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Effects of Grape Pomace Antioxidant Extract on Oxidative Stress and Inflammation in Diet Induced Obese Mice

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Norton grape is one of the most important wine grapes in Southern and Midwestern states and generates massive pomace byproducts. The objective of this study is to characterize the antioxidant compounds and activity in Norton grape pomace extract (GPE) and further assess the potential health promoting properties of Norton GPE using an animal disease model. The total phenolic content and anthocyanins in Norton GPE were 475.4 mg of gallic acid equiv/g and 156.9 mg of cyanidin 3-glucoside equiv/g, respectively. Catechin and epicatechin in GPE were 28.6 and 24.5 mg/g, respectively. Other major antioxidants in GPE included quercetin (1.6 mg/g), *trans*-resveratrol (60 μg/g), gallic acid (867.2 μg/g), coutaric acid (511.8 μg/g), *p*-hydroxybenzoic acid (408.3 μg/g), and protocatechuic acid (371.5 μg/g). The antioxidant activity of GPE was evaluated by oxygen radical absorbance capacity (ORAC) and was 4133 μmol of Trolox equiv/g. Male diet-induced obese (DIO) mice were randomly divided to three treatment groups (n = 12): a normal diet (ND group), a high fat diet (HF group), and the high fat diet supplemented with GPE (HFGPE group). After 12-week treatment, mice in the high fat diet groups gained 29% more weight than the ND group. The GPE supplementation (estimated 250 mg/kg bw/d) lowered plasma C-reactive protein levels by 15.5% in the high fat diet fed mice ($P < 0.05$), suggesting a potential anti-inflammatory effect by dietary GPE. However, dietary GPE did not improve oxidative stress in DIO mice as determined by plasma ORAC, glutathione peroxidase, and liver lipid peroxidation. The results showed that GPE contained significant antioxidants and dietary GPE exerted an anti-inflammatory effect in diet induced obesity.

KEYWORDS: Grape pomace; antioxidants; obesity; oxidative stress; inflammation

INTRODUCTION

In the US, nearly two-thirds of adults and one-third of children are overweight or obese and the rates show no sign of decline. Obesity has become the nation's major public health problem (*1*). During the past decade, it has become clear that elevated levels of oxidative stress and inflammation are the key features of obesity (*2*). Obesity has been associated with a low degree pro-inflammatory state, in which impairments in the oxidative stress and antioxidant mechanism could be involved (*3*). The chronic inflammation and oxidative stress have been attributed to a causal role in multiple forms of obesity-associated complications such as insulin resistance, CVD, and type-2 diabetes (*4*). The mechanisms that underlie observed associations between obesity and elevated inflammation and oxidative stress are unclear. It has been postulated that increased adiposity in obesity is a key mediator of inflammation and oxidative stress, contributing to the production of proinflammatory cytokines and reactive oxygen species (*5*). The chronic inflammation itself is a source of oxidative stress, and in turn, elevated oxidative stress enhances the inflammatory response by activating redox-sensitive nuclear transcription factors (*6*). Consequently, the synergy between the elevated oxidative stress and inflammation phenomena plays a major role in the development of comorbidities in obesity (*4*). Therefore, an effective strategy to promote health and reduce disease risk in obese individuals could be the prevention and treatment of chronic oxidative stress and inflammation associated with obesity. Dietary antioxidants may provide a cost-effective strategy to promote health in obesity through the treatment of oxidative stress and chronic inflammation. Indeed, the protection that fruits and vegetables provide against chronic disease, including cancer and cardio- and cerebrovascular diseases, has been attributed to the various antioxidants contained in them (*7*). Although intervention trials examining the effect of antioxidant supplementation on oxidative stress in healthy adults showed a general lack of benefit (*8*), antioxidant supplementation of obese individuals demonstrated a consistent improvement in oxidative stress reduction (*9*). The anti-inflammatory effect of dietary antioxidants has been highlighted by the studies demonstrating that the total antioxidant capacity of the diet and plasma levels of antioxidants are inversely related to inflammation markers in both healthy adults (*10*) and patients with myocardial infarction (*11*).

