Effects of Chronic Administration of Fruit Extract (Citrus unshiu Marc) on Endothelial Dysfunction in Streptozotocin-Induced Diabetic Rats

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We investigated the effects of chronic administration of fruit extract (Citrus unshiu Marc) on the endothelial dysfunction seen in aorta from streptozotocin (STZ-)induced diabetic rats. A ten-week administration of this fruit extract preserved acetylcholine (ACh)-induced endothelium-dependent relaxation, but not sodium nitroprusside (SNP)-induced endothelium-independent relaxation, in the diabetic aorta. In age-matched control rats, chronic administration of the fruit extract had no influence on the ACh- or SNP-induced aortic relaxation. The increased total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride levels seen in STZ-induced diabetic rats were not normalized by fruit-extract treatment. These results suggest that Citrus unshiu Marc extract preserves endothelial function in the aorta in STZ-induced diabetic rats without lowering plasma cholesterol. This beneficial effect may be due to this extract protecting of nitric oxide against inactivation by oxygen free radicals.

Key words acetylcholine; aorta; Citrus unshiu; diabetes; endothelium; relaxation

Diabetes mellitus is an important risk factor for the development of atherosclerosis.1) Normally, the endothelium preserves vascular integrity and prevents atherosclerosis by modulating vasomotor tone, platelet activity, thrombosis, and inflammation.2) In diabetes, endothelial dysfunction plays a pivotal role in the pathogenesis of diabetic vascular disease.1,3—8) It has been suggested that the excessive elevations in plasma glucose, low-density lipoprotein (LDL) cholesterol, and reactive oxygen species that occur in diabetes are involved in the development of this dysfunction in several blood vessels.4,7,9—13)

The results of epidemiological studies suggest that a high consumption of fruits reduces the risk of degenerative diseases such as cardiovascular disease, several types of cancer, and neurological diseases.14—17) There is accumulating evidence that these effects of fruits are attributable to antioxidants such as vitamins and phenolic phytochemicals,18) and indeed naturally occurring antioxidants have been reported to play a key role in ameliorating the oxidative damage induced by the free radicals that cause several human diseases. Citrus fruits contain sugar, organic acids, and a number of physiologically active components such as citric acid, ascorbic acid, minerals, coumarins, and flavonoids (e.g., naringin, hesperidin, neohesperidin, rutin, naringenin, hesperetin, nairutin, and tangeretin).19,20) Although direct supplementation with citrus fruits has been found to be beneficial in several conditions,21,22) no study has examined the effects of administering these fruits for prolonged periods on the endothelial dysfunction seen in the diabetic state.

In the present study, we investigated whether chronic treatment with citrus fruit extract might ameliorate the endothelial dysfunction seen in an experimental diabetic state. We previously reported that acetylcholine (ACh)-induced endothelium-dependent relaxation was impaired in the aorta at 10—12 weeks after streptozotocin (STZ) injection (which creates a model of Type 1 diabetes).7,11,12) Here, to investigate the possible effect of Citrus unshiu Marc extract we started chronic treatment with this extract immediately after the STZ injection, and continued it for 10 weeks.

MATERIALS AND METHODS

Materials Streptozotocin (STZ), (−)-norepinephrine hydrochloride (NE), Nω-nitro-1-arginine, and sodium nitroprusside (SNP) were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), while acetylcholine chloride (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan). All drugs were dissolved in saline, except where otherwise noted. The chow containing fruit extract was prepared as follows. The concentrated Citrus unshiu Marc juice was freeze-dried, and the resultant powder was mixed with normal commercial diet (Oriental Yeast, Tokyo, Japan) at 1, 3, 10 w/w% (fruit extract-containing diet).

Animals and Experimental Design Male Wistar rats (8 weeks old and 180—230 g body weight) were divided into the following eight groups: 1%, 3%, or 10% (wt/wt) fruit extract (Citrus unshiu Marc)-treated diabetic or age-matched control groups, plus normal diet-fed diabetic or age-matched control groups. To generate diabetes, some rats received a single injection via the tail vein of STZ 75 mg/kg dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. After STZ or buffer injection, the rats were either fed standard powdered rodent chow (Oriental Yeast, Tokyo, Japan) or the same chow containing fruit extract [1, 3, or 10% (wt/wt)], and they were all maintained in an environmentally controlled room under a 12:12-h light–dark cycle for ten weeks. Food and water were allowed ad libitum. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan).

