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Anti-obese and Blood Flow Improvement Activities of Ginseng Berry on the 45%Kcal High Fat Diet Supplied Mouse

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Abstract

Objectives : The present study investigated the anti-obese and blood flow improvement activities of aqueous extracts of ginseng berry (GBe) on the mild diabetic obese mice as compared with metformin.

Methods : After end of 56 days of continuous oral administrations of GBe 150, 100 and 50 mg/kg, or metformin 250 mg/kg, anti-obese and blood flow improvement effects - the changes of body weights, body and abdominal fat density by *in live* dual-energy x-ray absorptionmetry (DEXA), tail bleeding time, prothrombin time (PT), activated partial thromboplastin time (aPTT), serum total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL) levels, aorta and serum cyclic guanosine monophosphate (cGMP), nitric oxide (NO) and endothelin (ET)-1 levels, aorta phosphorylated PI3K (pPI3K), phosphorylated Akt (pAkt) and phosphorylated p38 MAPK (pp38 MAPK) levels were systemically analyzed. In addition, aorta vascular dilation and constriction related gene mRNA expressions - PI3K, Akt, eNOS, p38 MAPK and ET-1 were also analyzed by *realtime* RT-PCR.

Results : The obesity and related blood flow impairment, induced by 84 days of continuous HFD supply, were significantly inhibited by 56 days of continuous oral treatment of GBe 150, 100 and 50 mg/kg, dose-dependently, and they also dramatically normalized the changes of the aorta vascular dilation and constriction related gene mRNA expressions, also dose-dependently. Especially, GBe 150 mg/kg constantly showed favorable inhibitory activities against type II diabetes related obesity and vascular disorders through PI3K/Akt pathway and p38 MAPK mediated cGMP, NO and ET-1 expression modulatory activities, as comparable to those of metformin 250 mg/kg in HFD mice.

Conclusion : By assessing the key parameters for anti-obese and blood flow improvement activities on the HFD-induced mild diabetic obese mice, the present work demonstrated that GBe 150, 100 and 50 mg/kg showed favorable anti-obese and blood flow improvement effects in HFD-induced type II diabetic mice,

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through PI3K/Akt pathway and p38 MAPK mediated cGMP, NO and ET-1 expression modulatory activities.

Key words : Ginseng berry, 45%Kcal High fat diet, mouse, Anti-obese, Blood improvement, *realtime* RT-PCR, Serum biochemistry.

I. Introduction

Obesity contributes to the etiologies of a variety of comorbid conditions, such as cardiovascular disease, hypertension, and type II diabetes[1]. In addition to storing lipid for energy, adipose secretes a variety of adipokines, many of which affect metabolism and inflammation in adipose and nonadipose tissues. Modulation of the endocrine functions of adipose tissue can contribute to a chronic state of inflammation, which leads to the pathogenesis of associated disorders, specifically insulin resistance[2]. Recently, there has been a worldwide increase in the incidence of obesity associated with a metabolic syndrome known as type II diabetes, the development of which seems to be as a result of high-caloric diet intake and physical inactivity[3] and predicted estimates suggest that the population with this syndrome may double to over 300 million by the year 2025[4]. One of the critical determinants for the development of this obesity may be an increase in the regional distribution of body fat, especially abdominal obesity. The latter often shows clustering of atherogenic risk factors[5], like hypertension, dyslipidemia, alterations in coagulation and inflammatory cytokine profiles, and hyperinsulinemic insulin resistance. As a consequence, there is anexpected increase in morbidity and mortality of cardiovascular disease (CVD)[6].

Atherosclerosis is the common pathological mechanism of CVD and cerebrovascular diseases, including angina pectoris, and has been the subject of numerous investigations over an extended period of time[7]. In the past 100 years, the global death ratio caused by CVD has increased from 1/10 in the early 1900s to 1/3 in the early 2000s, becoming the leading cause of death worldwide[8, 9]. Therefore, it is essential to find therapeutic drugs to treat atherosclerosis to improve the long-term prognosis of patients with angina pectoris[7]. Atherosclerosis is one of the major causes leading to mortality of dysfunctional cardiovascular events in developed countries[10]. The atherosclerotic lesions are mostly characterized by the transformation of macrophages to foam cells through uptake of lipoprotein-derived cholesterols, which secrete various inflammatory cytokines in the arterial intima[11], suggesting a critical role for macrophages in the development of atherosclerosis[12, 13]. Atherosclerosis is generally characteristic features of metabolic syndrome[14]. Endothelial dysfunction is a key pathophysiological step in the early stage of vascular diabetic complications[15-17]. Previous studies have demonstrated that vascular dysfunction in the setting of diabetes is associated with increased vascular oxidative stress and low-grade inflammation[18-20].

The root of ginseng (*Panax ginseng* CA Meyer, Araliaceae) is a commonly used herbal medicine and alternative therapeutic material; however, very little work has been done to evaluate the effect of the ginseng berry (GB). Several studies have reported that the GB contains higher concentrations of biologically active ginsenosides than other ginseng parts[21-24]. As the interest in the GB has increased recently, it's pharmacological activities have also been reported such as: anti-diabetic[25-27], anti-cancer[22, 28], anti-oxidant, anti-aging[29-31], anti-stress[32], and anti-allergic[23, 33]effects, and the active

components were purified and identified as ginsenoside Re[23]. Especially, it also reported that ginsenoside-free molecules from steam dried GB promote ethanol metabolism and can be applied as an alternative choice for an alcohol hangover[24], and aqueous extracts of GB (GBe) showed favorable anti-obese and blood flow improvement activities in HFD supplied rats, through their potent antioxidant effects[34]. However, the action mechanism, the molecular target of GBe on anti-obese and blood flow improvement activities, were unclear, upon our knowledge. Therefore, the present study investigated the anti-obese and blood flow improvement activities of Gbeon the mild diabetic obese mice, the 45%Kcal HFD supplied mice[35-37].

II. Materials and methods

1. Animals and husbandry

Total eighty-five female SPF/VAF CrljOri:CD1 [ICR] mice (6-wk old upon receipt; OrientBio, Seungnam, Korea) were used after acclimatization for 7 days. Animals were allocated four to fiveper polycarbonate cage in a temperature (20-25℃) and humidity (40-45%) controlled room. Light : dark cycle was 12hr : 12hr, and standard rodent chow (Cat. No. 38057; Purinafeed, Seungnam, Korea) and water were supplied free to access. Adapt animals to HFD were selected at 4 weeks of adapt periods as six groups based on the body weights. All laboratory animals were treated according to the national regulations of the usage and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea) prior to animal experiment [Approval No. DHU2017-080].