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Animal and human clinical trials further demonstrated an improvement of systemic inflammation after antioxidant supplementation (*9*, *12*). Collectively, these findings suggest a beneficial role of dietary antioxidants in treating oxidative stress and inflammatory conditions.

Grape pomace is typically regarded as a waste product generated in the winemaking industry. Large amounts of grape pomace accumulate annually, which leads to a waste-management issue (*13*). On the other hand, grapes are known to be a rich source of dietary bioactive compounds in particular antioxidants with potential health promoting and disease protective qualities (*14*). For example, wine, grapes, and grape seed extracts are a major source of polyphenolic components such as anthocyanins, flavanols, catechins, and proanthocyanidins (*15*). Because grape skins and seeds are the predominant constituents in the pomace, this biomass is speculated to be one of richest and the most cost-effective sources of natural dietary antioxidants (*16*). However, no study has evaluated the effect of grape pomace-derived antioxidants on the elevated oxidative stress and inflammation associated with obesity.

The Norton grape has grown in popularity, especially in Southern and Midwestern states such as Virginia, Missouri, and Arkansas (*17*). This particular grape variety has good wine characteristics and is highly adaptable to local growing conditions, primarily humid regions with comparatively long growing seasons (*18*). Moreover, Norton grape is generally resistant to the primary fungal diseases, which has enhanced its attractiveness to wine growers due to increasing concerns on environmental protection and pesticide avoidance. Norton grape is therefore an important local wine grape and results in massive pomace byproducts.

Research has shown that the high calorie, low fiber dietary pattern of the diet was significantly associated with the increased risk of obesity (*19*). The C57BLK/6J mouse is a widely used diet-induced obese (DIO) animal model that develops obesity when fed a high fat diet formulated to approximate a typical human diet in Western cultures (*20*). The purpose of this study was to evaluate the effect of the dietary supplementation of grape pomace-derived antioxidants on oxidative stress and inflammation status in C57BLK/6J mice fed a high fat diet.

MATERIALS AND METHODS

Materials. Mouse insulin enzyme linked immunoassay (ELISA) kit was purchased from Millipore Corporation (St. Charles, MO), mouse CRP ELISA kit was obtained from Immunology Consultants Laboratory Inc. (Newberg, OR), cytosolic glutathione peroxidase (cGPx) kit was purchased from Cayman Chemical Company (Ann Arbor, MI), and bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotechnology (Rockford, IL). Folin−Ciocalteu reagent, Trolox, fluorescein, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from the Sigma Chemical Co. (St. Louis, MO). Phenolic standards including cyanidin 3-glucoside, catechin, epicatechin, quercetin, *trans*-resveratrol, caffeic acid, gallic acid, *p*-coumaric acid, syringic acid, and ferulic acid were also purchased from Sigma Chemical Co. The organic solvents for grape pomace extraction and HPLC analysis were HPLC grade (Fisher Scientific Co. Atlanta, GA).

Grape Pomace Extract (GPE) Preparation. Red wine grape pomace was obtained from a local Virginia vineyard (Blackstone, VA). The pomace sample was immediately freeze-dried upon receipt. The dried extract was then ground to a fine powder by a Thomas Wiley minimill (Swedesboro, NJ) and extracted with 80% ethanol at 1:10 ratio (m/v) under overnight shaking. The extract was filtered through Whatman No. 4 filter paper to remove unwanted residues. After evaporating off the organic solvent, the filtrates were frozen and lyophilized to obtain GPE. GPE was shipped to Research Diets Inc. (New Brunswick, NJ) for incorporation into a pelleted high fat diet.

Total Phenolic Content (TPC). The TPC of GPE was determined using Folin−Ciocalteu reagent with gallic acid as the phenolic standard (*21*). The TPC value was expressed as micrograms of gallic acid equivalents per gram of GPE (μg GAE/g).

