Hepatoprotective Effect of Fresh Grape Juice Prepared by a Low-Speed Masticating Juicer in db/db Mice

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ABSTRACT: This study investigated the hepatoprotective effect of fresh grape juice prepared using a low-speed masticating (LSM) juicer or a high-speed centrifugal (HSC) juicer in mice. Six-week-old db/db mice were fed on an AIN-93G diet or a diet containing 1% freeze-dried LSM or HSC grape juice for 7 weeks. Treatment with LSM grape juice significantly decreased hepatic triglycerides, serum aspartate transaminase activities, and homeostasis model assessment for insulin resistance values, whereas HSC juice did not significantly influence these parameters. The LSM grape juice showed higher antioxidant and anti-inflammatory activities than HSC juice. The benefits of LSM grape juice are probably due to a much higher proanthocyanidin content than that of HSC juice. These results suggest that LSM grape juice can exert hepatoprotective effects in db/db mice, partly through improving insulin resistance and promoting antioxidant and inflammatory activities.

Keywords: grape juice, low-speed masticating juicer, hepatoprotective effect, antioxidant activity, insulin resistance

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinical syndrome characterized by fat accumulation in liver cells that unrelated to excessive alcohol consumption (1-3). The spectrum of NAFLD ranges from benign steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis, and liver fibrosis (1). NAFLD is the most prominent form of chronic liver disease worldwide (3), of which the prevalence is increasing (4). However, there is not currently an approved medical therapy for NAFLD (4,5).

The ‘two-hit hypothesis’ is the prevailing model for the etiology for NAFLD (6,7). The ‘first hit’ is characterized by accumulation of fat in hepatocytes. It has been suggested that the first hit is related to insulin resistance, obesity, and abnormal fatty acid metabolism. The “second hit” consists of hepatocyte inflammation and necrosis. Oxidative stress plays a crucial role in triggering the second hit, since reactive oxygen species (ROS) inhibit enzymes in the mitochondrial respiratory chain, cause lipid peroxidation, and elevate the release of pro-inflammatory cytokines, contributing to liver injury (8). Therefore, improved insulin resistance and oxidative stress could contribute to preventing and delaying progression of NAFLD.

Grapes are one of the most widely consumed fruits worldwide. Grapes can be eaten whole (mainly the flesh) or processed to make juice, wine, and sauce using the flesh, skin, and seeds. Grapes contain polyphenols, including proanthocyanidins, resveratrols, anthocyanins, catechins, and epicatechins, which have antioxidant and anti-inflammatory activities (9). In a grape, the seed is the major source of proanthocyanidins, and the skin contains most of the resveratrols, anthocyanins, and catechins (10).

Since grape skin and seed, rather than flesh, contain phenolic phytochemicals, grape juice prepared from whole grapes might be a better source of antioxidant phytochemicals than table grapes, which are generally consumed after removal of skin and seed. Fresh grape juice can be prepared using a conventional juicer (high-speed centrifugal juicer; HSC juicer) or a screw-type juicer (low-speed masticating juicer; LSM juicer), which was recently developed (11). The HSC juicer uses a flat blade disk rotating at 8,000∼12,000 rpm to grind and filter the grapes, whereas the LSM juicer produces juice by squeezing the fruit with a vertical helical screw (an auger) rotating at 40∼80 rpm. Therefore, the LSM juicer can re-
duce oxidation and minimize heat generation during the juicing process compared with the HSC juicer. Kim et al. (12) reported that fresh grape juice prepared with the LSM juicer shows stronger antioxidant activity in vitro and has a higher total polyphenol content than juice prepared with the HSC juicer, and that the antioxidant activity of grape flesh is lower than that of both LSM and HSC juice. Reductions in oxidation and heat production during production of LSM grape juice may contribute to a higher antioxidant activity than that of HSC grape juice.

Of the grape polyphenols with antioxidant and anti-inflammatory activities, proanthocyanidins show hepatoprotective effects (13-15), protecting against carbon tetrachloride-induced steatosis and liver injury in rats via antioxidant activities (13). A proanthocyanidin-rich extract from grape seeds alleviates hepatic steatosis in a high-fat-fructose-diet-induced rat model of NAFLD (14). The consumption of grape seed extract for 3 months further alleviates fatty liver and improves liver function in patients with NAFLD (15). Therefore, fresh grape juice made from the grape flesh, skin, and seeds could be beneficial for alleviating hepatic steatosis, since it provides antioxidant and anti-inflammatory polyphenols, including proanthocyanidins. However, there are few reports on the protective effects of fresh grape juice in NAFLD.

In this study, we investigated the benefits of fresh grape juice, prepared using LSM and HSC household juicers, on hepatic steatosis in genetically leptin-resistant db/db mice, which develop obesity and insulin resistance and serve as an animal model of NAFLD (16). Furthermore, we investigated the underlying mechanisms for the hepatoprotective effects.

