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Mixture of Ethanol Extract of Grape Pomade and Omija Fruit Prevents Hyperglycemia and Alleviates Oxidative Stress in Mice Fed an Obesogenic Diet

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Abstract

Background: A high-fat diet (HFD) is thought to be one of the main environmental factors for obesity in which oxidative stress is an important pathogenic mechanism of obesity-associated metabolic syndrome including diabetes. Polyphenol-rich food plants are known to improve obesity-related diseases. The aim of this study was to investigate the effects of grape pomace (GP) and GP plus omija fruit (GPOF) on hyperglycemia and oxidative stress in diet-induced obese (DIO) mice.

Methods: Male C57BL/6J mice were fed an HFD with GP (0.5%, *w/w*) or GPOF (0.5% GP plus 0.05% omija fruit, *w/w*) for 12 weeks. One gram of GP contains 0.26 mg of total flavonoid and 0.475 mg of total polyphenol, while 1 g of GPOF contains 0.2635 mg of total flavonoid and 0.491 mg of total polyphenol.

Results: GP and GPOF significantly lowered fasting blood glucose level and insulin/glucagon ratio compared to HFD but increased plasma glucagon level. Supplementation with GP or GPOF improved glucose tolerance and the expressions of pancreatic insulin and glucagon while preserving α- and β-cells. GPOF in particular seems to improve insulin sensitivity by reducing plasma insulin and homeostasis model assessment of insulin resistance levels. Erythrocyte glutathione peroxidase and glutathione reductase activities were significantly lower in the GPOF group than in the HFD group along with decreases in erythrocyte hydrogen peroxide and thiobarbituric acid-reactive substance levels.

Conclusion: These results suggest that GPOF, which is rich in flavonoids, may be beneficial in preventing an increase in the risk factors for diabetes in obesity, including fasting hyperglycemia, glucose intolerance, and oxidative stress.

Keywords: Grape pomace; Omija fruit; Hyperglycemia; Oxidative stress; Obesity; Diet-induced obese mice; High-fat diet

Abbreviations: AUC: Area Under the Curve; DIO: Diet-induced Obese; GP: Grape Pomade; GPOF: Grape Pomade Plus Omija Fruit; GR: Glutathione Reductase; GSH-Px: Glutathione Peroxidase; H_2O_2: Hydrogen Peroxide; HFD: High-Fat Diet; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; IPGTT: Intraperitoneal Glucose Tolerance Test; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; TBARS: Thiobarbituric Acid-Reactive Substance

Introduction

Obesity is associated with genetic and environmental factors [1]. A high-fat diet (HFD), which has high energy density, is thought to be one of the main environmental factors [1]. Obesity is associated with various diseases including insulin resistance, diabetes, coronary heart disease, certain types of cancer, and sleep breathing disorders [2].

Insulin resistance and hyperinsulinemia are common features of obesity that are associated with an increased risk of diabetes [1]. Insulin resistance in obesity is characterized by an impaired ability of insulin to inhibit glucose output from the liver and promote glucose uptake by muscle and fat cells [3]. Dysfunction of pancreatic islet β-cells in obesity, which involves failure to compensate for decreased insulin sensitivity, is critical in the development of type 2 diabetes [4].

Increased oxidative stress is an important pathogenic mechanism of obesity-associated metabolic syndrome, which includes diabetes, hyperlipidemia, and hepatic steatosis. It has been reported that increased reactive oxygen species (ROS) production and lipid peroxidation from accumulated fat lead to increased oxidative stress in the blood and hazardously affect other organs [5]. Oxidative stress in the diabetic state interrupts insulin secretion from pancreatic β-cells and glucose uptake by muscle and fat cells [6,7].

Polyphenols, which are richly available in various edible plants, have been known to improve metabolic diseases. They can exhibit antioxidant, anti-obesity, anti-inflammatory, and anti-diabetes effects [8]. For example, polyphenol-rich grapes and their by-products reportedly reduce obesity-mediated type 2 diabetes, lipid peroxidation, and inflammation by multiple mechanisms, thereby preventing

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metabolic diseases [9]. Omija (*Schisandrachinensis*), another polyphenol-rich plant that has been used in traditional Chinese medicine and pharmacotherapy of European and other countries, plays an important role in lowering blood glucose level and regulating the endocrine and immune systems and antioxidant enzyme activities [10,11]. We previously demonstrated that combined extracts of grape pomace (GP) and GP plus omija fruit (GPOF) showed anti-obesity and anti-inflammatory effects in diet-induced obese (DIO) mice [12] and an anti-diabetes effect in *db/db* mice [13]. The aims of the present study were to examine whether GPOF has blood glucose– and oxidative stress–lowering effects in mice fed an obesogenic diet and compare its effects with those of GP.