Measurement of Plasma Glucose, Cholesterol, and
Triglyceride Levels  Ten weeks after the administration of STZ (diabetic groups) or buffer (control groups), plasma glucose was determined using a commercially available enzyme kit (Wako Pure Chemical Ind., Ltd., Osaka, Japan), which made use of the O-toluidine method.23) Plasma total cholesterol and triglyceride levels were determined using a commercially available enzyme kit (Wako Pure Chemical Ind., Ltd., Osaka, Japan), the plasma triglyceride level being assayed by the method described by Spayd et al.24) High-density lipoprotein (HDL) cholesterol was measured following phosphotungstic-MgCl₂ precipitation of apolipoprotein B containing very low density lipoprotein (VLDL) (Wako Pure Chemical Ind., Ltd., Osaka, Japan). The LDL level was derived from the above data using the Friedewald formula: \[ \text{LDL cholesterol} = \text{total cholesterol} - \text{HDL} - \left( \frac{1}{5} \right) \text{triglyceride}. \]

Measurement of Isometric Force  Rats from the eight groups mentioned above were anesthetized with diethyl ether and euthanized by decapitation 10 weeks after treatment with STZ or buffer. A section of the thoracic aorta from between the aortic arch and the diaphragm was then removed and placed in ice-cold, oxygenated, modified Krebs–Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO₃, 1.8 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgSO₄, and 11.0 dextrose. The aorta was cleaned of loosely adhering fat and connective tissue and cut into helical strips 3 mm in width and 20 mm in length. The tissue was placed in a well-oxygenated (95% O₂–5% CO₂) bath of 10-ml KHS at 37 °C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g (determined to be optimum in preliminary experiments). During this period, the KHS in the tissue bath was replaced every 20 min. For the relaxation studies, the NE-inhibited contraction had reached a plateau level, ACh (10⁻⁶ M) or SNP (10⁻⁷ M) was added. This concentration produced 75—80% of the maximal response, with each strip developing a tension of 1.0 g (determined to be optimum in preliminary experiments).

Statistical Analysis  Data are expressed as the mean±S.E.M. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after a one-way analysis of variance (ANOVA), a probability level of \( p<0.05 \) being regarded as significant. Statistical comparisons between concentration–response curves were made using a two-way ANOVA, with Bonferroni’s correction for multiple comparisons being performed post hoc \( (p<0.05 \text{ again being considered significant})\).

RESULTS

Blood Glucose and Animal Body Weights  As reported previously,7,11,12 at the time of the experiment all STZ-treated rats exhibited hyperglycemia, their blood glucose concentrations being significantly higher than those of theagematched nondiabetic control rats (Table 1). Moreover, the body weights of the diabetic rats were significantly lower than those of the age-matched control rats at the time of the experiment (Table 1). There was no evidence of an influence of the chronic citrus fruit extract treatments (1, 3, or 10%) on either body weight or plasma glucose level in the diabetic and age-matched control rats (Table 1).

Plasma Cholesterol and Triglyceride Levels  As shown in Table 2, the plasma total cholesterol and triglyceride levels were significantly higher in normal diet-fed STZ-induced diabetic rats than in the normal diet-fed age-matched controls. The plasma HDL level did not differ significantly.

### Table 1. Body Weights and Plasma Glucose in Citrus unshiu Marc. Extract-Fed Controls and STZ-Induced Diabetic Rats

<table>
<thead>
<tr>
<th>Type</th>
<th>Body weight (g)</th>
<th>Plasma glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>503.3±8.2 (5)</td>
<td>125.9±4.3 (5)</td>
</tr>
<tr>
<td>1%</td>
<td>488.5±7.0 (6)</td>
<td>127.3±3.1 (6)</td>
</tr>
<tr>
<td>3%</td>
<td>486.0±12.5 (6)</td>
<td>122.9±11.4 (6)</td>
</tr>
<tr>
<td>10%</td>
<td>502.8±9.7 (6)</td>
<td>108.0±13.9 (6)</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>235.1±13.5 (5)*</td>
<td>418.0±21.0 (5)*</td>
</tr>
<tr>
<td>1%</td>
<td>260.6±17.1 (5)*</td>
<td>468.2±27.9 (5)*</td>
</tr>
<tr>
<td>3%</td>
<td>244.0±12.1 (6)*</td>
<td>421.4±11.9 (5)*</td>
</tr>
<tr>
<td>10%</td>
<td>238.3±11.0 (6)*</td>
<td>493.6±18.0 (5)*</td>
</tr>
</tbody>
</table>

Number of determinations is shown within parentheses. Normal, animals fed the normal diet; 1%, 3%, and 10%, animals fed a diet containing 1%, 3%, or 10% Citrus unshiu Marc. extract, respectively. \(* p<0.001\) vs. Normal-diet controls.

### Table 2. Changes in Various Plasma Parameters in Citrus unshiu Marc. Extract-Fed Controls and STZ-Induced Diabetic Rats

<table>
<thead>
<tr>
<th>Type</th>
<th>Plasma parameters (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>145.0±6.6 (5)</td>
</tr>
<tr>
<td>1%</td>
<td>130.7±7.6 (6)</td>
</tr>
<tr>
<td>3%</td>
<td>137.7±4.8 (6)</td>
</tr>
<tr>
<td>10%</td>
<td>132.8±4.8 (6)</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>266.4±24.3 (5)**</td>
</tr>
<tr>
<td>1%</td>
<td>208.0±29.9 (5)</td>
</tr>
<tr>
<td>3%</td>
<td>217.3±23.7 (6)*</td>
</tr>
<tr>
<td>10%</td>
<td>254.5±23.0 (6)*</td>
</tr>
</tbody>
</table>

Number of determinations is shown within parentheses. Normal, animals fed the normal diet; 1%, 3%, and 10%, animals fed a diet containing 1%, 3%, or 10% Citrus unshiu Marc. extract, respectively. \(* p<0.05\), \]** p<0.01\) vs. Normal-diet controls.
between these two groups. The plasma LDL cholesterol level tended to be higher in the normal diet-fed diabetic rats than in the corresponding controls. Following chronic treatment with citrus fruit extract (1, 3, or 10%), there were no significant alterations in these parameters in either the diabetic rats or the age-matched control rats.