MATERIALS AND METHODS

Preparation of the fresh grape juice

Grapes (Vitis labrusca, Campbell Early) were purchased from a local market in Gimhae, Korea. The grapes were removed from their stems, washed with tap water, and drained. The LSM and HSC grape juices were prepared using LSM and HSC juicers, respectively, according to the manufacturers’ instructions (12), and were freeze-dried. We obtained 15 g of freeze-dried material from 100 g of either grape juice.

Analyses of proximate compositions and proanthocyanidin

The proximate compositions of the freeze-dried LSM and HSC grape juices were analyzed according to the standard American Organization of Analytical Chemists procedure (official method number 926.5 for moisture, 935.38 for fat, 950.36 for protein, 930.22 for ash, and 958.29 for dietary fiber) (17). The proanthocyanidin contents of the grape juices were determined according to the method described by Li et al. (18), with some modification. Each sample (0.1 g) was extracted with 2 mL of 80% methanol (Sigma, St. Louis, MO, USA) and kept at room temperature for 2 h, before they were centrifuged at 850 g for 10 min at 4°C. A 0.1 mL aliquot of the supernatant was mixed with 0.6 mL of 4% vanillin-methanol solution and 0.3 mL hydrochloric acid, and was kept at room temperature for 15 min. The absorbance of the reaction mixture was measured at 500 nm, and the result was expressed as catechin equivalents. All samples were analyzed in triplicate.

Animals and diets

The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee at Inje University, Korea (Approval no. 2013-49). Male 5-week-old C57BL/KsJ-db/db mice (n=24) were purchased from the Korean Research Institute of Bioscience and Biotechnology, Ochang, Korea. The animals were housed individually in plastic cages and kept on a 12-h light/dark cycle at a controlled temperature (19 ~ 23°C) and humidity (50 ~ 60%). Mice were fed with standard chow and tap water ad libitum for 1 week for adaptation, before they were divided randomly into three groups. The mice were maintained on either an AIN-93G diet (control group) or a diet containing 1% (w/w) of the freeze-dried LSM or HSC grape juice (LSM and HSC groups, respectively) ad libitum for 7 weeks (Table 1). Based on the proximate composition of the freeze-dried LSM or HSC grape juice (Table 2), the experimental diets were adjusted so that the protein, fat, and dietary fiber contents were the same as those in the AIN-93G diet. The body weight of each mouse was recorded once, and food intake was recorded three times a week.

Table 1. Composition of basal and experimental diets

| Ingredients                          | Control (%) | LSM (%) | HSC (%) |
|--------------------------------------|-------------|---------|---------|
| Corn starch                          | 39.8        | 39.8    | 39.8    |
| Casein                               | 20.0        | 19.96   | 19.97   |
| Dextrinized cornstarch                | 13.2        | 13.2    | 13.2    |
| Sucrose                              | 10.0        | 9.06    | 9.04    |
| Soybean oil                          | 7.0         | 6.99    | 7.0     |
| Alpha-cellulose                      | 5.0         | 4.98    | 5.0     |
| Mineral mixture                      | 3.5         | 3.5     | 3.5     |
| Vitamin mixture                      | 1.0         | 1.0     | 1.0     |
| L-Cystine                            | 0.3         | 0.3     | 0.3     |
| Choline bitartrate                   | 0.25        | 0.25    | 0.25    |
| tert-Butyl hydroquinone              | 0.0014      | 0.0014  | 0.0014  |
| LSM juice                            | –           | 1.0     | –       |
| HSC juice                            | –           | –       | 1.0     |

LSM, low-speed masticating juicer; HSC, high-speed centrifugal juicer.
Biochemical analyses of serum and liver tissue

On completion of the experiment, mice were sacrificed by heart puncture after an overnight fast, and blood and liver tissues were collected immediately. The blood samples were centrifuged at 3,000 g for 15 min to obtain serum. Serum and liver tissues were stored at −70°C until analysis. Serum triglyceride, total cholesterol, high-density lipoprotein (HDL)-cholesterol, and glucose were determined enzymatically using commercial assay kits (Asan Pharmaceutical, Seoul, Korea). The serum insulin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the following equation (19):

\[
\text{HOMA-IR} = \frac{\text{fasting serum insulin (ng/mL)} \times \text{fasting serum glucose (mg/dL)}}{405}
\]

The serum aspartate transaminase (AST) and alanine transaminase (ALT) activities were determined spectrophotometrically using commercial assay kits (Youngdong Pharmaceutical, Yongin, Korea). Serum tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein-1 (MCP-1) were quantified using ELISA kits (eBioscience, Vienna, Austria).