Materials and Methods

Preparation of extracts

GP (skin and stem) and omija fruits (*FructusSchisandrae*) were used in this study. The grapes (*Vitisvinifera*, Muscat bailey Aspecies) and omija (*Schisandrachinensis*, Baillon) were purchased from Gyeongsangbuk-do, Korea. Samples were prepared by adding 2 L of 80% and 50% ethanol to 100 g of dried GP and omija fruit, respectively; extraction was performed at 80°C for 2 h after which the solution was cooled. It was then filtered (Whatman paper No. 2), concentrated with a rotary vacuum evaporator, and stored at -70°C. The final weight of the GP ethanol extract was 19.9 g (recovery rate: 19.9%) and that of the omija fruit ethanol extract was 39.7 g (recovery rate: 39.7%). A 1 g sample of GP ethanol extract contains 0.2 mg of resveratrol, 52 mg of total flavonoid, and 95 mg of total polyphenol. The same amount of omija fruit ethanol extract contains 8 mg of schizandrin, 7 mg of total flavonoid, and 32 mg of total polyphenol.

HPLC anlaysis of index compounds

Chemical concentrations of index were simultaneously determined using the HPLC method. The apparatus used was an Agilent 1100/1200 system (Agilent, Waldbronn, Germany) consisting of a binary pump, thermostated auto sampler (injection volume = 30 Ml and 20 µL, grape pomace ethanol extract and omija fruit ethanol extract, respectively), column oven (set=35°C), and a DAD detector (306 and 254 nm, grape pomace ethanol extract and omija fruit ethanol extract, respectively). The column was a Eclipse XDB-C18, 250 mm × 4.6 mm, 5 µm (Agilent). Eluent was acetonitrile and water with 0.05% (v/v) TFA gradient. Flow rate were 0.6 mL/min and 0.8 mL/min, running time were 25 min, grape pomace ethanol extract and omija fruit ethanol extract, respectively. The HPLC chromatograms of resveratrol standard, grape pomace extract, schizandrin standard and omija fruit extract are illustrated in Figures 1C,1D, 2C and 2D.

Animal and diets

Male mice (strain C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 4 weeks of age. The animals were individually housed with a constant temperature (24°C) and 12-h light/dark cycle and fed a pelletized commercial non-purified diet for 1 week after arrival. The mice were then randomly divided into 3 groups (n=10) and fed the high-fat diet and experimental diets for 12 weeks: HFD (20% HFD based on AIN-76 diet plus 1% cholesterol, *w/w*), GP extract (HFD plus 0.5% GP extract, *w/w*), and the combined extracts of GP and omija fruit (GPOF, GP diet plus 0.05% omija fruit extract, *w/w*). The composition of the diets is presented in Table 1. The mice had free access to food and distilled water during the experimental period and their body weights were measured weekly.