Table 1. Macronutrient Content of the Diet per 10 MJ (megajoules)*

nutrient	high fat diet (HF and HFGPE groups)	normal diet (the ND group)
protein (g)	93.5 (17% of energy)	126.5 (23% of energy)
fat (g)	106.2 (41% of energy)	44.0 (17% of energy)
carbohydrate (g)	240.9 (43% of energy)	336.2 (60% of energy)

* Calculations were based on the diet composition data provided by Research Diets Inc. (New Brunswick, NJ).

Total Anthocyanin Content (TAC). The TAC of GPE was determined using a pH differential method with cyanidin 3-glucoside as the standard (*22*). TAC was expressed in milligrams of cyanidin 3-glucoside equivalents per gram of GPE (mg CGE/g).

Oxygen Radical Absorbance Capacity (ORAC). The ORAC assay was conducted to kinetically measure the peroxyl radical scavenging activity of GPE with Trolox as the antioxidant standard according to a method reported previously (*23*). Fluorescein (FL) was used as the fluorescent probe, and the peroxyl radicals were generated from AAPH in 75 mM phosphate buffer (pH 7.4). The fluorescence of the reaction mixture was monitored and recorded every minute (λ_{ex} = 485 nm and λ_{em} = 535 nm) and maintained at 37 °C until the reading had declined to less than 5% of the initial reading with a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland). Results for ORAC were determined by using a regression equation relating Trolox concentrations and the net area under the kinetic fluorescein decay curve. The ORAC value was expressed in micromoles of Trolox equivalents per gram of GPE (μmol TE/g).

Determination of Antioxidant Compounds in GPE by High Performance Liquid Chromatography−Mass Spectrometry (HPLC−MS). Individual phenolic compounds in GPE were characterized using HPLC−MS. GPE solution (20 μL) was injected onto a Luna C18 column (150 × 2.1 mm, 4 μm dp; Phenomenex, Torrance, CA) with a Thermo Survey autosampler (San Jose, CA) maintained at 10 °C. Mobile phase A consisted of 1% aqueous formic acid, and mobile phase B consisted 1% (v/v) formic acid in acetonitrile. The mobile phase was delivered to the HPLC column at a flow rate of 0.2 mL/min. The gradient elution program was as follows: 0 min, 97/3 A/B; 40 min, 70/30 A/B; 45 min, 0/100 A/B; 55 min, 0/100 A/B; 60 min, 97/3 A/B; 5 min postrun equilibration). The HPLC column effluent was pumped directly without any split into a Finnigan LCQDUO mass spectrometer (Thermo Scientific, Waltham, MA), which was operated in positive electrospray ionization at a temperature of 350 °C and a voltage of 4 kV. The mass spectrometer mass scan spectra were measured from *m*/*z* 150 up to *m*/*z* 1500 at 1000 amu/s. Individual phenolic compounds were then identified and quantified by comparing their MS spectra to the corresponding phenolic standards.

Animals and Diets. Male 6 week old C57BLK/6J mice (National Cancer Institute, Frederick, MD) were used to conduct this 12-week supplementation study. Animal husbandry, care, and experimental procedures were conducted in compliance with the "Principles of Laboratory Animal Care" NIH guidelines, as approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. Mice were housed in cages (4 mice/cage) at a regulated temperature (20−23 °C) and alternating 12 h light/dark cycles with access to mouse chow and water *ad libitum*. Mice were fed a standard rodent chow (Harlan Tekland Global Diets 2018 rodent diet, Madison, WI) for 2 weeks during the initial quarantine and acclimatization phase. After the acclimatization period, mice were randomly divided to three dietary treatment groups (n=12): a healthy normal diet (ND), a high fat diet (*24*), the high fat diet + GPE (HFGPE). The pelleted high fat diet (D12079B; Research Diets Inc., New Brunswick, NJ) contained 41% of calories from fat, 43% calories from carbohydrate, and 17% of calories from protein, and had a vitamin mixture containing α-tocopherol (3350 mg/kg feed) (**Table 1**). This diet resembles a typical high fat Western diet and has been shown to induce obesity and diabetes in mice (*25*). The dosage of the GPE in the feed was incorporated into pellet form at a mixture of 2.4 g/kg of feed in order to dose each mouse at approximately 250 mg GPE/kg body weight (bw) per day. Fresh feed, stored at 4 °C in light and air protected containers, was weighed and dispensed every three days. Body weight and feed intake were determined at weeks 0, 2, 4, 6, 8, 10, and 12. At termination (week 12), blood samples