2. Preparations and administration of test substances

GBe were prepared and supplied by Aribio Co. Ltd. (Seungnam, Korea), and stored at −20℃ in a refrigerator to protect from light and humidity until used as follows. GBe used in this study contained ginsenoside Re as specific ingredients as 30 mg/g by high-performance liquid chromatography (HPLC) analysis (Fig 1). White powders of metformin hydrochloride (Wako, Osaka, Japan) were used as reference recommendation drugs.

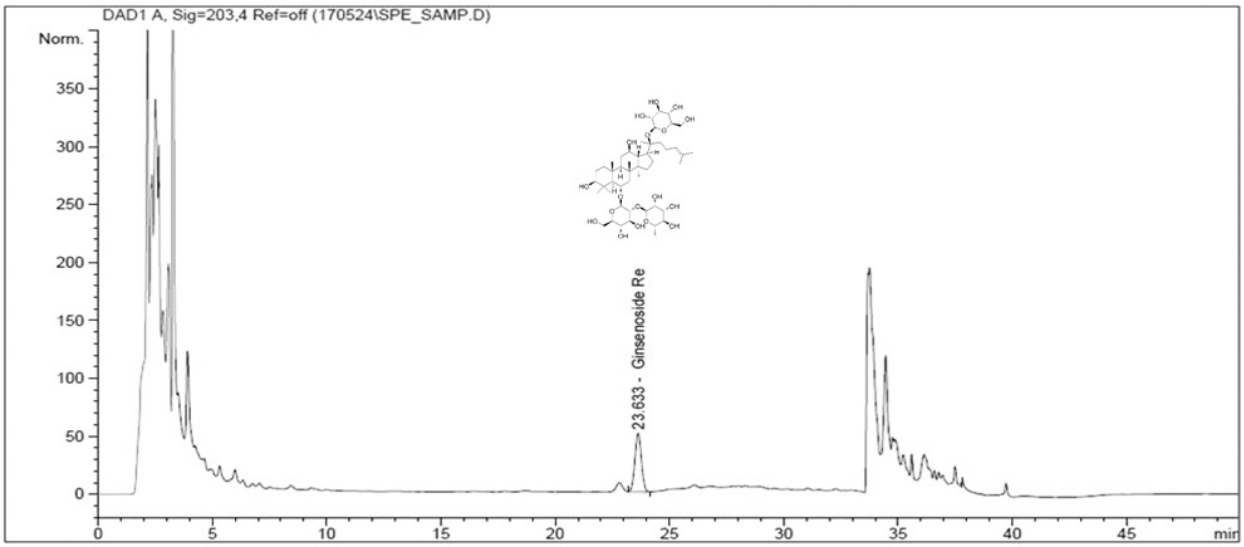

Figure 1. Representative HPL Cof Ginsenoside Re in GBe used in this study. HPLC = High performance liquid chromatography; GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae); GBe = GB aqueous extracts.

Appropriate amounts of GBe were dissolved in distilled water as 15, 10 and 5 mg/ml concentration, and were orally administered once a day for 56 days in a volume of 10 ml/kg (equivalence to 150, 100 and 50 mg/kg), using a stainless zonde attached to 1 ml syringe, from 4 weeks after HFD supply. In addition, metformin HCl was dissolved in distilled water as 25 mg/ml concentrations and also orally administered in a volume of 10 ml/kg, as equivalence to 250 mg/kg, once a day for 56 days from 4 weeks after initial HFD supply. The dosage of GBe was selected as same as previous HFD supplied rat study[34]. In intact vehicle and HFD control mice, equal volumes of distilled water were also orally administered, instead of test substances to provide same restrain stresses from gastric gavages, respectively.

3. HFD supply

Animals were supplied 45%Kcal HFD (Cat. No. D12451; Research Diet, New Brunswick, NJ, USA) free to access listed in Table 1 after 7 days of acclimatization. In intact control mice, NFD (Cat. No. 38057; Purinafeed, Seungnam, Korea) was supplied free to access instead of HFD. Adapt animals to HFD were selected at 4 weeks (27 days) of adapt periods as six groups (eight mice in each groups, total 40 HFD supplied mice and 8 NFD supplied mice) based on the body weights.

4. Changes in body weights

Changes of body weights were measured at 28 days (at just immediately before start of HFD supply) and 1 day before initiation of administration, initial administration day, and then weekly to 24 hrs after last 56th administration using an automatic electronic balance (XB320M, Precisa Instrument, Zuerich, Switzland). At initiation of administration and at a termination, all experimental animals were overnight fasted (water was not; about 12 hrs) to reduce the differences from feeding. In addition, body weight gains were

Table 1. Formulas of normal and high fat diets used in this study

Compositions*	Normal pellet diets	High fat diets
Ingredient (g/kg)		
Casein	200	200
L-Cystein	3	3
Corn starch	150	72.8
Sucrose	500	172.8
Cellulose	50	50
Soybean Oil	50	25
Lard	0	177.5
Mineral mixture	35	35
Vitamin mixture	10	10
Choline bitartrate	2	2
Energy (kcal/g)	4.00	4.73
Protein (% kcal)	20	20
Carbohydrate (% kcal)	64	35
Fat (% kcal)	16	45

* 45%Kcal/Fat pellet diets (Cat. No. D12451; Research Diet, New Brunswick, NJ, USA) were used as high fat diet (HFD) and normal rodents pellet diet (Cat. No. 38057; Purinafeed, Seungnam, Korea) were used as normal fat pellet diets

additionally calculated during adapt periods (from 28 days to 1 day before initial test article administration) and administration periods (from the day of initial test article administration to sacrifice, 24 hrs after last 56th test article administration)

5. Estimation of body fat density: Total and abdominal fat mass (%)

The mean fat densities on the total body and abdominal cavity regions of each mouse were detected by in live DEXA (InAlyzer; Medikors, Seungnam, Korea), once at end of 84 days continuous treatment of test substances, according to our previous report[38].