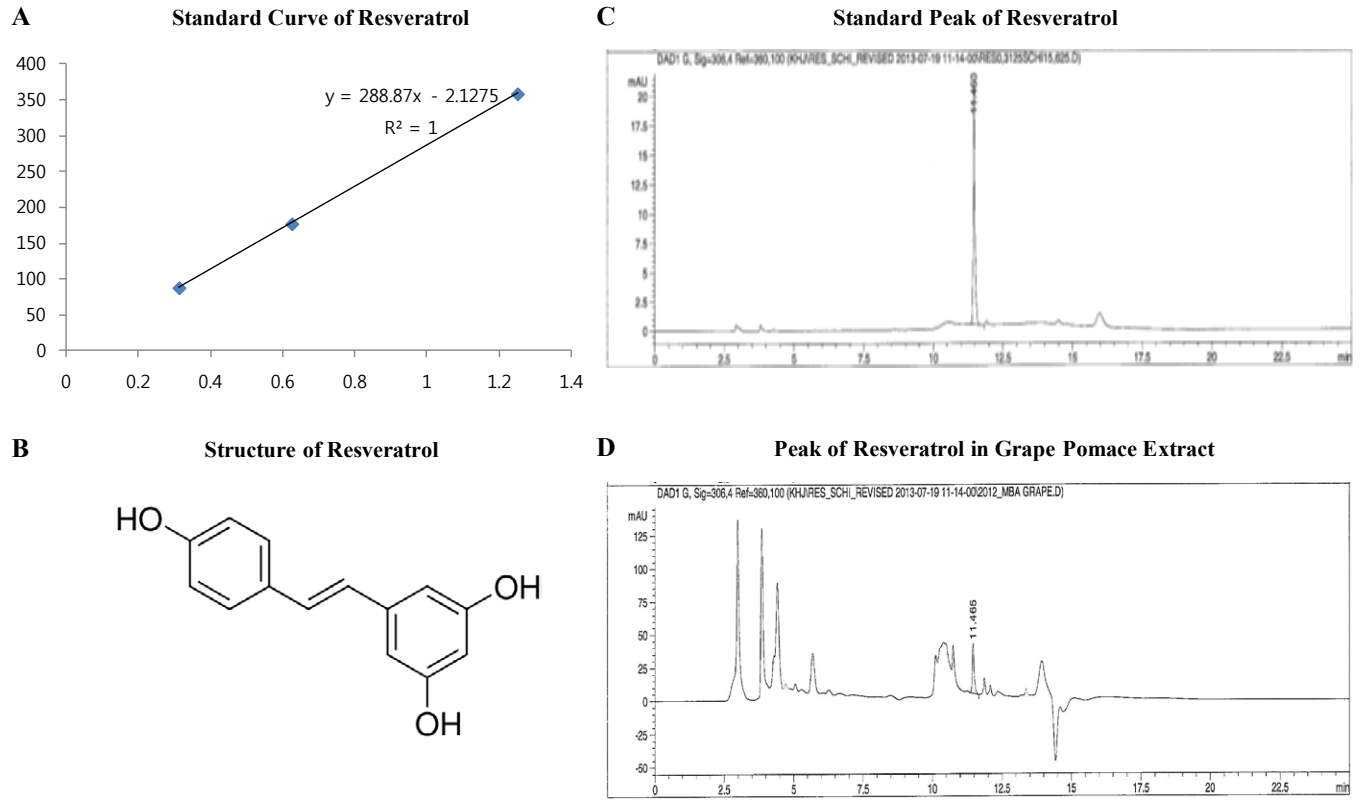

Figure 1: HPLC chromatogram of resveratrol in grape pomace extract. (A) Standard curve of resveratrol, (B) Structure of resveratrol, (C) Standard peak of resveratrol, (D) Peak of resveratrol in grape pomace extract.

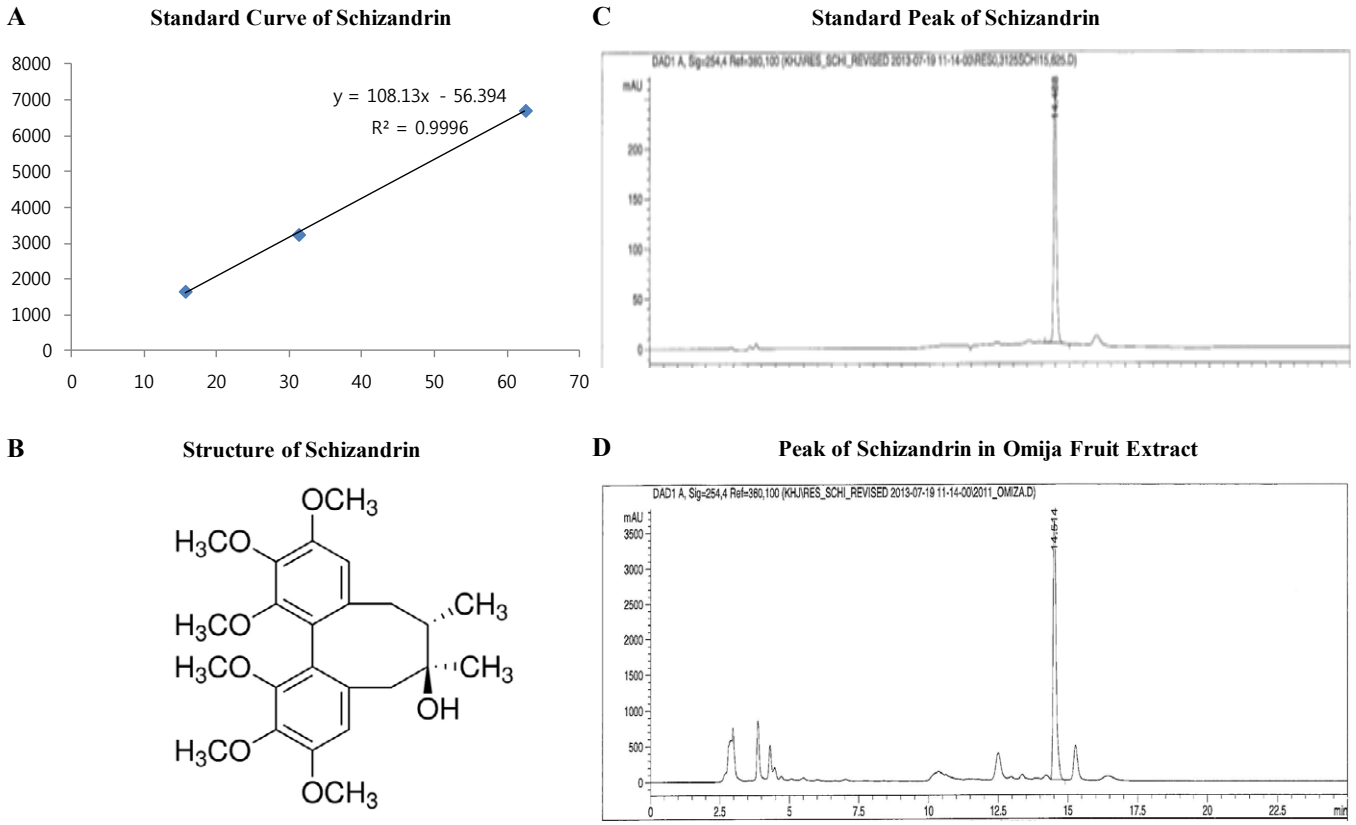

Figure 2: HPLC chromatogram of schizandrin in omija fruit extract. (A) Standard curve of schizandrin, (B) Structure of schizandrin, (C) Standard peak of schizandrin, (D) Peak of schizandrin in omija fruit extract.