were collected into ice chilled heparinized tubes from the retro orbital venous plexus while under anesthesia to obtain plasma for measurement of oxidative stress and inflammatory biomarkers. All mice were then sacrificed with cervical dislocation under anesthesia. Liver organs were immediately excised, washed/chilled in ice-cold 0.9% NaCl, weighed, and then snap frozen with liquid nitrogen. The plasma and liver samples were stored for 2 weeks at −20 °C and −80 °C, respectively (*26*).

Plasma C-Reactive Protein (CRP). An immunoperoxidase assay for determination of CRP at the termination time point (week 12) was used. The plasma CRP level was measured by ELISA using a plate reader at an absorbance of 450 nm and expressed as ng/mL.

Plasma Cytosolic Glutathione Peroxidase. The plasma antioxidant enzyme glutathione peroxidase (GPx) activity was measured with an assay kit following the manufacturer's instructions. The GPx assay was measured spectrophotometrically with the plate reader at an absorbance of 340 nm (nmol/min/mL). The bicinchoninic acid (BCA) protein assay was used for the determination of the total protein (μg/mL) in each plasma sample following manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The GPx values were expressed as nmol/min/g protein.

Plasma ORAC. Plasma ORAC was measured to determine the influence of grape phenolic supplementation on oxidative stress status following the 12-week study. The ORAC assay was conducted using the appropriate dilutions of the plasma samples compared to the Trolox solutions (*27*). The assay conditions were the same as described above for ORAC measurement of GPE. The ORAC value of each plasma sample was expressed as micromoles of Trolox equivalents per liter of the plasma sample (μmol TE/L).

Liver Lipid Peroxidation. Liver lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) and was expressed as micrograms of malondialdehyde (MDA) per gram of liver specimen using an established protocol with modification (*28*). In brief, equal amounts of liver tissue from each mouse were weighed (0.8 g) and homogenized in 10 mL of distilled deionized water with 0.002% propyl gallate and 0.004% ethylenediaminetetraacetic acid (EDTA), 0.1 mL of 2.0% sodium dodecyl sulfate (SDS) using a Virtishear homogenizer (Gardiner, NY). Next, 9 mL of distilled water was used to rinse the homogenizer head and then mixed into the homogenate with vortexing. For each sample, 1.0 mL of the homogenate was transferred into 4.0 mL of 0.38% TBA solution (0.51% SDS and 9.38% acetic acid (HF: v)). After vortexing, each sample mixture was incubated in a 95 °C water bath (Fisher Scientific, Pittsburgh, PA) for 60 min and cooled to room temperature. After cooling, 5.0 mL of 1-butanol and pyridine solution at a ratio of 15:1 was added to each sample mixture, thoroughly vortexed, and then centrifuged at 10 °C at 3000 rpm for 15 min. The absorbance of the upper organic layer of the centrifuged solution was measured at a wavelength of 532 nm with a spectrophotometer (Milton Roy Co., Rochester, NY). The final unit of TBARS value was calculated and expressed as milligrams of malondialdehyde (MDA) equivalents per kilogram of liver (mg MDA equivalent/kg).

Statistical Analysis. All data are expressed as the mean ± standard deviation (SD). The data were analyzed using one-way analysis of variance followed by post hoc Duncan's multiple range tests for comparison of differences among all groups (SPSS for Windows, Version 13.0, SPSS Inc., Chicago, IL). A significant difference was defined as a $P < 0.05$.