6. Tail bleeding time

Tail bleeding times were measured using the method described previously[39, 40]. Briefly, mice were anesthetized by 25 mg/kg intraperitoneal injection of Zoletile mixture (Zoletile 50™ Virbac Lab., Carros, France) at 24 hrs after end of last 56th test material administration with overnight fasting. Tails of mice were transected by surgical scissor at 4 mm from their tips. Then, transected tail tips were immersing into 37℃ saline at a right angle. Bleeding time was defined as the time elapsed until bleeding stopped. When the bleeding time exceeded 15 min, bleeding time was recorded as 15 min for the analysis.

7. Coagulation assays

At end of 56 days continuous test article treatment, bloods were collected from vena cava, under inhalation anesthesia with 2 to 3% isoflurane (Hana Pharm. Co., Hwasung, Korea) in the mixture of 70% N_2O and 28.5% O_2, using rodent inhalation anesthesia apparatus (Surgivet, Waukesha, WI, USA) and rodent ventilator (Model 687, Harvard Apparatus, Cambridge, UK), and plasma for the coagulation assays was obtained by centrifugation (3,000 rpm, 10 min under room temperature) using 3.2% sodium citrate contained vaccutainer (Becton Dickinson, Franklin Lakes, NJ, USA). PT and aPTT were measured within 2 hrs of sample collection using automated coagulation analyzers (Sysmex CA-540, Sysmex Co., Kobe, Japan) according to the manufacturer's instructions.

8. Measurement of serum biochemistry

Some collected bloods from vena cava at 56 days after initial test substance treatment under inhalation anesthesia, were deposited into clotting activated serum tubes (Becton Dickinson, Franklin Lakes, NJ, USA), and centrifuged at 15,000 rpm for 10 min under room temperature for separating the serum to TC, TG, LDL and HDL measurement. Serum biochemistrical levels were measured using automated blood analyzer (Model Dri-Chem NX500i; Fuji Medical System Co., Ltd., Tokyo, Japan), after stored in −150℃ an ultra deep freezer (MDF-1156, Sanyo, Tokyo, Japan).

9. Serum cGMP, NO and ET-1 concentration detection

Serum concentrations of cGMP, NO and ET-1 were measured using specific ELISA according to previous reports[41-43]. cGMP ELISA Kit (Colorimetric) (MBS168636, MyBioSource, San Diego, CA, USA), Mouse total NO ELISA kit (MBS720290, MyBioSource, San Diego, CA, USA) and Mouse ET-1 (EDN1) ELISA kit (MBS722206, MyBioSource, San Diego, CA, USA) were used in this measurement. All ELISA procedures were performed according to the manufacturer's instructions, with the exception of the dilution ratio. After being thawed to room temperature, standards and samples (100 μL) were added to the appropriate wells and in-

cubated for 2 hrs at 37℃. The wells were aspirated and 1×biotin-labeled antibody (100 μL) was added to each well and incubated for 1 hr at 37℃. The wells were aspirated, washed three times, and incubated with 1×horseradish peroxidase-conjugated avidin (100 μL) for 1 hour at 37℃. 3,3',5,5'-Tetramethylbenzidine substrate (90 μL) was added to each well and incubated for approximately 15~30 min at 37℃. Stop solution (50 μL) was added to each well, and the absorbance at 450 nm was measured using a microplate Reader (Tecan, Männedorf, Switzerland). Duplicate readings were performed for allsamples, and the average reading was recorded.

10. Measurement of aorta cGMP, NO, ET-1, pPI3K, pAKT and pp38 MAPK levels

At sacrifice, 24 hrs after end of last 56th GB 150, 100 and 50 mg/kg or metformin 250 mg/kg oral administration, some aorta tissues were separated and collected at 1 mm apart from aortic arch to end of thoracic aorta, diaphragm attached site, respectively. Separated aorta tissues were weighed and homogenized in ice-cold 0.01M Tris-HCl (pH 7.4) with bead beater (Taco™Pre, GeneResearch Biotechnology Corp., Taichung, Taiwan) and ultrasonic cell disruptor (KS-750, Madell Technology Corp., Ontario, CA, USA), and then centrifuged, at 12,000 × g for 15 min under 4℃ conditions as described by other investigators[41-43]. cGMP ELISA Kit (Colorimetric) (MBS 168636, MyBioSource, San Diego, CA, USA), Mouse total NO ELISA kit (MBS720290, MyBioSource, San Diego, CA, USA), Mouse ET-1 (EDN1) ELISA kit (MBS722206, MyBioSource, San Diego, CA, USA), pPI3K ELISA Kit (ab207485, Abcam, Cambridge, UK), RayBioTM Human, Mouse and Rat pAKT (Ser473) ELISA Kit (PEL-AKT-S473, RayBiotech, Norcross, GA, USA), and Mouse pP38 MAPK ELISA kit (MBS 722905, MyBioSource, San Diego, CA, USA) were used in this measurement. Tissue homogenates were stored in an ultradeep freezer under -150℃ until analysis. The concentrations of aorta cGMP, NO, ET-1, pPI3K, pAKT and pp38 MAPK levels were determined by a microplate Reader (Tecan, Männedorf, Switzerland) at absorbance 450 nm. Conents of total protein were measured by previous method[44] using bovine serum albumin (Invitrogen, Carlsbad, CA, USA) as internal standard.

11. *Realtime* RT-PCR analysis

Aorta vascular dilation and constriction related gene mRNA expressions - PI3K, Akt, eNOS, p38 MAPK and ET-1 To elucidate the mechanisms by which GBe exert blood flow impairment effects, we investigated aorta vascular dilation and constriction related gene mRNA expressions - PI3K, Akt, eNOS, p38 MAPK and ET-1 through *realtime* RT-PCR analysis, according to the previous reports[7, 45]. Briefly, RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentrations and quality was determined by CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). To remove contaminating DNA, samples were treated with recombinant DNase I (DNA-free; Ambion, Austin, TX, USA). RNA was reverse transcribed using the reagent High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Analysis was carried out using ABI Step One Plus Sequence Detection System (Applied Biosystems, Foster City, CA, USA), and their expression levels were calculated as relative to vehicle control. The following thermal conditions were applied as 10 min at 94℃ and 39 cycles of 15 sec at 94℃, 20 sec at 57℃ and 30 sec at 72℃. The data were normalized by GAPDH mRNA expression, using comparative threshold cycle method[46]. The sequences of the PCR oligonucleotide primers were as follows: PI3K, 5'- TCC