Ingredients	HFD	GP	GPOF
Casein	20	20	20
D, L-Methionine	0.3	0.3	0.3
Sucrose	36.996	36.496	36.446
Cellulose	5	5	5
AIN-mineral[1)]	4.2	4.2	4.2
AIN-vitamin[2)]	1.2	1.2	1.2
Choline bitartrate	0.2	0.2	0.2
Cornstarch	11.1	11.1	11.1
Lard	17	17	17
Corn oil	3	3	3
Cholesterol	1	1	1
Tert-butylhydroquinone	0.004	0.004	0.004
Grape pomace extract		0.5	0.5
Omija fruit extract			0.05
Total	100	100	100

HFD, high-fat diet; GP, HFD plus grape pomace extract (0.5%, *w/w*); GPOF, GP plus omija fruit extract (0.05%, *w/w*). *n*=10 animals per group.

Table 1: Composition of the experimental diets (unit: % of diet).

At the 12th week, the mice were anesthetized with diethyl ether and sacrificed after a 12-h fast. Blood was taken from the inferior vena cava and then centrifuged at 1000 × *g* for 15 min at 4C, and the plasma was separated for analysis of the plasma biomarkers. After blood collection, the pancreas was removed, rinsed, and fixed in 1% hydrogen peroxide (H_2O_2). This animal study protocol was approved by the Ethics Committee for animal studies at Kyungpook National University, Republic of Korea (approval No. KNU 2011 - 28).

Blood glucose and intraperitoneal glucose tolerance test (IPGTT)

The blood glucose concentration was measured in the whole blood obtained from the tail veins after withholding of food for 12 h using a GlucDrsupersensor glucose analyzer (Allmedicus). The IPGTT was performed at the 10th week. After a 12-h fast, the mice were injected intraperitoneally with glucose (0.5 g/kg body weight). The blood

glucose levels were determined from the tail vein at 0, 30, 60, and 120 min after the glucose injection. The cumulative changes in blood glucose responses were quantified based on the incremental area under the curve (AUC).

Plasma insulin level, glucagon level, and homeostasis model assessment of insulin resistance levels

The levels of plasma insulin and glucagon were determined with a multiplex detection kit (Bio-Rad). All samples were assayed in duplicate and analyzed with a Luminex 200 Labmap system (Luminex). Data analyses were done with the Bio-Plex Manager software version 4.1.1 (Bio-Rad). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as previously described: HOMA-IR = [fasting insulin concentration (mU/L)] × [fasting glucose concentration (mg/dL) × 0.05551]/22.5.

Immunohistochemical analysis of the pancreas

For the immunohistochemical analysis of the pancreatic α- and β-cells, the islets were sectioned, fixed in 1% hydrogen peroxide, and washed in 0.01 M citrate buffer (pH 6.0). These sections were treated with blocking reagent (Ultra Tech horseradish peroxidase [HRP]) to prevent nonspecific binding and incubated with monoclonal antibodies against insulin and glucagon (Santa Cruz Biotech, Inc.). Antibody reactivity was detected using HRP-conjugated biotin–streptavidin complexes and developed with diaminobenzidinetetrahydrochloride as the substrate. Stained areas were viewed using an optical microscope with a magnifying power of ×200.

H_2O_2 and lipid peroxidation assay

The H_2O_2 level in the erythrocytes was measured by Wolff's method [14]. FOX 1 reagent consisted of 100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM H_2SO_4. Fifty microliters of the test sample was added to 950 μL of FOX 1 reagent, vortexed, and incubated at room temperature for a minimum of 30 min, at which point color development is virtually complete. The absorbance was read at 560 nm, and the standard was linear in the 0-5 μM concentration range. The erythrocyte thiobarbituric acid-reactive substance (TBARS) concentration, as a marker of lipid peroxide production, was measured spectrophotometrically using the method of Ohkawa et al. [15].

Antioxidant enzymes activities

Paraoxonase activity was measured using the method developed by Mackness et al. [16] with slight modification, and the activity expressed as the produced *p*-nitrophenol in nmol/min/mg protein. Superoxide dismutase (SOD) activity was spectrophotometrically measured by the inhibition of pyrogallol autoxidation at 420 nm for 10 min according to the method of Marklund and Marklund [17]. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. Catalase activity was measured using Aebi's method [18] with slight modifications, in which the disappearance of H_2O_2 was monitored spectrophotometrically at 240 nm for 5 min. A molar extinction coefficient of 0.041 $mM^{-1}\,cm^{-1}$ was used to determine catalase activity.

