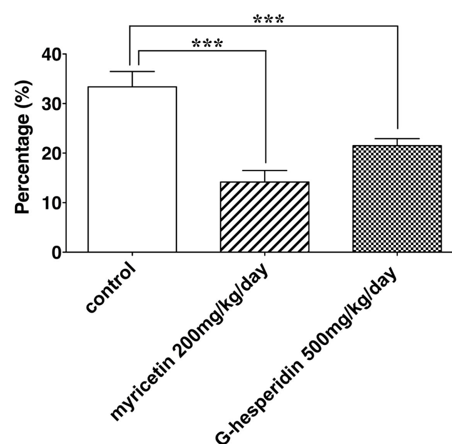


Figure 5. Image analysis of dissected aortas of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout mice treated with myricetin and G-hesperidin for 8 weeks. Percentages of Oil-Red-O stained area to entire area of dissected aorta were calculated using NIH Image. Statistical significance was assessed by one-way ANOVA and by the post hoc test of Dunnett. ***: $p < 0.001$.

*Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ double knockout mice fed high fat diet for eight weeks accumulated lipid over approximately about one third of the entire aorta, and that the flavonoids significantly prevented this deposit of lipid.

Therapeutic flavonoids including quercetin, kaempferol, myricetin, apigenin, and luteolin are well known to reduce the incidence of coronary disease in elderly humans [11]. Atherosclerosis is widely considered to be a chronic inflammatory disease, and excess ROS, generated by oxidative stress, has emerged as a critical common mechanism involved in vascular injury in both the development and progression of atherosclerosis [20]. It is generally believed that the potent antioxidant activity of polyphenols and their inhibitory effects on LDL oxidation support antiatherogenic properties [4] [10]. In addition, Bertin *et al.* reported that myricetin appeared to be the most active of selected plant-derived polyhydroxyl compounds, with prominent capacities against oxidized LDL and ROS production [40]. Our finding that the inhibition of both thrombotic tendency and atherogenesis by myricetin and G-hesperidin, were in keeping with their strong antioxidant potential, although other factors also might be involved. In this context, Sun et al, recently suggested that hesperidin moderated atherosclerosis not only by anti-oxidative and anti-inflammatory action but also by other pleiotropic effects, including improvement of insulin resistance, amelioration of lipid profiles and inhibition of macrophage foam cell formation [41]. Our results provide convincing direct evidence that myricetin and G-hesperidin inhibited thrombogenesis and atherogenesis. Precise mechanisms underlying this important inhibitory action remain to be fully characterized.

## 4. Conclusion

Myricetin and G-hesperidin demonstrated significant inhibitory effects on the development of atherosclerosis and thrombosis in a sensitive murine model. The

findings provided new evidence on the mechanism of action of myricetin and hesperidin those are well known to reduce the incidence of coronary disease in elderly humans. Our findings strongly suggested that the prophylactic, daily intake of flavonoids offers a promising strategy for the prevention of cardiovascular diseases and the maintenance of human health.

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## Author Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this article.

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