AAATACCAGCAGGATCA −3' and 5'−ATGCTTCG ATAGCCGTTCTT−3' Akt, 5'−TACTCATTCCAGACC CACGA−3' and 5'−GAGGTTCTCCAGCTTCAGGT−3' eNOS, 5'−TTTGTCTGCGGCGATGT−3' and 5'−GTG CGTATGCGGCTTGT−3' P38MAPK, 5'−CGTTGTT TCCTGGTACAGACC−3' and 5'−CCATTTCTTCTTG GTCAAGGG−3' ET−1, 5'−TGCCTCTGAAGTTAGC CGTG−3' and 5'−AGTTCTCCGCCGCCTTTTTA−3' GAPDH, 5'−CATCTTCCAGGAGCGAGACC−3' and 5'−TCCACCACCCTGTTGCTGTA−3'.For quantitative analysis, the intact control aorta tissue was used as the control, and the relative expression of PI3K, Akt, eNOS, p38 MAPK and ET−1 was calculated using the $2^{-\Delta\Delta Ct}$ method[47].

12. Statistical analyses

All numerical values are expressed mean ± standard deviation (SD) of eight mice. Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test followed by least−significant differences multicomparison(LSD) test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity were observed at Levene test, a non−parametric comparison test, Kruskal−Wallis H test was conducted. When a significant difference is observed in the Kruskal−Wallis H test, the Mann−Whitney U (MW) test with Bonferroni's correction was conducted to determine the specific pairs of group comparison, which are significantly different. Statistical analyses were conducted using SPSS for Windows (Release 14.0K, IBM−SPSS Inc., Chicago, IL, USA).

Ⅲ. Results

1. Effects on the body weight changes

We selected only adapted mice shows regular body weight increases as compared with intact (normal diet supplied) control during 4 weeks of HFD supply (intact control: mean 32.70±1.09 g, ranged in 31.50~34.20 g; HFD supplied group:

Table 2. Changes on body weight gains in NFD or HFD supplied mice

Groups \ Times	Body weights (g) at days after initial test substance treatment				Body weight gains during	
	28 days before[A]	1 day before [B]	0 day* [C]	56 days* [D]	Adapt period [B−A]	Administration period [D−C]
Controls						
NFD	27.98±0.67	32.70±1.09	30.78±1.13	34.26±0.94	4.73±0.63	3.49±0.62
HFD	27.91±0.54	42.96±0.68[a]	41.06±0.74[a]	54.00±3.03[a]	15.05±0.55[a]	12.94±3.07[a]
Reference						
Metformin	27.94±0.48	43.01±0.45[a]	40.98±0.71[a]	45.31±1.48[ab]	15.08±0.34[a]	4.34±1.19[b]
Test material − GBe						
150 mg/kg	27.96±0.60	43.00±0.60[a]	41.05±0.60[a]	45.16±1.25[ab]	15.04±0.29[a]	4.11±1.36[b]
100 mg/kg	27.90±0.46	42.95±0.71[a]	40.98±0.65[a]	47.13±1.43[ab]	15.05±0.33[a]	6.15±1.38[ab]
50 mg/kg	27.99±0.48	43.04±0.62[a]	41.13±0.68[a]	48.93±1.26[ab]	15.05±0.18[a]	7.80±1.52[ab]

Values are expressed as Mean ± SD of eight mice. NFD = Normal pellet diet HFD = 45%Kcal high fat diet GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae) GBe = GB aqueous extracts. Metformin were administrated at a dose level of 250 mg/kg. * All animals were overnight fasted. [a]$p<0.01$ as compared with intact control [b]$p<0.01$ as compared with HFD control.

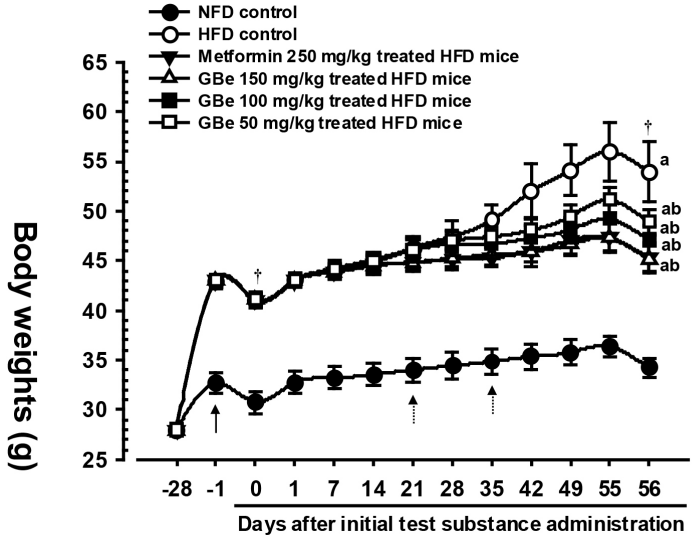

Figure 2. Body weight changesin NFD or HFD supplied mice. We selected only adapted mice shows regular body weight increases as compared with NFD supplied intact control during 4 weeks of HFD supply, consequently, HFD control mice showed significant increases of body weights as compared with intact control mice from 27 days after HFD supply (arrow). However, significant decreases of the body weights were detected in metformin 250 mg/kg and GBe 150mg/kg treated mice from 21 days after start of administration, and from 35 days after initial administration in GBe 100 and 50 mg/kg treated mice as compared with HFD control, respectively (dot arrow). Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated mice also showed obvious dose-dependent decreases of body weights during 56 days of administration periods as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in the present study. Values are expressed as Mean ± SD of eight mice. NFD = Normal pellet diet HFD = 45%Kcal high fat diet; GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae) GBe = GB aqueous extracts. All animals were overnight fasted before initial test substance administrations and sacrifice (†). $^{a}p<0.01$ as compared with intact control; $^{b}p<0.01$ as compared with HFD control.

mean 42.99±0.59 g, ranged in 42.10~44.00 g), consequently, HFD control mice showed significant ($p<0.01$) increases of body weights as compared with intact mice from 27 days after HFD supply, and accordingly the body weight gains during 27 days of HFD adaption and 56 days of administration periods were also significantly ($p<0.01$) increased as compared with intact control, respectively. However, significant ($p<0.01$ or $p<0.05$) decreases of the body weights were detected in metformin 250 mg/kg and GBe 150 mg/kg treated mice from 21 days after start of administration, and from 35 days after initial administration in GBe 100 and 50 mg/kg treated miceas compared with HFD control, and accordingly the body weight gains during 56 days of administration were also significantly ($p<0.01$) decreased in metformin 250 mg/kg, GBe 150, 100 and 50 mg/kg treated mice as compared with HFD control, respectively. Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated micealso showed obvious dose-dependent decreases of body weights and body weight gains during 56 days of administration periods as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in the present study(Table 2, Fig 2).