Glutathione peroxidase (GSH-Px) activity was measured using Paglia and Valentine's method [19] with slight modifications. The reaction mixture contained 1 mmol/L glutathione, 0.2 mmol/L NADPH, and 0.24 units of glutathione reductase in a 0.1 mol/L Tris-HCl (pH 7.2) buffer. The reaction was initiated by the addition of 0.25 mmol/L H_2O_2 and the absorbance was measured at 340 nm for 5 min. A molar extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$ was used to determine the activity. Glutathione reductase (GR) activity was determined using the method of Pinto and Bartley [20] by monitoring the oxidation of NADPH at 340 nm. The reaction mixture contained 1 mM EDTA and 1 mM GSSG in 0.1 M potassium phosphate buffer (pH 7.4). The activity was expressed as oxidized NADPH in nmol/min/mg protein. The hemoglobin concentration was estimated in an aliquot of the hemolysate using a commercial assay kit (Sigma-Aldrich).

Statistical analysis

The statistical analyses were performed with the Statistical Package for Social Science software program (SPSS, Inc.). Significant differences between the means were determined by one-way ANOVA. Duncan's multiple-range test was performed if differences were identified between the groups at $p<0.05$. All data are expressed as mean with the standard error of the mean.

Results

Body weight and glycemiablood markers

Body weights were significantly lower at the 6th, 7th, 8th, and 11th weeks, although there was no significant difference in final body weight in the GPOF group compared to the HFD group (Figure 3A). Supplementation with GPOF also suppressed blood glucose increases from the 6th week and significantly decreased fasting blood glucose levels at the 8th, 10th, and 12th weeks (Figure 3B). Both the GP and GPOF groups had significantly lowered fasting blood glucose levels at the final week and significantly improved glucose tolerance on the IPGTT at 120 min after glucose load compared to the HFD group (Figures 3B and 3C). Moreover, GP- and GPOF-supplemented mice exhibited significant decreases in AUC compared to the HFD group (Figure 3D). Plasma insulin level was significantly lower in the GPOF group and tended to be lower in the GP group than in the HFD group, while plasma glucagon levels were significantly increased in these two groups (Figure 3E and 3F). Thus, insulin/glucagon ratios were significantly decreased in the GP and GPOF groups compared to those in the HFD group (Figure 3G). Supplementation with GPOF significantly lowered HOMA-IR level compared to the HFD group as well (Figure 3H).

Immunohistochemical analysis of the pancreas

We performed the immunohistochemical analysis of the pancreas using anti-insulin and anti-glucagon antibodies. Examination of the pancreas morphology using optical microscopes suggested that insulin and glucagon protein expression was better preserved in islets of GP- and GPOF-supplemented DIO mice than those of HFD mice, although further studies are required to validate these finding (Figures 4A and 4B).

Levels of H_2O_2 and TBARS and/or antioxidant enzyme activities in erythrocytes and plasma

There were no significant differences in the activities of erythrocyte SOD and catalase among the three groups (Figure 5A). However, erythrocyte GSH-Px and GR activities were significantly lowered in the GPOF group than in the HFD group. Plasma paraoxonase activity was significantly increased by GPOF supplementation in the DIO mice (Figure 5A). GPOF treatment significantly lowered erythrocyte H_2O_2 and lipid peroxidation levels compared to the HFD group (Figure 5B). The level of TBARS, which is formed as a byproduct of lipid peroxidation, was also significantly lowered in the GPOF group compared with that in the HFD and GP groups (Figure 5B). Supplementation with GP significantly decreased the TBARS level and GR activity in erythrocytes