To determine the hepatic fat content, a portion of the frozen liver samples was thawed and homogenized in saline with a Teflon homogenizer (Wheaton Science Products, Millville, NJ, USA). Total lipids were extracted according to the method described by Folch et al. (20). The triglyceride concentration of the liver was determined using a commercial kit (Asan Pharmaceutical, Seoul, Korea).

To determine the amounts of hepatic lipid peroxides, a liver sample was homogenized in 10 volumes of 10 mM sodium phosphate buffer (pH 7.4) using a glass Teflon homogenizer. Thiobarbituric acid reactive substances (TBARS) from the homogenate were measured according to the method described by Ohkawa et al. (21). To quantify hepatic glutathione (GSH), a liver sample was homogenized in 9 volumes of 0.1 mM phosphate buffer (pH 7.4) and was centrifuged at 10,000 g at 4°C for 30 min. GSH in the supernatant was measured using the method described by Ellman (22). The protein content was measured using the Bradford technique (23). The TBARS and GSH levels were expressed as nmol malondialdehyde/mg protein and nmol/mg protein, respectively.

To assess the activities of antioxidant enzymes, liver samples were homogenized in 10 volumes of 50 mM phosphate buffer (pH 7.4), and centrifuged at 700 g for 10 min. The supernatant was further centrifuged at 10,000 g for 10 min to pellet the mitochondria, and then centrifuged at 100,000 g (Centrifuge 5415R, Eppendorf, Hamburg, Germany) for 60 min to collect the final supernatant. Catalase (CAT) activity was measured in the mitochondrial pellets, as detailed by Aebi (24). Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities were measured in the final supernatant, as detailed by Marklund and Marklund (25) and Paglia and Valentine (26), respectively. All analyses were performed in triplicate.

Statistical analysis

All values are expressed as the mean±standard error (SE). Data were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey’s test (P<0.05).

RESULTS AND DISCUSSION

Proximate composition and proanthocyanidin contents of freeze-dried LSM and HSC grape juices

The fresh grape juice yield from the LSM juicer (79.1±1.7%) was significantly higher than that from the HSC juicer (45.0±1.3%, P<0.01, data not shown). In a previous study, a higher yield of tomato juice was reported from a LSM juicer compared with that from a HSC juicer, suggesting that the juice extracting mechanism may be the cause of the differing yields (11). The proximate compositions of the freeze-dried LSM and HSC grape juices are shown in Table 2. The proanthocyanidin content of the freeze-dried LSM grape juice (3.25±0.4 mg/g dry weight) was 20.3 times higher than that of the freeze-dried HSC grape juice (0.16±0.0 mg/g mg/g dry weight, P<0.01, Table 2). Grape proanthocyanidins are primarily located in the grape seed (10) and are candidates for alleviating NAFLD (14,15,27). The portion of grape seeds discarded with the remnant pulp after preparing juice from a HSC juicer is 6.7 times higher than the portion discarded from a LSM juicer (28). Therefore, more grape

| Proximate composition | LSM juice | HSC juice |
|-----------------------|-----------|-----------|
| Moisture (%)          | 11.7      | 11.8      |
| Crude fat (%)         | 0.6       | 0.4       |
| Crude protein (%)     | 3.6       | 2.9       |
| Crude ash (%)         | 2.2       | 2.0       |
| Total dietary fiber (%) | 1.9     | 0.5       |

Proanthocyanidin content

| Proanthocyanidin (mg/g dry weight) | 3.25±0.4 | 0.16±0.0* |

*P<0.01. LSM, low-speed masticating juicer; HSC, high-speed centrifugal juicer.
Fig. 1. Hepatic triglycerides and serum activities of aspartate transaminase (AST) and alanine transaminase (ALT) in db/db mice. 
(A) Hepatic triglycerides, (B) serum AST activity, and (C) serum ALT activity. Control, LSM, and HSC groups were fed AIN-93G diet, diet containing 1% freeze-dried grape juice prepared using a low-speed masticating juicer, and diet containing 1% freeze-dried grape juice prepared using a high-speed centrifugal juicer for 7 weeks, respectively. Values represent means±SE (n=8). Different letters (a-c) are significantly different at P<0.05.
0.05) than the control group (63.9±2.6 mg/g liver, Fig. 1A). Furthermore, the AST activity of the LSM group was 20% lower than the control group (Fig. 1B, P<0.05).

Consumption of LSM and HSC juices decreased serum ALT activity 32% and 16%, respectively, compared with the basal diet (Fig. 1C, P<0.05); the ALT activity of the LSM group was lower than that of the HSC group (P<0.05). Consumption of LSM juice for 7 weeks alleviated fatty liver and improved liver function in db/db mice without significantly influencing the serum lipid profiles. Polyphenols are reported to exert hepatoprotective effects through increasing fatty acid oxidation in the liver and improving insulin sensitivity (32). Kim et al. (12) reported that the total phenol content of LSM grape juice was 3.7 time higher than that of the HSC grape juice. Whilst there might have been insufficient amounts of phenols in the HSC grape juice to alleviate fatty liver in db/db mice, the LSM grape juice may have contained enough polyphenols to improve hepatic steatosis and liver function.