2. Effects on the body fat density: Total and abdominal fat mass (%)

Significant ($p<0.01$) increases of total body and abdominal fat densities were detected in HFD control as compared with intact control, respectively. On the contrary, significant ($p<0.01$)

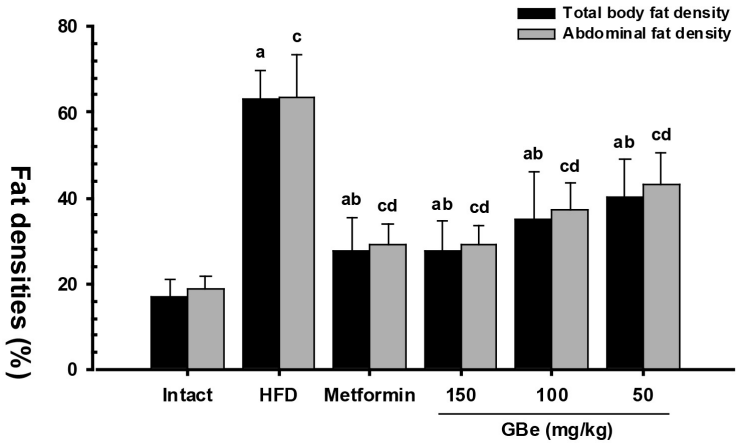

Figure 3. Total body and abdominal fat densities in NFD or HFD supplied mice. Values are expressed mean ± SD of eight mice. NFD = Normal pellet diet; HFD = 45%Kcal high fat diet; GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae); GBe = GB aqueous extracts; DEXA = Dual-energy x-ray absorptionmetry. Metformin were administrated at a dose level of 250 mg/kg. $^{a}p<0.01$ as compared withintact control; $^{b}p<0.01$ as compared with HFD control.

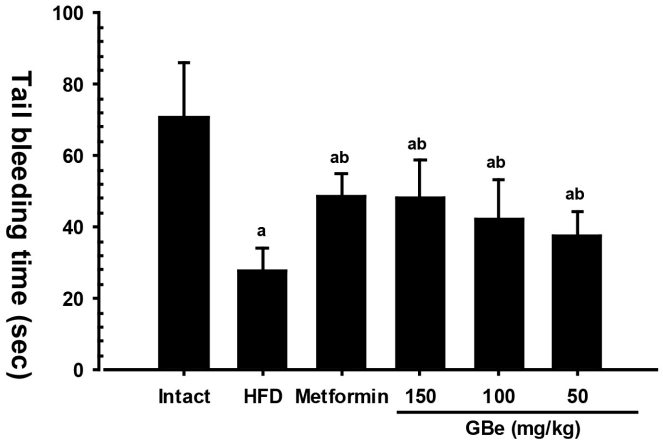

Figure 4. Tail bleeding time in NFD or HFD supplied mice. Values are expressed mean ± SD of eight mice. NFD = Normal pellet diet; HFD = 45%Kcal high fat diet; GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae); GBe = GB aqueous extracts. Metformin were administrated at a dose level of 250 mg/kg. $^{a}p<0.01$ as compared withintact control; $^{b}p<0.01$ as compared with HFD control.

decrease of total body and abdominal fat masses were detected in all test substances treated mice including GBe 150 mg/kg, at analysis of in live DEXA, respectively. Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated mice also showed clear dose-dependent decreases of total body and abdominal fat masses as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in the current study(Fig 3).

3. Effects on the tail bleeding time

Significant ($p<0.01$) decreases of the tail bleeding times were detected in HFD control as compared with intact control. However, these shortening of tail bleeding times were significantly ($p<0.01$) inhibited by treatment of all test substances including GBe 100 mg/kg, respectively. Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated mice also showed

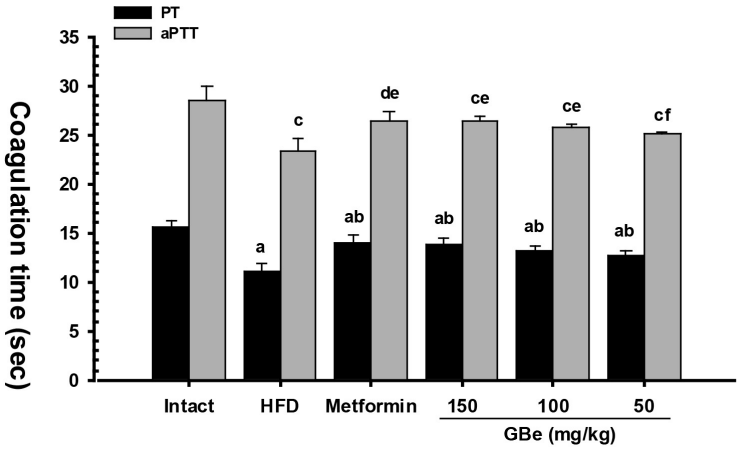

Figure 5. Blood PT and aPTT in NFD or HFD supplied mice. Values are expressed mean ± SD of eight mice. NFD = Normal pellet diet; HFD = 45%Kcal high fat diet; GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae); GBe = GB aqueous extracts; PT = Prothrombin time; aPTT = Activated partial thromboplastin time. Metformin were administrated at a dose level of 250 mg/kg. $^{a}p<0.01$ and $^{b}p<0.05$ as compared with intact control; $^{c}p<0.01$ and $^{d}p<0.05$ as compared with HFD control.

definitive dose-dependent prolongation of tail bleeding times as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in our study(Fig 4).

4. Changes on the coagulation factor: PT and aPTT

Similar to those of tail bleeding time, significant ($p<0.01$) shortening of PT and aPTT were detected in HFD control as compared with intact control, respectively. However, these decreases of PT and aPTT were significantly ($p<0.01$ or $p<0.05$) inhibited by treatment of all test substances including GBe 50 mg/kg, respectively. Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated mice also showed dose-dependent increases of the PT and aPTTas comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in the present experiment(Fig 5).