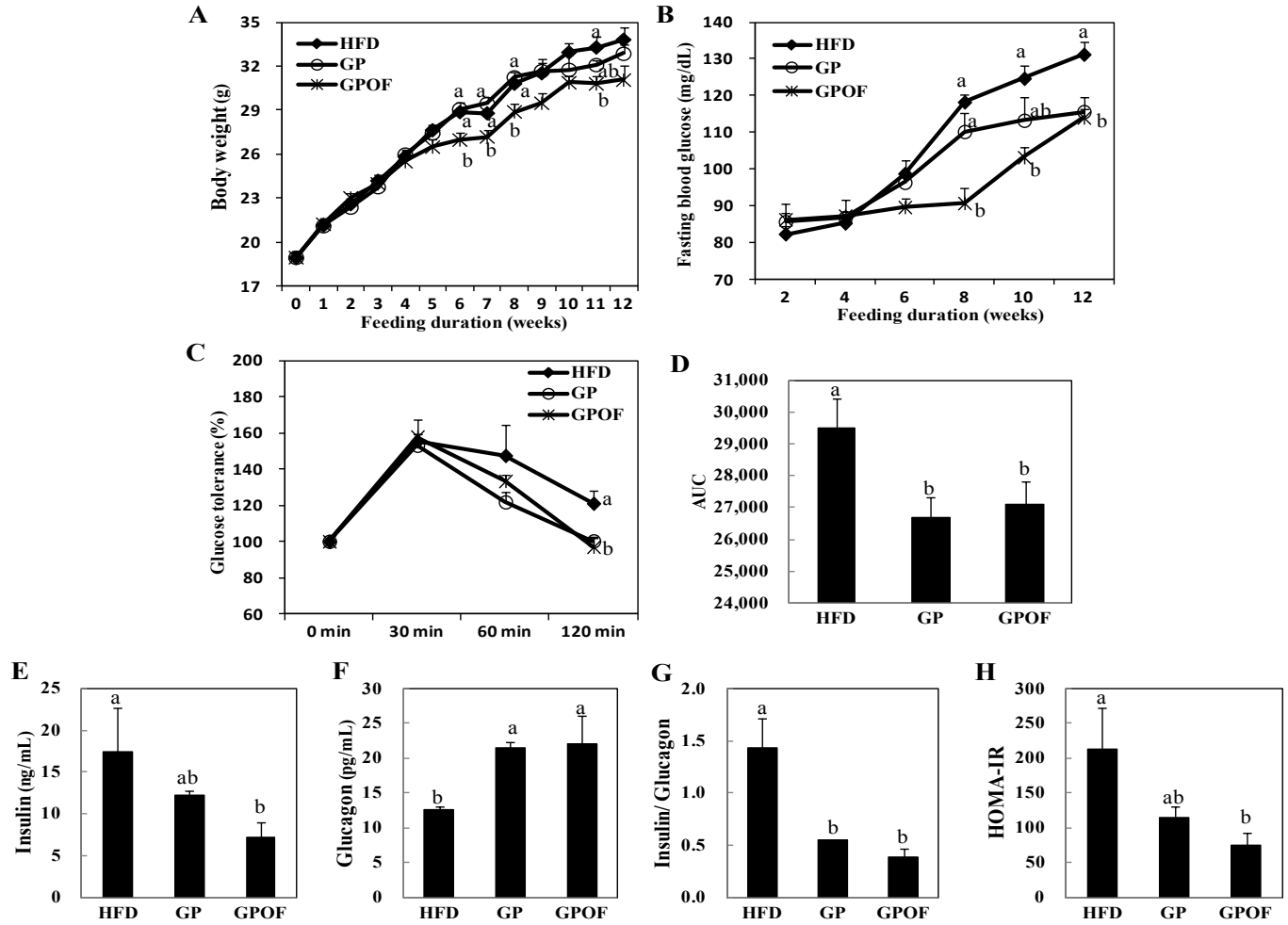

Figure 3: Effects of GP or GPOF on body weight (A), fasting blood glucose (B), IPGTT (C), AUC (D), plasma insulin (E), plasma glucagon (F), insulin/glucagon ratio (G), and HOMA-IR (H) levels in DIO mice. A-H: Data are the means ± SE (n=10).[a,b,c] Means not sharing a common letter are significantly different among the groups at p<0.05. HFD, high-fat diet; GP, HFD plus grape pomace extract (0.5%, *w/w*); GPOF, GP diet plus omija fruit extract (0.05%, *w/w*); IPGTT, intraperitoneal glucose tolerance test; AUC, area under the curve; HOMA-IR, homeostasis model assessment of insulin resistance; DIO, diet-induced obese.

and significantly increased plasma paraoxonase activity compared to the HFD group (Figures 5A and 5B).

Discussion

The objective of this study was to investigate the effects of GPOF supplementation on hyperglycemia and oxidative stress in high-fat fed DIO mice. C57BL/6J mice fed an HFD develop oxidative stress–associated obesity and diabetes [21]. Supplementation of GPOF amelioratedadipogenesis, hepatic steatosis, and inflammation in DIO mice, and its effects were stronger than those of GP alone as reported by our previous study [12]. In the present study, we have firstly demonstrated that GPOF alleviates hyperglycemia, insulin resistance, and oxidative stress in DIO mice.

GP alone exerted a significant anti-postprandial hyperglycemic effect in diabetic mice [22], while GPOF lowered fasting hyperglycemia in type 2 diabetic *db/db* [13]. It is important to identify that GPOF can prevent blood glucose rise in DIO condition in the early stage (12 weeks). We observed that GP and GPOF significantly decreased fasting blood glucose level and improved glucose tolerance in DIO mice. HFD-fed DIO mice generally develop hyperglycemia and impaired glucose tolerance [23]. In addition, DIO mice showed dramatic increases in circulating insulin level and HOMA-IR [23]. An elevated HOMA-IR index is compatible with the presence of insulin resistance in the mice fed an HFD [24]. GPOF significantly decreased plasma insulin and HOMA-IR levels in HFD-fed DIO mice, but GP aloneseemed to be less capable than GPOF as tended to lower plasma insulin concentration and HOMA-IR value.

