Chronic Administration of Satsuma Mandarin Fruit (Citrus unshiu MARC.) Improves Oxidative Stress in Streptozotocin-Induced Diabetic Rat Liver

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Fruits and vegetables contain numerous antioxidants such as carotenoids, vitamins, and phenolic phytochemicals. Recent studies have demonstrated that antioxidants may reduce the risk for diabetes or its complications. In this study, we investigated the effects of the chronic administration of Satsuma mandarin fruit on an antioxidant defense system in streptozotocin (STZ)-induced diabetic rat liver. After a ten-week administration of Satsuma mandarin, antioxidant enzymes and glutathione levels in the liver were evaluated. The superoxide dismutase (SOD), catalase, and glutathione-peroxidase (GPx) activities, and glutathione level in the STZ-induced diabetic rats liver decreased significantly compared with those in the age-matched normal rats. The glutathione-reductase (GR) activities did not differ significantly between these two groups. In contrast, the SOD, GR, and glutathione levels in the Satsuma mandarin (1% or 3%) diet-fed STZ-diabetic rat livers were significantly higher than those in the normal diet-fed STZ-diabetic rat livers. In addition, although the serum alanine aminotransferase and γ-glutamyl-aminotransferase concentrations of normal diet-fed STZ-diabetic rat livers were significantly higher than those of the age-matched normal rats, these increments of serum liver enzymes were diminished by the chronic administration of Satsuma mandarin. These results suggest that Satsuma mandarin may act as a suppressor against liver cell damage and inhibit the progression of liver dysfunction induced by chronic hyperglycemia.

Key words Satsuma mandarin; oxidative stress; hyperglycemia; liver

Recent epidemiologic studies have demonstrated that high dietary consumption of fruit and vegetables results in lower risks of certain cancers and cardiovascular disease.1,2 There is accumulating evidence that these effects of fruit and vegetables attribute to antioxidants nutrients such as carotenoids, vitamins, and phenolic phytochemicals. Recent studies have shown that antioxidant vitamins and carotenoids may have a protective effect against diabetes mellitus.3–6

Satsuma mandarin (Citrus unshiu MARC.) is the most frequently consumed domestic fruit in Japan.7 Numerous antioxidants such as β-cryptoxanthin, vitamins, and flavonoids exist in pulp of this fruit.8–10 Previously, we found inverse association of the prevalence of diabetes mellitus with frequency of Satsuma mandarin intake based on data from 6049 participants on a self-administered questionnaire in cross-sectionally.11 Furthermore, very recently, Kamata et al. reported that the chronic administration of Satsuma mandarin fruit extract improved endothelial dysfunction in the aorta in streptozotocin (STZ)-induced diabetic rats without lowering plasma cholesterol.12 Hyperglycemia increases the generation of free radicals by glucose auto-oxidation.13–15 Oxidative stress resulting from the increased production of reactive oxygen species plays a key role in the development of diabetic mellitus or its complications.16–20 Therefore, Satsuma mandarin would be expected to protect against the development of diabetes mellitus or the pathogenesis of diabetic complications.

On the other hand, the liver plays an important role in glucose metabolism, and it is a major site of insulin clearance.21,22 Oxidative stress induced by hyperglycemia may lead to liver cell damage. Wohaib et al. found that the antioxidant enzyme activities and glutathione level in STZ-induced diabetic rat liver significantly decreased compared with those in control rats and that the impairment of the antioxidant defense system in STZ-induced diabetic rat liver was reversed by insulin treatment.23 These results indicate that oxidative stress induced by hyperglycemia may lead to liver dysfunction.

In this study, we expected that Satsuma mandarin would inhibit liver injury induced by hyperglycemia and investigated the effects of the chronic administration of Satsuma mandarin on the antioxidant defense system: i.e., the superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione levels in an STZ-induced type-1 diabetic model rat liver.

MATERIALS AND METHODS

Satsuma mandarin (Citrus unshiu MARC.) juice was purchased from the Ehime Beverage, Inc. (Ehime, Japan). The concentrated Satsuma mandarin juice was freeze-dried and mixed with a standard commercial diet (MF diet, Oriental Yeast, Tokyo, Japan) at 1, 3 w/w% (fruit extract-containing diet).