RESULTS AND DISCUSSION

Antioxidant Components and Activity of GPE. A growing body of epidemiological studies has associated the consumption of grapes, wine, and grape juice with a variety of health-promoting effects, particularly the reduced risk of cardiovascular diseases (*29*, *30*). The beneficial effects of grape and relevant grape derived food products are believed to be related to the antioxidant phenolic compounds. The *in vitro* experiments of this study were to characterize polyphenol constituents and antioxidant activity of Norton GPE. **Table 2** shows the major antioxidant phenolic constituents in Norton GPE. The TPC and TAC of Norton GPE were 475.4 mg GAE/g and 156.9 mg CGE/g GPE, respectively. The result revealed that the phenolic compounds account for approximately 48% of the dried weight extract, suggesting that Norton GPE contained substantial amounts of phenolic compounds in particular anthocyanins. A number of studies have reported significant phenolic compounds in different grape pomaces (*31*, *32*). The phenolic content in the Norton GPE was relatively higher than 35.7% reported in the methanol extract from var. Bangalore blue grape pomace (*33*).

Table 2. Phenolic Composition and Antioxidant Activity of Norton GPE

	mg/g GPE
TPC (mg GAE/g GPE)	475.4 ± 11.6
TAC (mg CGE/g GPE)	156.9 ± 6.3
catechin	28.6 ± 1.5
epicatechin	24.5 ± 1.3
quercetin	1.6 ± 0.1
trans-resveratrol	0.06 ± 0.00
ORAC (μmol TE/g GPE)	4133 ± 94

Table 3. Phenolic Acid Composition of Norton GPE

	μg/g GPE
caffeic acid	21.4 ± 0.5
coutaricacid	511.8 ± 10.1
ferulic acid	32.7 ± 0.9
gallic acid	867.2 ± 14.6
p-coumaric acid	36.4 ± 0.6
p-hydroxybenzoicacid	408.3 ± 4.2
protocatechuicacid	371.5 ± 3.9
syringic acid	13.2 ± 0.4

The individual phenolic compounds in Norton GPE were determined by HPLC−MS and quantified with the corresponding antioxidant standards. Catechin and epicatechin were two of major phenolic compounds in Norton GPE and their concentrations were 28.6 and 24.5 mg/g GPE, respectively (**Table 2**). Norton GPE also contained detectable amounts of quercetin and *trans*-resveratrol (1.6 and 0.06 mg/g GPE, respectively). Phenolic acids have also been reported in a variety of grape pomaces (*34*, *35*). Our results showed that gallic acid, coutaric acid, *p*-hydroxybenzoic acid, and protocatechuic acid were the predominant phenolic acids in Norton GPE where their concentrations were 867.2, 511.8, 408.3, and 371.5 μg/g GPE, respectively. Other phenolic acids detected in Norton GPE included caffeic acid, ferulic acid, *p*-coumaric acid, and syringic acid but in remarkably lower concentrations (**Table 3**).

Antioxidant activity of Norton GPE was evaluated by peroxyl radical scavenging activity as determined by ORAC. Norton GPE had an ORAC value of 4133 μmol TE/g (**Table 2**), which was remarkably higher than the reported ORAC values of 5−92 μmol TE/g fresh weight of common fruits and vegetables (*36*). We previously reported an ORAC value of 22.9 μmol TE/g fresh weight of Norton grape (*23*). It appears that Norton GPE exerted approximately 180 times higher peroxyl radical scavenging activity than the fresh Norton grapes. The ORAC value of Norton GPE was also comparable to the commercial grape seeds-derived antioxidant products with the ORAC values of 2.71 to 26.4 μmol TE/mg (*37*). Collectively, the *in vitro* analysis showed that Norton GPE contained substantial amount of antioxidant phenolic compounds in particular anthocyanins, catechin, epicatechin, quercetin, and several phenolic acids.

Estimated Daily Feed Intake and GPE Dosage. Table 4 shows the average daily feed intake of three dietary treatment groups. The higher feed intake of the ND group (3.7 g/d) can be attributable to its lower caloric density than the high fat dietary pattern of HF and HFGPE groups. There was no significant difference between the feed intake of HF group (3.05 g/d) and HFGPE group (3.16 g/d), suggesting that the GPE was well