Use of the HFD in specific rodent strains (for example, C57BL/6J mice and Wistar rats) also results in decreased pancreatic insulin expression and β-cell mass but persistently high plasma insulin levels [25,26]. The β-cell is able to increase its insulin secretory response in the early state of insulin resistance in obese individuals to compensate for insulin resistance. However, once this compensatory mechanism fails, impairment of β-cell secretory function is accompanied by β-cell loss and apoptosis in C57BL/6J mice fed an HFD for 12 weeks [25]. An increased plasma insulin level inhibits glucagon secretion [27]. However, the present study demonstrated that GPOF decreased plasma insulin level and increased plasma glucagon levels in DIO mice. Although GPOF was more effective at maintaining blood glucose concentration, both GPOF and GP preserved pancreatic

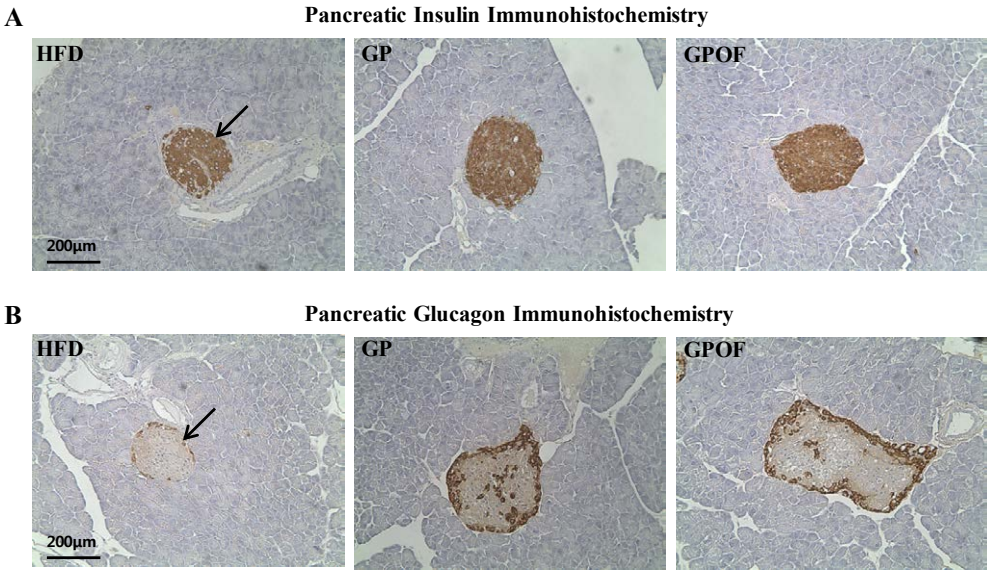

Figure 4: Effects of GP or GPOF on immunohistochemistry staining for pancreatic insulin (A), and glucagon (B) in DIO mice. A, B: Representative photomicrographs of the pancreas are shown at 200× magnification (n = 10). Arrows, α- (B) and β-cells (A). HFD, high-fat diet; GP, HFD plus grape pomace extract (0.5%, *w/w*); GPOF, GP diet plus omija fruit extract (0.05%, *w/w*); DIO, diet-induced obese.

