Anti-proliferative effect of the extract of Guangzao (Fructus Choerospondiatis) on cultured rat cardiac fibroblasts

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**Abstract**

**OBJECTIVE:** To ascertain if total flavonoids of Guangzao (Fructus Choerospondiatis) (TFFC) extracted from Guangzao (Fructus Choerospondiatis) can inhibit angiotensin II-induced proliferation of cardiac fibroblasts (CFs).

**METHODS:** CFs were cultured by the differential attachment method. A model of cell proliferation was established by stimulation with Ang II. Cardiac fibroblasts growth was determined using a hemocytometer. Cell proliferation was detected by methyl thiazole tetrazolium. Lactate dehydrogenase activity was measured by chemical colorimetric method.

**RESULTS:** Proliferation of TFFC-treated (25, 50, 100 mg/L) fibroblasts was significantly less than that of cells in the angiotensin II group (P < 0.01), and TFFC inhibited proliferation in a dose-dependent manner. These inhibitory effects were partly blocked by pretreatment with NG-nitro-L-arginine methyl ester (L-NAME) and 1H-[1,2,4]-oxadiazole-[4,3-a]-quinoxalin-1-one (ODQ).

**CONCLUSION:** TFFC inhibited angiotensin II-induced proliferation of cardiac fibroblasts via a mechanism that probably involves activation of the NO-cyclic guanosine monophosphate signaling pathway.

**Key words:** Flavones; Guangzao (Fructus Choerospondiatis); Angiotensin II; Fibroblasts; Nitric oxide; Cyclic GMP

**INTRODUCTION**

Proliferation of cardiac fibroblasts and accumulation of excessive amounts of proteins in the extracellular matrix are the basic pathologic processes of myocardial fibrosis. Angiotensin II (Ang II) is considered to be an important factor in the autocrine and paracrine functions of cardiac fibroblasts. Furthermore, Ang II activates a series of signaling molecules to induce cardiac fibrosis.

Guangzao (Fructus Choerospondiatis) is a Chinese herb believed to have therapeutic potential against cardiovascular diseases. The main component extracted from this herb, total flavonoids of Guangzao (Fructus Choerospondiatis) (TFFC), has been reported to have cardioprotective effects. Thus, TFFC could be a therapeutic agent for a multitude of cardiovascular diseases. Here, we investigated if TFFC can inhibit Ang II-induced proliferation of cardiac fibroblasts.
MATERIALS AND METHODS

This study was conducted in accordance with the institutional guidelines for the care and use of laboratory animals set by Zhejiang University (Zhejiang, China). Ethical approval was given by the Medical Ethics Committee of Zhejiang University.

Animals and reagents

Six male 3-day-old Sprague-Dawley (SD) rats were purchased from the Laboratory Animal Center of Zhejiang University. L-NG-Nitroarginine methyl ester (L-NAME), 3-[(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1H-[1,2,4]oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), and Ang II were purchased from Sigma-Aldrich (Saint Louis, MO, USA). A Lactate Dehydrogenase (LDH) Reagent kit was purchased from Jincheng Bio-engineering Institute (Nanjing, China). HP-100 resin was purchased from Mitsubishi Chemicals (Tokyo, Japan).

Preparation of ethanol extracts of TFFC

Ripe fruits of Guangzao (Fructus Choerospondiati) were collected in April 2010 from Baotou (Inner Mongolia, China). Extracts were obtained by refluxing the dried, powdered fruit (2.7 kg) of Guangzao (Fructus Choerospondiati) with 50% ethanol for 60 min twice. Resulting solutions were combined and filtered. After solvent removal, fruit extracts were centrifuged. Supernatants (12.6 L) were concentrated using a HPD100 macroporous resin for enrichment and then fractionated using water (0% ethanol) and increasing concentrations of ethanol (20%, 50%, 70%, and 95%). The eluates obtained with 50% ethanol were concentrated under vacuum to produce a powder.

Analyses of constituents of fruit extracts using high-performance liquid chromatography (HPLC)

Major constituents in active fractions of Guangzao (Fructus Choerospondiati) extracts were identified using HPLC, which was undertaken using an Alltima™ C18 Reversed-phase Analytical Column and determination of absorbance at 508 nm. Rutin was used as an external standard. Finally, dry crude total flavonoids were obtained (11.83 g) from fractions eluted in 50% ethanol. Total flavonoid content (53.65%) was determined using water (0% ethanol) and increasing concentrations of ethanol (20%, 50%, 70%, and 95%). The eluates obtained with 50% ethanol were concentrated under vacuum to produce a powder.