Table 4. Daily Feed Intake and Doses of Norton GPE, Antioxidants, and ORAC Values in Different Dietary Treatment Groups[a]

daily dietary intake	ND group (n = 12)	HF group (n = 12)	HFGPE group (n = 12)
feed intake (g/d)	3.7 ± 0.3	3.1 ± 0.2	3.1 ± 0.2
GPE (mg/kg bw/d)			249.6 ± 16.1
TPC from GPE (mg/kg bw/d)			118.6 ± 7.7
TAC from GPE (mg/kg bw/d)			39.2 ± 2.5
catechin from GPE (mg/kg bw/d)			7.1 ± 0.5
epicatechin from GPE (mg/kg bw/d)			6.1 ± 0.4
ORAC value from GPE (μmol TE/kg bw/d)			1031.5 ± 66.5

[a] Daily feed intake was determined based on the total weekly intake per cage (4 mice/cage). GPE dose was calculated based on its supplemented concentration in HFGPE diet (2.4 g/kg in diet). The content of catechin in GPE is based on LC/MS measurement.

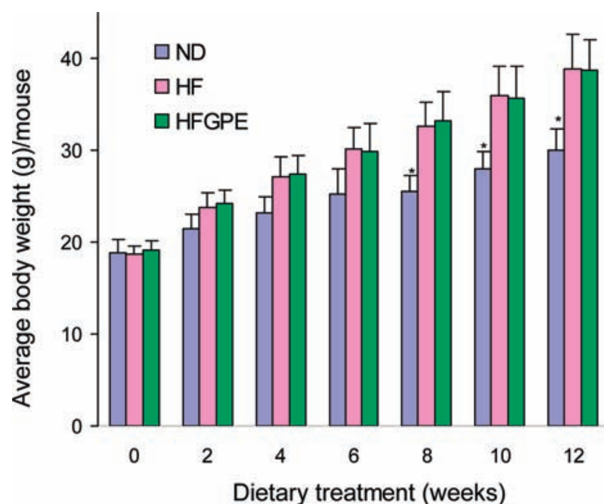

Figure 1. Effect of different dietary treatments on mouse body weight. Values are expressed as the mean ± SD (n = 12). * represents significant difference (P < 0.05) between the normal diet group (the ND group) and the high fat groups (the HF and HFGPE groups).

tolerated by the DIO mice. Based on the daily feed intake and GPE concentration in the HFGPE diet (2.4 g/kg), the daily dose of GPE was 250 mg/kg body weight (bw). The GPE dosage was in line with our expectation of GPE intake between 200 and 300 mg/kg bw. Such a dosage range has been practiced in a number of published reports in antioxidant supplementation in different animal models (*38*, *39*). The ORAC value associated with the daily GPE was 1031.8 μmol TE/kg bw. Since catechin and epicatechin were two of the major phenolic compounds in Norton GPE, we determined the daily catechin and epicatechin intake by DIO mice in HFGPE group were 7.1 and 6.1 mg/kg bw, respectively.

Effect of Different Dietary Treatments on Body Weight. Figure 1 shows the body weight changes of the mice by three dietary treatments. The fat content of the diet exerted notable effect on mouse body weight. At week 8, the high fat diet in the HF and HFGPE groups induced significantly more weight gain than the normal diet (the ND group). However, no significant difference was detected between the body weight of the HF and HFGPE groups, suggesting that incorporation of the GPE in the high fat diet did not affect body weight. By week 12, the average body weight in the HF and HFGPE groups was 38.9 and 38.7 g, respectively, which values were approximately 29% higher than that of the ND group (30.1 g).

Effect of GPE Supplementation on Oxidative Stress. Three biomarkers including plasma ORAC, cGPx, and liver lipid oxidation were measured to determine the effect of GPE supplementation on mouse oxidative stress. Plasma ORAC reflects the potential to reduce oxidative deterioration of lipid macromolecules. An enhanced antioxidant capacity translates to an improvement in oxidative stress status by detoxifying the reactive oxygen species (ROS). A previous study has shown the enhanced serum ORAC values in humans after 4 weeks of daily consumption of grape-derived antioxidants (*40*). However, in our study, 12 weeks of GPE supplementation did not show a significant increase in plasma ORAC values in high fat diet fed DIO mice (**Figure 2A**). Our results also showed that 12 weeks of the high fat diet did not lower plasma ORAC values in mice (the HF group) compared to the normal diet (the ND group).