Male Wistar rats (5 weeks old and with a 150–170 g body weight) were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animals were maintained in an environmentally controlled room under a 12:12-h light–dark cycle and fed a standard MF diet for one week before use. To generate diabetes, some rats received a single injection via the tail vein of STZ 75 mg/kg dissolved in a citrate buffer (pH 4.5). Age-matched normal rats were injected with the buffer alone. The rats were kept on a standard MF diet for three days after the STZ injection and then divided into the following three groups: 0%, 1%, or 3% (wt/wt) Satsuma mandarin-treated diabetic groups. Rats in the age-matched normal group were fed the standard MF diet. Food and water were allowed ad libitum. This study was conducted in accordance with the
Hemoglobin A1C (HbA1C) was measured by an auto-analyzer using commercial kits (Kyowa-Medics, Inc., Tokyo, Japan). The livers of the animals were removed under diethyl ether anesthesia and stored at −80 °C until analyses. The livers were homogenized with a homogenizer (Ultra Turrax T25, Rose Scientific Ltd., Edmonton, Canada) in 10 volumes of a 50 mM sodium phosphate buffer (pH 7.4) at 4 °C. Homogenates were centrifuged (Beckman, U.S.A.) at 15000g for 10 min, and the supernatant obtained was used for the following antioxidant enzyme measurements. Antioxidant enzyme activities in the liver were assayed using commercial kits (SOD assay kit BIOXYTECH SOD-525 for SOD activity, Oxis International, Oregon, U.S.A.; catalase assay kit for catalase activity, Cayman CHEMICAL, Michigan, U.S.A.; glutathione peroxidase assay kit BIOXYTECH GPx-340 for GPx activity, Oxis International, Oregon, U.S.A.; and glutathione reductase assay kit BIOXYTECH GR-340 for GR activity, Oxis International, Oregon, U.S.A.). The protein concentrations of the supernatants were determined using a commercial kit (Bio-Rad protein assay kit, California, U.S.A.). Another 0.25 g aliquot of each liver tissue was homogenized in 3.75 ml ice-cold 0.1 M sodium phosphate–5 mM EDTA (pH 8.0), and then 1 ml 25% phosphoric acid was added. After vortexing for 10 s, the sample was centrifuged at 15000×g for 30 min, and the supernatant obtained was used for the measurement of the glutathione levels. Measurements of reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were carried out according to previous report.24 Data were expressed as the mean±S.E.M. Statistical significance was analyzed using Dunnett’s multiple range tests. In the test for a linear trend, the associations among continuous variables across three groups (0%, 1%, 3%) were carried out by regression analysis.

RESULTS

The body weight of the STZ-diabetic rats was significantly lower than that of the age-matched normal rats after STZ injection; however, there was no evidence of any influence from the chronic Satsuma mandarin treatments (1 or 3%) on body weight in the diabetic rats during the experimental period (Table 1). Furthermore, diet intakes were not different among the three STZ-diabetic rat groups (0%, 1%, 3%) during the experimental period (data not shown). As shown in Table 1, the HbA1c, serum ALT, and γ-GTP levels were significantly higher in the normal diet-fed STZ-induced diabetic rats than in the age-matched normal rats. The serum AST level was slightly higher in the normal diet-fed diabetic rats than in the age-matched normal rats; however, the difference was not statistically significant. Following chronic administration of Satsuma mandarin (1 or 3%), these blood parameters tended to be low in a dose-dependent manner, except for HbA1c. Table 2 shows the result of the antioxidant defense

### Table 1 Effects of Chronic Administration of Satsuma Mandarin on Body Weigh, HbA1c, and Serum Liver Enzyme Concentrations in Streptozotocin-Induced Diabetic Rat