5. Serum biochemistry: TC, TG, LDL and HDL level changes

Significant ($p<0.01$) increases of serum TC, TG and LDL levels, and decreases of serum HDL levels were detected in HFD control as compared with intact control. However, the serum TC TG and LDL levels were significantly ($p<0.01$) decreased in all test substances including metformin 250 mg/kg, as compared with HFD control, respectively. On the contrary, the serum HDL levels were significantly ($p<0.01$) increased in all test substances including GBe 150 mg/kg, as compared with HFD control, respectively. Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated mice also showed dose-dependent decreases of the serum TC,TG and LDL levels, and increases of serum HDL levelsas comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in this measurement(Table 3).

6. Changes on the serum cGMP, NO and ET-1 contents

Significant ($p<0.01$) decreases of serum NO contents, and increases of serum ET-1 levels were detected in HFD control as compared with intact control. However, these HFD-induced serum NO and ET-1 content changes were significantly ($p<0.01$) normalized by treatment of all test substances including GBe 150 mg/kg, as compared

Table 3. Changes on the serum lipid levels in NFD or HFD supplied mice

Items / Groups	Serum levels (mg/dl)			
	Total cholesterol	Triglyceride	Low density lipoprotein	High density lipoprotein
Controls				
NFD	104.25±26.50	58.13±15.46	16.13±2.90	90.38±19.49
HFD	281.38±42.09[a]	211.63±34.60[a]	80.63±11.78[a]	21.13±7.08[a]
Reference				
Metformin	176.38±26.67[ab]	112.38±22.24[ab]	38.38±13.02[ab]	66.25±16.95[ab]
Test material − GBe				
150 mg/kg	173.63±27.98[ab]	114.00±17.35[ab]	40.38±11.92[ab]	65.75±12.91[ab]
100 mg/kg	195.00±23.38[ab]	127.38±15.55[ab]	48.88±13.65[ab]	55.38±17.65[ab]
50 mg/kg	211.38±25.77[ab]	166.88±15.95[ab]	58.50±11.77[ab]	44.63±11.50[ab]

Values are expressed as Mean ± SD of eight mice. NFD = Normal pellet diet; HFD = 45%Kcal high fat diet; GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae); GBe = GB aqueous extracts. Metformin were administrated at a dose level of 250 mg/kg. [a]$p<0.01$ as compared with intact control; [b]$p<0.01$ as compared with HFD control.

Table 4. Changes on the serum cGMP, NO and ET-1 levels in NFD or HFD supplied mice

Items / Groups	Serum levels		
	cGMP (fM/ml)	NO (μM/L)	ET-1 (ng/L)
Controls			
NFD	9.71±1.30	55.21±11.24	3.61±0.52
HFD	10.60±2.32	29.28±5.97[a]	6.07±0.87[a]
Reference			
Metformin	10.00±3.81	44.24±6.14[bc]	4.23±0.47[bc]
Test material − GBe			
150 mg/kg	9.68±1.98	45.44±5.26[bc]	4.15±0.42[bc]
100 mg/kg	10.05±2.62	40.22±2.52[bc]	4.47±0.42[ac]
50 mg/kg	9.87±2.63	37.71±4.73[ac]	5.02±0.41[ac]

Values are expressed as Mean ± SD of eight mice. NFD = Normal pellet diet; HFD = 45%Kcal high fat diet GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae); GBe = GB aqueous extracts cGMP = Cyclic guanosine monophosphate; NO = Nitric oxide; ET-1 = Endothelin-1, preproendothelin-1. Metformin were administrated at a dose level of 250 mg/kg. [a]$p<0.01$ and [b]$p<0.05$ as compared with intact control [c]$p<0.01$ as compared with HFD control.

with HFD control, respectively. Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated mice also showed dosedependent increases of the serum NO levels, and decreases of serum ET-1 levels as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in this measurement. No significant changes on the serum cGMP contents were demonstrated in HFD control mice as compared to those of intact control mice, and also no significant changes on the serum cGMP contents were observed in all test article administered mice including GBe 100 mg/kg, as compared to those of HFD control mice, in the current measurement(Table 4).

Table 5. Changes on the aorta cGMP, NO, ET-1, pPI3K, pAkt and pp38 MAPK levels in NFD or HFD supplied mice

Items / Groups	Aorta contents					
	cGMP (fM/mg protein)	NO (μM/mg protein)	ET-1 (pM/mg protein)	pPI3K (pg/mg protein)	pAkt (pg/mg protein)	pp38 MAPK (pg/mg protein)
Controls						
Intact	10.44±1.60	53.04±11.57	1.71±0.27	1.41±0.34	1.29±0.28	0.87±0.23
HFD	4.65±0.98[a]	23.25±4.88[a]	3.61±0.48[a]	3.15±0.45[a]	2.55±0.33[a]	2.23±0.50[a]
Reference						
Metformin	7.04±0.90[ac]	38.49±6.55[bc]	2.35±0.52[bc]	2.12±0.26[ac]	1.63±0.19[ac]	1.32±0.28[ac]
Test material − GBe						
150 mg/kg	7.10±1.40[ac]	37.26±6.35[ac]	2.39±0.43[ac]	2.14±0.34[ac]	1.59±0.22[bc]	1.33±0.16[ac]
100 mg/kg	6.67±0.76[ac]	33.85±3.44[ac]	2.70±0.38[ac]	2.46±0.25[ac]	1.72±0.21[ac]	1.40±0.29[ac]
50 mg/kg	6.13±0.98[ad]	29.94±3.85[ad]	3.02±0.18[ac]	2.62±0.10[ac]	1.99±0.17[ac]	1.63±0.26[ac]

Values are expressed as Mean ± SD of eight mice. NFD = Normal pellet diet; HFD = 45%Kcal high fat diet; GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae); GBe = GB aqueous extracts; cGMP = Cyclic guanosine monophosphate; NO = Nitric oxide ET-1 = Endothelin-1, preproendothelin-1; pPI3K = Phosphorylated phosphoinositide 3-kinase; pAkt = Phosphorylated protein kinase B; pp38 MAPK = Phosphorylated p38 mitogen-activated protein kinases. Metformin were administrated at a dose level of 250 mg/kg. [a]$p<0.01$ and [b]$p<0.05$ as compared with intact control [c]$p<0.01$ and [d]$p<0.05$ as compared with HFD control.