Enzymatic antioxidant activity, such as glutathione peroxidase (GPx), is a biomarker of lipid peroxidation and reported to be lower in animals with chronic diseases (*41*). GPx is a selenium dependent enzyme that catalyzes the formation of GSSG and water from hydrogen peroxide within the body. As a result, GPx prevents oxidative damage of tissues and reduces oxidative stress. In our study, the plasma GPx was not significantly influenced by any of the dietary treatments (**Figure 2B**). Dietary intake of grape antioxidant extracts has been reported to promote GPx activation in animal supplementation studies (*42*, *43*). Despite the similar supplement dosage and experimental period, our results indicated that GPE supplementation did not result in improved plasma GPx activity in high fat diet fed DIO mice.

Lipid peroxidation within the body acts with cell components such as proteins, RNA, and DNA and is associated with cell dysfunctions and chronic diseases such as cancer, diabetes, atherosclerosis etc. (*44*). Lipid peroxidation is frequently initiated by ROS which can react with double bonds of polyunsaturated fatty acids to yield lipid hydroperoxides. Malondialdehyde (MDA) is one of the major secondary products of lipid peroxidation and is widely used as a biomarker for the assessment of oxidative stress (*45*). MDA is usually determined spectrophotometrically as thiobarbituric acid reactive substances (TBARS). In this study, lipid peroxidation in DIO mice was determined by measuring the levels of TBARS in mouse liver tissues. The result showed that dietary GPE supplementation exerted no effect on liver MDA (TBARS) levels of DIO mice (**Figure 2C**). In fact, liver peroxidation of the DIO mice was not affected by any of the dietary treatments during the 12-week experimental period. A previous study reported a decrease in liver lipid peroxidation after feeding mice a high fat diet for 4 weeks compared to a normal diet (*46*). These authors suggested that lipid peroxidation was not necessarily related to the development of metabolic conditions such as obesity (*46*).

Collectively, the results showed that there was no significant difference in the three oxidative biomarkers among the ND, HF, and HFGPE groups, suggesting that 12 weeks of the high fat diet did not induce an elevated oxidative stress in DIO mice despite that the high fat fed mice weighed 29% higher than the normal diet fed mice. The DIO mice in the HFGPE group were consuming a significant amount of grape pomace derived antioxidants (daily intake of 1031.5 μmol TE/kg bw/d, **Table 4**). However, 12 weeks of dietary GPE supplementation showed no significant improvement on oxidative stress of the high fat diet fed mice (**Figure 2**). Our results were inconsistent with other studies which reported supplementation of grape derived antioxidants significantly reduced oxidative stress in different animal models (*47*, *48*). In our study, the high fat diet induced significant body weight gain at week 8 and our dietary treatments ended at week 12. The inconsistency may be because our experimental duration was not sufficient to allow for the DIO mice to develop significantly elevated levels of oxidative stress associated with obesity, therefore, the GPE effects on oxidative stress were not noticeable.

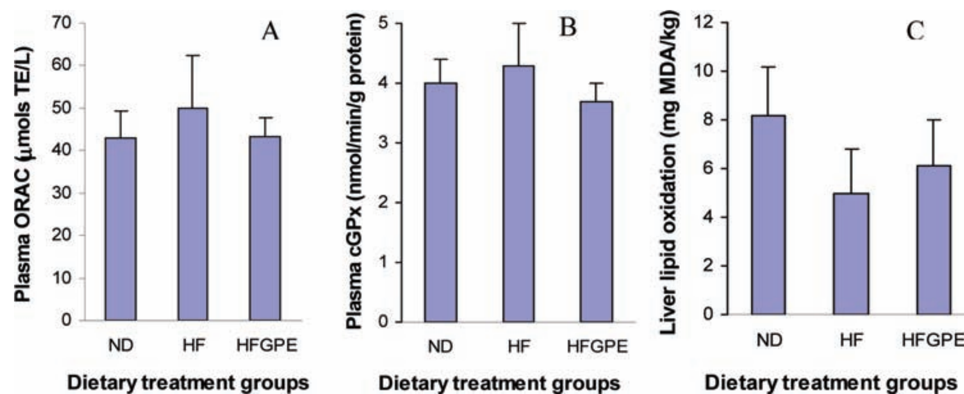

Figure 2. Effect of different dietary treatments on oxidative stress biomarkers including plasma ORAC (**A**), plasma cGPx (**B**), and liver lipid oxidation (**C**). Values are expressed as the mean $\pm$ SD ($n = 12$).