**Relaxation Response** To investigate endothelium-dependent relaxation, we added ACh (10^{-9}–10^{-5} M) cumulatively to strips precontracted by NE (5×10^{-5}–3×10^{-7} M) (Figs. 1A, B). In strips from age-matched control rats, ACh (10^{-9}–10^{-5} M) induced a concentration-dependent relaxation, with the maximum response at 10^{-7} M. The relaxation induced by ACh was significantly weaker in strips from STZ-induced diabetic rats (Fig. 1B). This relaxation did not differ significantly among the various control groups (which had been fed either the normal diet or one of the citrus fruit extracts (1, 3, or 10%) (Fig. 1A, Table 3). Interestingly, aortic strips from STZ-induced diabetic rats chronically treated with 10% citrus fruit extract relaxed in a normal way to ACh (Fig. 1B, Table 3). In contrast, the endothelium-independent relaxation induced by SNP did not differ significantly among the various groups (Fig. 2, Table 3). The relaxation induced by ACh was completely inhibited by endothelial denudation or by pretreatment with 10^{-4} M N^\text{G}-nitro-L-arginine (a nitric oxide synthase inhibitor) in all groups (data not shown).

**DISCUSSION**

The main conclusion to be drawn from the present study is that in rats with STZ-induced diabetes, chronic feeding with citrus fruit extract (10% of the diet by weight) prevents the development of the endothelial dysfunction otherwise seen in aortas isolated from established diabetic rats without changing the plasma level of cholesterol or triglyceride.

It is well known that fruits contain not only various minerals, but also sugar, such as fructose. From this point, excessive fruit intake seems to be undesirable. Although high-fructose diets induce insulin resistance in animal models, including mice and rats, most studies of chronic fructose ingestion have been shown some benefits of the substitution of...
fructose for other carbohydrates to diabetic patients, 28,29) and low-dose fructose improves the glycemic response to an oral glucose load in adults with type 2 diabetes, and this effects is not a result of stimulation of insulin secretion. 30) In the present study, when citrus fruit extract was fed for 10 weeks to diabetic and age-matched control rats, plasma glucose and cholesterol levels showed no significant alterations compared with those in the respective normal diet-fed rats, nor indeed did body weight (Tables 1, 2). Furthermore, it was noteworthy that such fruit-extract administration, even at 10% of the matched control rats. These data suggest that our administration of citrus fruit extract had no influence on food intake or on cholesterol metabolism, and had no other adverse effect that we could detect. Also, we suggested that the 10% fruit extract supplementation was not excessive intake (i.e. moderate amount as supplement) in this model.

We and others have reported that the relaxation responses induced in blood vessels by endothelium-dependent agents are weak in STZ-induced diabetic rats. 3-8,13,31-34) Moreover, a considerable body of evidence now suggests that the impairment of endothelium-dependent relaxation seen in diabetes and atherosclerosis may involve inactivation of NO by oxygen-derived free radicals. 4,33,35-37) Production of superoxide anion leads to inactivation of NO 35,38) and dismutation of free radicals has generally 33,36,37) been found to improve impaired endothelium-dependent relaxation in experimental models of diabetes. Indeed, we recently reported that NO is metabolized by O2− to NO2− not just to NO3−, and that the resulting rapid inactivation of NO may be responsible for the impairment of endothelium-dependent relaxation that is seen in aortic strips from diabetic rats. 35) In the present study, chronic administration of 10% citrus fruit extract effectively prevented the attenuation of the ACh-induced endothelium-dependent relaxation otherwise seen in aortae from STZ-induced diabetic rats (Fig. 1B). This may suggest that NO metabolism is normalized by such treatment. Although our data suggest that citrus fruit administration may preserve endothelial function by alleviating oxidative stress in diabetic rats, we did not directly investigate which ingredient(s) of these fruits might exert this beneficial effect. Indeed, citrus fruits contain antioxidants such as vitamin C. 21,39) polyphenol, 40) flavonoids 41) and β-cryptoxanthin. 42) Further investigation is required on this point.

In conclusion, our findings suggest that chronic supplementation of the diet with citrus fruit extract (e.g., Citrus unshiu MARC) might prevent the development of the endothelial dysfunction involved in diabetic vasculopathy. We believe that our findings should stimulate further interest in whether an increased intake of citrus fruits might help to prevent diabetic complications, or possibly even alleviate existing ones.

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REFERENCES