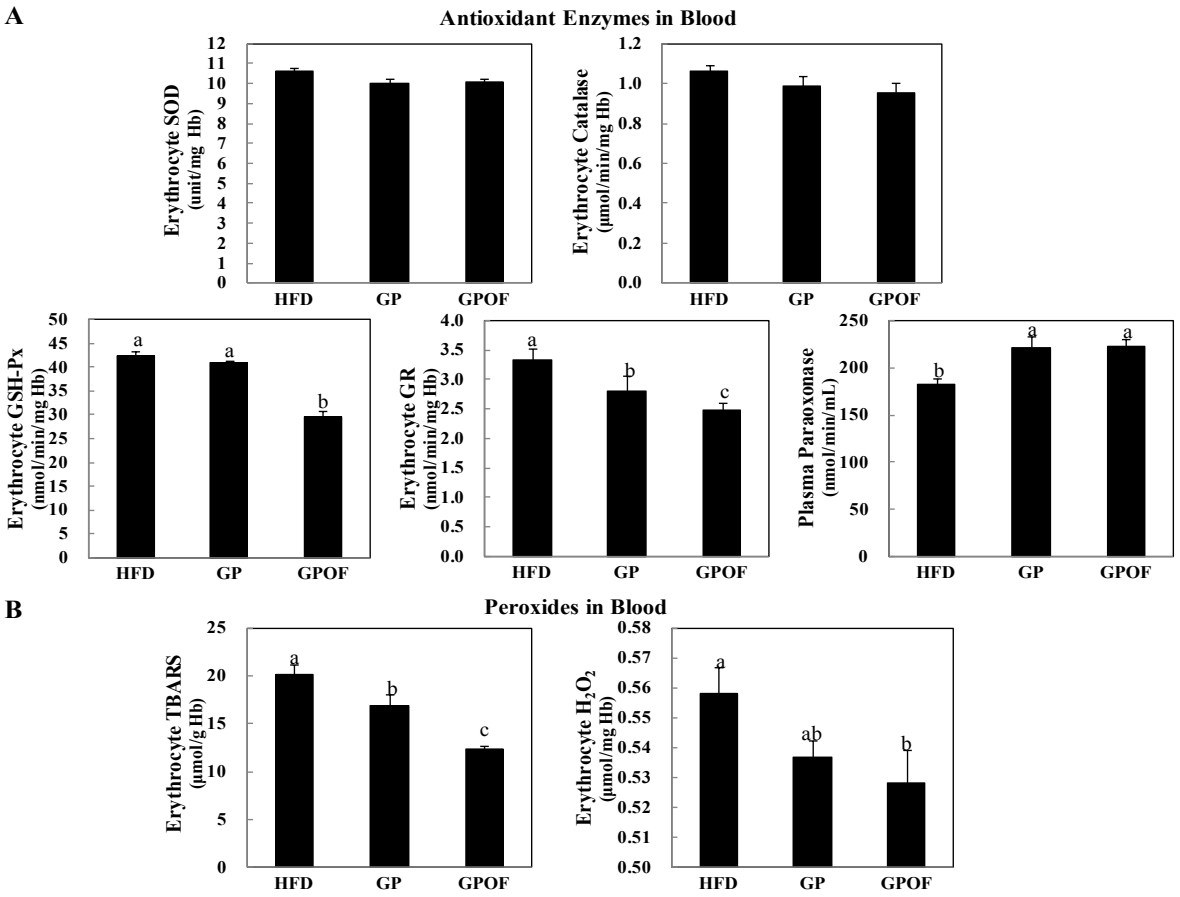

Figure 5: Effects of GP or GPOF on antioxidant enzymes activities (A) and peroxides (B) in the blood of DIO mice. A, B: Data are the means ± SE (n = 10).[a,b,c] Means not sharing a common letter are significantly different among the groups at p<0.05. HFD, high-fat diet; GP, HFD plus grape pomace extract (0.5%, *w/w*); GPOF, GP diet plus omija fruit extract (0.05%, *w/w*); SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; GR, glutathione reductase; TBARS, thiobarbituric acid-reactive substance; DIO, diet-induced obese.

islets architecture with increased pancreatic expressions of insulin and glucagon in HFD-induced obese mice. This study also suggests that the supplementation of GP or GPOF with an HFD could reduce the plasma insulin/glucagon ratio, which is positively associated with hyperlipidemia and hyperglycemia in obese mice [28]. We suppose that GPOF can protect against increased fasting blood glucose and plasma insulin levels, and insulin/glucagon ratio, which are observed in HFD-obese mice, by improving insulin resistance. Another possibility is due to the preserved islet after supplementation of GPOF.

The increased production of ROS such as H_2O_2 caused by fat accumulation may cause dysregulation of insulin sensitivity and adipocytokines [5]. Excessive and long-term exposure to ROS impairs glucose and lipid metabolism while reducing insulin sensitivity [5]. Malondialdehyde is used as a biomarker for the assessment of oxidative stress–induced lipid peroxidation and is usually determined spectrophotometrically as TBARS. Supplementation with GPOF significantly decreased H_2O_2 and TBARS levels in the erythrocytes, while GP only decreased the erythrocyte TBARS level in DIO mice.

Grape products are major sources of polyphenolic components such as anthocyanins, flavanols, catechins, and proanthocyanidins, which are known to be rich sources of dietary antioxidant compounds with potential health-promoting and disease-preventing effects [29]. Several animal studies have demonstrated that the dietary intake of grape extracts promotes antioxidant enzymes activities [30,31]. Furthermore, in some cases, an HFD did not change antioxidant enzymes activities or up-regulate antioxidant genes compared with a normal diet [32,33]. In the present study, however, erythrocyte GSH-Px and GR activities were significantly lowered in the GPOF group, although erythrocyte SOD and catalase activities was not increased in the GPOF-treated group compared to the HFD group. GSH-Px plays an important role in protecting erythrocytes against H_2O_2 by catalyzing H_2O_2 using GSH as a hydrogen donor by which it is oxidized. The regeneration of GSH is catalyzed by GR [34]. It is plausible that the decreased activities of GSH-Px and GR observed in the GPOF group were due to the decreased H_2O_2 concentrations in the erythrocytes. Since GP and GPOF treatments significantly increased plasma paraoxonase activity, a high-density lipoprotein-associated esterase [35], they could promote reversal of the cholesterol transport function of high-density lipoprotein in lowering the risk of coronary artery disease. Paraoxonase not only prevents low-density lipoprotein oxidation functioning as an antioxidant but also prevents insulin resistance and diabetes [36].

In conclusion, GP and GPOF reduced fasting hyperglycemia, glucose intolerance, and insulin/glucagon ratio and preserved pancreatic insulin and glucagon expressions in DIO mice. In particular, GPOF also improved hyperinsulinemia and HOMA-IR. The tandem use of GP and GPOF ameliorated the oxidative stress that is induced by obesity and insulin resistance. Therefore, the addition of GPOF is superior to GP alone in preventing obesity-associated hyperglycemia.

Acknowledgements

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