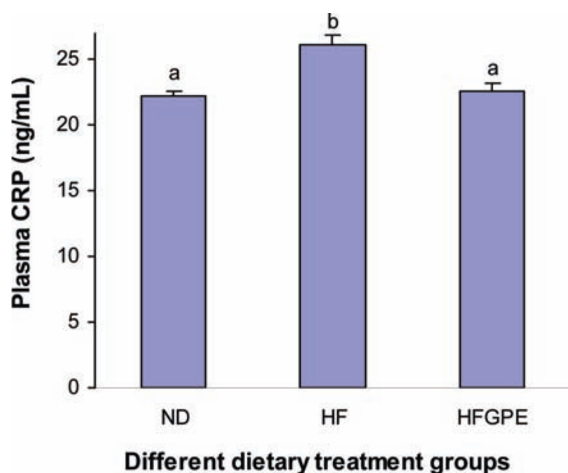

Figure 3. Effect of different dietary treatments on plasma CRP levels. Values were expressed as the mean $\pm$ SD ($n = 12$). Means with different letters are significantly different, $P < 0.05$.

Effect of GPE Supplementation on Inflammation. Hotamisligil et al. provided the first clear link between obesity and chronic inflammation by demonstrating that tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is overexpressed in the adipose tissue of obese mice (*49*). The inflammatory biomarker CRP levels have been shown to be significantly higher in obese populations compared to lean humans (*50*, *51*). The mechanisms that underlie observed associations between obesity and elevated levels of inflammation are unclear. It has been postulated that increased adiposity in obesity is a key mediator of inflammation, contributing to the production of proinflammatory cytokines (*5*). Our results showed that the average plasma CRP level in the HF group (26.1 ng/mL) was 17.6% higher than that in the ND group ($P < 0.05$, **Figure 3**), suggesting that the high fat diet induced a significant inflammatory response in DIO mice. However, the supplementation of GPE into the high fat diet for 12-week dietary treatment reduced the CRP levels in the mice by 15.5% ($P <$ 0.05), suggesting a potential anti-inflammatory effect by GPE supplementation. Moreover, the CRP levels in the HFGPE group were comparable to those in the ND group ($P > 0.05$), suggesting that GPE supplementation was able to restore the elevated CRP levels in the high fat diet fed mice to a normal range. Similar to our findings, improvements in inflammatory immune response have been reported from grape seed extracts (*52*, *53*). A recent study showed that high fat diet fed rats supplemented with grape seed procyanidins (7 mg/d for 19 weeks) resulted in a significant decrease in plasma CRP (*52*). Additional evidence comes from a recent double blind randomized trial demonstrating that dietary supplementation of grape seed extract (600 mg/d for 4 weeks) significantly improved markers of inflammation in obese Type 2 diabetic subjects (*53*). The anti-inflammatory effect of grape extracts was originally believed to be attributed to their antioxidant protection, but recent research suggests that the grape compounds may also act on human differentiated adipocytes and foam cell formation to exert anti-inflammatory activity (*54*, *55*). In our study, GPE supplementation showed an anti-inflammatory but not an antioxidant effect in high fat diet fed DIO mice, suggesting that GPE may involve additional protective mechanisms to exert anti-inflammatory activity.

In summary, our *in vitro* experiments showed that Norton GPE contained significant amounts of anthocyanins, catechin, epicatechin, quercetin and a few phenolic acids. Norton GPE exerted comparable antioxidant activity (ORAC) to the commercial antioxidant products. The dietary GPE supplementation (250 mg/kg bw/d for 12 weeks) showed significant anti-inflammatory activity in high fat diet induced obese mice.

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