7. Changes on the aorta cGMP, NO, ET-1, pPI3K, pAkt and pp38 MAPK contents

Significant ($p<0.01$) decreases of aorta cGMP and NO contents, and increases of aorta ET-1, pPI3K, pAkt and pp38 MAPK contents were detected in HFD control as compared with intact control. However, these HFD-induced aorta cGMP, NO, ET-1, pPI3K, pAkt and pp38 MAPK content changes were significantly ($p<0.01$ or $p<0.05$) normalized by treatment of all test substances including GBe 50 mg/kg, as compared with HFD control, respectively. Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated mice also showed dose-dependent increases of the aorta cGMP and NO contents, and decreases of aorta ET-1, pPI3K, pAkt and pp38 MAPK contents as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in the present analysis(Table 5).

8. Aorta vascular dilation and constriction related gene − PI3K, Akt, eNOS, p38 MAPK and ET-1 mRNA expressions

Significant ($p<0.01$) increases of aorta PI3K, Akt, p38 MAPK and ET-1 mRNA expressions, and decreases of aorta eNOS mRNA expressions were detected in HFD control as compared with intact control. However, these HFD-induced abnormal aorta PI3K, Akt, eNOS, p38 MAPK and ET-1 mRNA expression changes were significantly ($p<0.01$) normalized by treatment of all test substances including metformin 250 mg/kg, as compared with HFD control, respectively. Especially, all three different dosages of GBe 150, 100 and 50 mg/kg treated mice also showed dose-dependent decreases of the aorta PI3K, Akt, p38 MAPK and ET-1 mRNA expressions, and increases of aorta eNOS mRNA expressionsas comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, in the current *realtime* RT-PCR analysis

Table 6. Changes on the aorta vascular dilation and constriction related gene mRNA expressions in NFD or HFD supplied mice, *realtime* RT-PCR analysis

Items / Groups	Aorta mRNA expressions (Relative to control/GAPDH mRNA expressions)				
	PI3K	Akt	eNOS	p38 MAPK	ET-1
Controls					
Intact	0.99±0.07	1.01±0.13	1.00±0.06	1.05±0.08	1.010.11
HFD	2.88±0.29[a]	2.73±0.65[a]	0.39±0.09[a]	4.58±0.90[a]	2.64±0.39[a]
Reference					
Metformin	1.52±0.23[ab]	1.47±0.18[ab]	0.79±0.11[ab]	2.03±0.45[ab]	1.52±0.28[ab]
Test material - GBe					
150 mg/kg	1.62±0.27[ab]	1.42±0.19[ab]	0.76±0.08[ab]	1.93±0.19[ab]	1.64±0.29[ab]
100 mg/kg	1.95±0.21[ab]	1.73±0.34[ab]	0.68±0.10[ab]	2.61±0.33[ab]	1.88±0.21[ab]
50 mg/kg	2.14±0.27[ab]	1.97±0.22[ab]	0.57±0.06[ab]	3.19±0.58[ab]	2.01±0.31[ab]

Values are expressed as Mean ± SD of eight mice. NFD = Normal pellet diet; HFD = 45%Kcal high fat diet; GB = Ginseng berry (Berries of *Panax ginseng* CA Meyer, Araliaceae); GBe = GB aqueous extracts; PI3K = Phosphoinositide 3-kinase; Akt = Protein kinase B; eNOS = Endothelial nitric oxide synthase, nitric oxide synthase 3; p38 MAPK = P38 mitogen-activated protein kinases; ET-1 = Endothelin-1, preproendothelin-1; GAPDH = Glyceraldehydes3-phosphate dehydrogenase. Metformin were administrated at a dose level of 250 mg/kg. [a]$p<0.01$ as compared with intact control [b]$p<0.01$ as compared with HFD control.

(Table 6).

Ⅳ. Discussion

The accumulation or increases of fat deposition in body is major characteristics of obesity, and cellular hypertrophy appeared to be the major mode of expansion of the intra-abdominal adipose tissue in rodents[35, 36, 48, 49]. Adipose tissue is currently known to work not simply as an organ for energy storage, but also as an endocrine and secretory organ[35, 36]. Adipose tissues secret adipokines and changes in the expression, secretion, and action of the adipokines in obesity are possibly implicated in the development of various diseases including insulin resistance[49-51]. In the present study, 56 days continuous oral treatment of GBe 150, 100 and 50 mg/kg significantly and dose-dependently inhibited the accumulation of total body and abdominal fat masses as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, at analysis of in live DEXA, respectively. These results are considered as reliable evidences that GBe 150, 100 and 50 mg/kg have favorable anti-obese effectsin HFD mice, as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, at least in a condition of the present DEXA and histopathological analysis.

Obesity is characterized by multiple hemostasis disorders of blood coagulation including strengthening of platelet activation[52], increasing concentration and enhancing activity of plasma coagulation factors[53-55]. In addition, systematic inflammation[56, 57], plasma proteins originating from the adipose tissue, such as endocannabinoids, leptin and adiponectin[58], disturbance of lipid and glucose metabolism also induce the coagulation in obesity[59, 60]. Blood coagulation times measured in the present study are aPTT and PT, the most commonly used clotting time assays in mammals[40, 61]. aPTT, PT and TT assess the function of the intrinsic pathway, the extrinsic pathway and the common pathway, respectively[61]. Prolongation of aPTT suggests the inhibition of intrinsic and/or common pathway, and PT prolongation suggests the inhibition of extrinsic

and/or common pathway.[39, 40] In addition, tail bleeding time assay is one of the most commonly used in vivo coagulation assays, and shortening of tail bleeding time indicated the impairment of blood flow[39, 40].

Noticeable shortening of PT, aPTT and tail bleeding time have been detected in HFD supplied rodents as blood flow impairment[61-63]. In the current study, significantly decreases of PT, aPTT and tail bleeding time were also demonstrated in HFD control mice as compared to those of intact control mice, but they were significantly and dose-dependently normalized by oral administration of GBe 150, 100 and 50 mg/kg as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg. These findings on the coagulation assays are considered as reliable evidences that GBe 150, 100 and 50 mg/kg showed favorable blood flow improvement effects in HFD-induced type II diabetic mice as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, at least, in a condition of this study.