The preventive effect of LSM grape juice against hepatic steatosis may be predominantly due to proanthocyanidins. Tsuruta et al. (33) reported that feeding db/db mice a polyphenolic extract from lotus root containing proanthocyanidins decreased liver triglyceride contents without significantly impacting the serum lipid profile. Grape seed proanthocyanidins reduce mRNA expression of sterol-regulatory element-binding protein 1-c, a lipogenic transcription factor (14). In addition, consumption of LSM grape juice reduced the HOMA-IR value, a parameter used for insulin resistance in db/db mice in this study (Table 4). Insulin resistance is strongly associated with accumulation of fat in hepatocytes, the “first hit” of NAFLD (6,7). Therefore, LSM grape juice may be able to alleviate fatty liver in db/db mice by reducing lipogenesis in the liver and improving insulin sensitivity.

**Effect on hepatic antioxidant status and serum proinflammatory cytokines**

The hepatic TBARS level for the LSM group was 17% lower than that for the control group (P<0.05, Table 5). The GSH content of the liver was increased 36% for the LSM group compared with the control group (P<0.05). LSM grape juice showed stronger DPPH-radical-scavenging activity and SOD-like activity than the HSC grape juice in vitro (12). Proanthocyanidins may be responsible for the antioxidant in vivo effect of LSM grape juice in our study. Proanthocyanidins can scavenge free radicals and quench singlet oxygen (34), and GSH acts as an antioxidant and can scavenge ROS and remove lipid peroxides (35).

SOD and GSH-Px activities were significantly increased in the LSM group compared with the control (Table 5). There were no significant differences in CAT activities for the control, LSM, and HSC groups. SOD catalyzes the conversion of superoxide radicals into H₂O₂, which can be detoxified to water by CAT in the peroxisomes or by GSH-Px in the cytosol (36).

Serum levels of TNF-α were significantly lower in the LSM group compared with the control, whereas TNF-α in the HSC group did not significantly differ from either other group (Table 5). In addition, there were no significant differences in levels of serum MCP-1 between the three groups. Procyanidins, oligomeric proanthocyanidins from grape seed, reduced mRNA expression of TNF-α and interleukin-6, inflammatory cytokines induced in Zucker fa/fa rats with a high-fat diet (37). Therefore proanthocyanidins in LSM grape juice could contribute to the reduction in TNF-α observed in this study. TNF-α mediates insulin signaling, leading to insulin resistance (38), and is involved in inflammatory and metabolic alterations leading to aggravation of NAFLD (39). Since oxidative stress and inflammation are important factors that can trigger the second hit of NAFLD (8), LSM grape juice could be beneficial for decreasing oxidative stress and inflammation to inhibit progression of NAFLD.

To conclude, in this study we showed that consumption of LSM grape juice induces hepatoprotective effects in db/db mice, in part by improving insulin resistance and exerting antioxidant and anti-inflammatory activities.

### Table 5. Biomarkers associated with oxidative stress and inflammation

| Liver | Control | LSM | HSC |
|-------|---------|-----|-----|
| TBARS (nmol/mg protein) | 3.0±0.1b | 2.5±0.1a | 2.7±0.1ab |
| GSH (nmol/mg protein) | 8.1±0.4a | 11.1±0.7b | 8.7±0.5a |
| SOD activity (U/mg protein) | 15.1±0.7a | 18.2±0.8b | 17.1±0.9ab |
| CAT activity (U/mg protein) | 9.1±0.5bc | 10.3±0.6 | 8.5±0.6 |
| GSH-Px activity (U/mg protein) | 20.6±1a | 24.6±1.1b | 21.4±1.1ab |

| Serum | Control | LSM | HSC |
|-------|---------|-----|-----|
| TNF-α (pg/mL) | 28.2±1.7b | 19.8±1.3a | 24.7±1.4ab |
| MCP-1 (pg/mL) | 111.1±8.1b | 94.8±6.3 | 97.4±6.8 |

Values represent mean±SE (n=8). Different letters (a, b) are significantly different at P<0.05.

1) Control, LSM, and HSC groups were fed AIN-93G diet, diet containing 1% freeze-dried grape juice prepared using a low-speed masticating juicer, and diet containing 1% freeze-dried grape juice prepared using a high-speed centrifugal juicer for 7 weeks, respectively.

2) TBARS, thiobarbituric acid reactive substances; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemotactic protein-1.

3) Not significant.
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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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