<table>
<thead>
<tr>
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<th>Normal rats</th>
<th>Satsuma mandarin supplemented diabetic rats</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>366.0 (4.6)</td>
<td>200.2 (8.3)**</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>1.3 (0.5)</td>
<td>8.8 (0.3)**</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>113.4 (8.0)</td>
<td>150.9 (36.8)</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>41.1 (7.6)</td>
<td>110.3 (24.0)*</td>
</tr>
<tr>
<td>γ-GTP (IU/l)</td>
<td>0.0 (0.0)</td>
<td>13.6 (2.6)**</td>
</tr>
</tbody>
</table>

Data are mean (S.E.). *p<0.05 and **p<0.01 vs. normal rats by Dunnet’s multiple comparison test. †p for trend tests were carried out by regression analysis about the associations among continuous variables across three groups (0%, 1%, 3%).

### Table 2 Effects of Chronic Administration of Satsuma Mandarin on Antioxidant Enzyme Activities and Glutathione Levels in the Streptozotocin-Induced Diabetic Rats Liver

<table>
<thead>
<tr>
<th></th>
<th>Normal rats</th>
<th>Satsuma mandarin supplemented diabetic rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0%</td>
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<tr>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Superoxide dismutase (U/mg protein)</td>
<td>23.2 (1.2)</td>
<td>13.3 (0.5)**</td>
</tr>
<tr>
<td>Catalase (U/mg protein)</td>
<td>198.2 (10.6)</td>
<td>151.2 (4.8)*</td>
</tr>
<tr>
<td>Glutathione peroxidase (mU/mg protein)</td>
<td>1216.8 (45.0)</td>
<td>909.7 (49.3)***</td>
</tr>
<tr>
<td>Glutathione reductase (mU/mg protein)</td>
<td>29.2 (1.0)</td>
<td>31.5 (1.1)</td>
</tr>
<tr>
<td>Glutathione (nmol/mg protein)</td>
<td>Reduced glutathione</td>
<td>25.9 (0.5)</td>
</tr>
<tr>
<td></td>
<td>Oxidized glutathione</td>
<td>3.8 (0.4)</td>
</tr>
</tbody>
</table>

Data are mean (S.E.). *p<0.05, **p<0.01 and ***p<0.001 vs. normal rats. †p<0.05 and ††p<0.01 vs. normal diet-fed STZ-diabetic rats by Dunnet’s multiple comparison test. †p for trend tests were carried out by regression analysis about the associations among continuous variables across three groups (0%, 1%, 3%).
system in the liver. In the normal diet-fed STZ-diabetic rat livers, the SOD, catalase, GPx, GSH, and GSSG levels significantly decreased compared with those in the age-matched normal rats. In contrast, the GR activities did not differ significantly between these two groups. Following chronic administration of Satsuma mandarin (1 or 3%), the SOD and GR activities were significantly higher than those in the normal diet-fed STZ-diabetic rat livers. Furthermore, the GSH and GSSG levels in the Satsuma mandarin-supplemented STZ-diabetic rat livers were also significantly higher than those in the normal diet-fed STZ-diabetic rats.

DISCUSSION

In this study, we tested the hypothesis that Satsuma mandarin fruit prevents hyperglycemia-induced oxidative stress in the liver using STZ-induced type 1 diabetic rat models. Our results showed that the chronic administration of Satsuma mandarin fruit improves impairments of SOD activity and glutathione homeostasis in STZ-induced diabetic rat liver without lowering the blood glucose level. Furthermore, the serum liver enzyme concentrations in Satsuma mandarin diet-fed STZ-diabetic rats tended to be low in a dose-dependent manner. These results suggest that Satsuma mandarin may act as a suppressor against liver cell damage and inhibit the progression of liver dysfunction induced by chronic hyperglycemia.