As chronic progress of diabetes in HFD mice, hyperlipidemia was also, generally occurred[64]. Since the most critical problem in hyperlipidemia is increases of serum LDL, TG and TC levels with decrease of HDL levels[35, 36, 48], the efficacy of hypolipidemic agents generally evaluated based on the decrease of serum LDL, TG and TC with increase of HDL levels[48, 49]. In the present study, GBe 150, 100 and 50 mg/kg effectively and dose-dependently decreased the serum LDL, TG and TC levels, and increased the serum HDL levels as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, respectively. These results are once again, considered as direct evidences that GBe 150, 100 and 50 mg/kg have favorable hypolipidemic effects and related blood flow improvement activities in HFD mice, as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, at least in a condition of the current serum biochemistrical analysis.

Obesity is associated with endothelial dysfunction, leading to reduced levels of NO and consequentially cGMP, which may causatively manifests as impaired blood vessel relaxation and hypertension[65, 66]. Dramatic decreases of aorta cGMP and NO, and serum NO levels have been demonstrated in HFD supplied animals without significant changes on serum cGMP levels[43, 66]. ET-1, a potent vasoconstrictor[67], was also involved in lipid metabolism[68, 69] and has been implicated in the pathogenesis of obesity-associated hypertension[70, 71]. Noticeable upregulation of ET-1 also has been detected in HFD supplied type II diabetic mice serum and aorta[41, 42]. In our study, significant decreases of aorta cGMP and NO, serum NO levels, increases of serum and aorta ET-1 contents, and upregulation of aorta ET-1 mRNA were also demonstrated in HFD control without significant changes on the serum cGMP levels, as HFD-induced blood flow impairment. However, these abnormal changes on serum and aorta cGMP, NO and ET-1 were significantly and dose-dependently normalized by oral administration of GBe 150, 100 and 50 mg/kg as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg. These findings are considered as obvious evidences that GBe 150, 100 and 50 mg/kg showed favorable blood flow improvement effects in HFD-induced type II diabetic mice as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, through cGMP, NO and ET-1 modulatory expressions, at least partially, in a condition of the present experiment.

Insulin is not only a principal regulator of glucose homeostasis but also a vasoactive hormone involved in modulation of vascular tone[72]. In the vasculature, insulin exerts both vasodilator and vasoconstrictor effects by promoting the endothelial production of NO and the release of ET-1[73]. Insulin-stimulated NO production in endothelial cells is mediated by the PI3K/Akt signaling cascade, which in turn phosphorylates

and activates eNOS[74]. On the other hand, insulin-induced expression and secretion of the vasoconstrictor ET-1 is mediated by the extracellular signal-regulated kinase (ERK) 1/2 MAPK signaling pathway in vascular endothelium[75]. Activation of ERK1/2 increases both mRNA expression and secretion of ET-1 in endothelial cells[74]. The balanced endothelial production of NO and ET-1 is critical in maintaining both metabolic and hemodynamic homeostasis under the healthy condition[72]. Vascular insulin resistance, manifested by impaired vasodilator effects and augmented vasoconstrictor actions of insulin, is a key phenomenon linking obesity, diabetes, and CVD[76, 77]. In insulin-resistant states such as ageing and obesity, insulin-induced activation of PI3K/Akt signaling is selectively impaired, and also in MAPK pathway[78, 79]. Endothelial dysfunction is not only a well-established antecedent of hypertension and atherosclerosis but also an important contributor to metabolic insulin resistance by reducing the capillary recruitment and blood flow in skeletal muscle[72, 79]. The p38 MAPK signaling pathway is an important member of MAPK family. As a kinase activated by oxidative stress, p38 MAPK primarily participates in apoptosis, immune regulation, cell transdifferentiation, and inflammatory response in response to oxidative stress[80]. The p38 MAPK signaling pathway is activated by many stimulating factors such as reactive oxygen species, inflammatory factors, high glucose, and angiotensin II, thus exacerbating CVD[81-83], and phosphorylation and upregulation of p38 MAPK also have been detected in human and animals of diabetes related CVD[80, 84, 85]. Significant increases of aorta pPI3K, pAkt and pp38 MAPK levels were demonstrated in HFD control of the current experiment, with significant increases of aorta PI3K, Akt and p38 MAPK mRNA expressions and also significant decreases of aorta eNOS mRNA expressions as compared to those of intact control at *realtime* RT-PCR analysis. However, these HFD-induced abnormal phosphorylation or expressions of PI3K, Akt, eNOS or p38 MAPK were significantly and dose-dependently normalized by oral administration of GBe 150, 100 and 50 mg/kg as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg. These findings are considered as definitive evidences that GBe 150, 100 and 50 mg/kg showed favorable blood flow improvement effects in HFD-induced type II diabetic mice as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, through PI3K/Akt pathway and p38 MAPK mediated cGMP, NO and ET-1 expression modulatory activities, at least partially, in a condition of this experiment, once again.

Ⅴ. Conclusion

In the current study, the anti-obese and blood flow improvement effects of GBe were observed on the HFD-induced mild diabetic obese mice, as compared to those of metformin, a representative anti-diabetic drugs for type II diabetes and related side effects including CVD through PI3K/Akt pathway modulation, at a dose level of 250 mg/kg. As results of GBe 56 days of continuous oral administration at dose levels of 150, 100 and 50 mg/kg, HFD supplement-induced obesity, and PI3K/Akt pathway and p38 MAPK dysregulation dependent diabetic vascular disorders - blood flow impairment were significantly inhibited, dose-dependently. Especially, GBe 150 mg/kg constantly showed favorable inhibitory activities against type II diabetes related obesity and vascular disorders through PI3K/Akt pathway and p38 MAPK modulations as comparable to those of metformin 250 mg/kg in HFD mice, respectively. These findings are considered as clear and direct evidences that GBe 150, 100 and 50 mg/kg showed favorable anti-obese and blood flow improvement effects in HFD-induced type

II diabetic mice, through PI3K/Akt pathway and p38 MAPK mediated cGMP, NO and ET-1 expression modulatory activities, as comparable to those of metformin 250 mg/kg in GBe 150 mg/kg, at least, partially in a condition of this experiment. It, therefore, is expected that GBe will be promising as a new potent refinement agent or medicinal food for various obese and blood flow impairments, in future.

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