On the other hand, it is well known that fruits contain numerous carbohydrates such as sucrose, fructose and glucose. In the present study, when Satsuma mandarin extract was fed for 10 weeks to STZ-diabetic rats, blood glucose and serum lipid levels showed no significant alterations among the three STZ-diabetic rat groups (0%, 1%, 3%) (data not shown). Furthermore, Kamata et al. reported that the 10% supplementation of this fruit extract had no influence on blood glucose, serum lipid levels, and body weight of STZ-diabetic rats.12) From these results, we suggest that chronic administration of this fruit extract had no influence on blood glucose level. Furthermore, we have no data about the changes of oxidative damage pathogenesis in the STZ-diabetic rat liver in detail. Secondly, we did not identify the intracellular levels of reactive oxygen spices in STZ-diabetic rat liver. Therefore, we could not discuss the associations of reactive oxygen spices with the pathogenesis in the STZ-diabetic rat liver in detail. Secondly, we have no data about the changes of oxidative damage markers in STZ-diabetic rat liver except for lipid peroxide. From these study limitations, it remains unclear whether chronic administration of Satsuma mandarin could diminish the oxidative stress in the STZ-diabetic rat liver.

In this study, the SOD and glutathione levels in the Satsuma mandarin diet-fed STZ-diabetic rat liver were significantly higher than those in the normal diet-fed STZ-diabetic rats. In addition, the GR activities in the Satsuma mandarin diet-fed STZ-diabetic rat livers were significantly higher than those in the normal diet-fed STZ-diabetic and age-matched normal rats. In contrast, the GPx and catalase activities did not differ among the three groups of STZ-diabetic rats. From these results, we assumed that Satsuma mandarin might mitigate the oxidative stress in the STZ-diabetic rat liver via the enhancement of SOD activity or glutathione homeostasis, and as a result, the increments of serum liver enzymes in the STZ-diabetic rat might be diminished.

We also measured lipid peroxide in the liver, and significant increment of lipid peroxide was noted in STZ-induced diabetic rat liver. In contrast, chronic administration of Satsuma mandarin slightly diminished the increment of lipid peroxide in the STZ-induced diabetic rat liver, but significant differences were not observed among the three STZ-diabetic rat groups (data not shown). Recently, many studies have been reported that carotenoids have antioxidant effects against lipid peroxidation in rat liver.28—30 It seems that diabetic model used in this study is not suitable for evaluating effects of Satsuma mandarin on lipid peroxidation in the liver because STZ induces an excessive hyperglycemia. We are planning to investigate the effect of Satsuma mandarin on lipid peroxidation levels in the liver using type-2 diabetic GK rats.

Satsuma mandarin fruit contains numerous antioxidants, such as β-cryptoxanthin, vitamins, and phenolic phytochemicals.8—10 We attribute the beneficial effects of Satsuma mandarin on liver dysfunction in the STZ diabetic rat to these antioxidant phytochemicals. Very recently, from the results of a population-based nutritional epidemiologic survey, we determined that the serum ALT and AST concentrations are higher in hyperglycemic subjects than in normal fasting glucose subjects and that these serum liver enzymes in hyperglycemic subjects are inversely associated with the serum β-cryptoxanthin concentrations.31 Beta-cryptoxanthin is a carotenoid pigment that is found in Satsuma mandarin fruit in particular.10 We are planning to investigate whether β-cryptoxanthin improves the antioxidant defense system in STZ-diabetic rat liver.

There are several limitations in the present study. Firstly, we did not identify the intracellular levels of reactive oxygen spices in STZ-diabetic rat liver. Therefore, we could not discuss the associations of reactive oxygen spices with the pathogenesis in the STZ-diabetic rat liver in detail. Secondly, we have no data about the changes of oxidative damage markers in STZ-diabetic rat liver except for lipid peroxide. From these study limitations, it remains unclear whether chronic administration of Satsuma mandarin could diminish the reactive oxygen spices and oxidative damage.

In conclusion, we tested the hypothesis that Satsuma mandarin fruit prevents hyperglycemia-induced oxidative stress in the liver using STZ-induced type-1 diabetic rat models. As a result, we found that the chronic administration of Satsuma mandarin fruit mitigates the liver dysfunction in STZ-diabetic rat via the improvement of the antioxidant defense sys-
tem. Although our data suggest that chronic administration of Satsuma mandarin fruit may improve liver dysfunction in STZ-diabetic rat, we did not try to determine which ingredient(s) of this fruit exerts this beneficial effect. Further study will be needed.

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REFERENCES