Cell culture

Cardiac fibroblasts were isolated from 3-day-old SD rats using a differential attachment method reported previously. Cells from the second to third passage were used for all experiments. Cardiac fibroblasts were distinguished from other cell types through immunohistochemical staining. Cardiac fibroblasts stained positive for the fibroblast marker fibronectin and stained negatively for a marker of muscle cells (α-actin).

Effect of TFFC on growth of cardiac fibroblasts

For culture of adherent cells, 500 μL of a suspension of cardiac fibroblasts (2.0 × 10^5 cells/mL) was added to each well of 24-well plates, and cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) for 24 h. Adherent cells were serum-starved for 24 h and then treated with various concentrations of TFFC (0, 25, 50, 100 mg/mL) in DMEM containing 0.2% FCS for 24, 48, and 72 h. At each time point, cells were digested using 0.25% trypsin for 6-8 min at 37 °C, stained with 0.4% trypan blue, and the number of viable cells calculated using a hemocytometer. Each experiment was done in triplicate.

Effect of TFFC on the Ang II–induced proliferation of cardiac fibroblasts

Cell proliferation was assessed by the MTT assay. Briefly, 200 μL of a suspension of cardiac fibroblasts (2.5 × 10^5 cells/mL) was added to each well of 96-well plates and cultured to obtain adherent cells. Then, cells were cultured in DMEM containing 10% FCS for 24, 48, and 72 h, starved by serum deprivation for 24 h, and then treated with various concentrations of TFFC (0, 25, 50, 100 mg/mL) for 24, 48, and 72 h. Ang II (100 nM) was added to a set of cultures from each treatment group 1 h before TFFC treatment. MTT (20 μL of 5 mg/mL) was added to each well, cells incubated for 4 h, the culture supernatant removed, and cells treated with 150 μL of dimethyl sulfoxide (DMSO) for 10 min. Absorbance at 490 nm was determined using a Microplate Spectrophotometer (Biorad, Model 680). The experiment was done in triplicate.

Effects of L-NAME and ODQ on proliferation of cardiac fibroblasts

A suspension of cardiac fibroblasts (200 μL; 2.5 × 10^5 cells/mL) was added to each well of 96-well plates and cultured in DMEM containing 10% FCS for 24 h to obtain adherent cells. Cells were then serum-starved for 24 h and treated with various concentrations of TFFC (0, 25, 50, 100 mg/mL) in DMEM containing 0.2% FCS for 72 h. L-NAME (100 μM) and ODQ (0.1, % v/v) as solvent A and acetonitrile: formic acid (100:25, 0.2% FCS) as solvent B. A gradient of 2%-30% solvent B over 60 min was used, and the flow rate was 1.0 mL/min. For quantification, absorbance at 254 nm was determined.
(10 μM) with or without 100 nM Ang Ⅱ were added to the culture 1 h before TFFC treatment. Then, MTT (20 μL of 5 mg/mL) was added to each well and incubated for 4 h, followed by removal of the culture supernatant and treatment of cells with 150 μL of DMSO for 10 min. Finally, absorbance at 490 nm was determined using a Microplate Spectrophotometer (Bio-Rad, Model 680).

**Effect of TFFC on LDH release**
Cardiac fibroblasts were seeded at 2.5×10⁴ cells/mL in 96-well plates and cultured in DMEM containing 10% FCS for 24 h. Cells were serum-starved overnight in 0.1% FCS and then treated with various concentrations of TFFC in DMEM containing 0.2% FCS. Ang Ⅱ (100 nM) was added to a set of cultures from each treatment group 1 h before treatment with TFFC. After 24, 48, and 72 h of incubation, culture media were collected and LDH activity assayed using a colorimetric method described previously. LDH activity was estimated by determining changes in the absorbance of pyruvic acid at 440 nm using a Spectrophotometer (Bio-Rad, Model 680).

**Data analyses**
Data are the mean ± standard deviation (±s). One-way analysis of variance followed by Bonferroni’s multiple comparison tests were employed using SPSS v10.0 (Higher Education Press, Beijing, China). P < 0.05 was considered significant.

**RESULTS**

**TFFC chromatogram**
Samples were quantified using an HP1100 series HPLC system maintained at 35℃ (Figure 1).

![HPLC-UV 254-nm chromatogram of ethanol extract produced from guangzao (Fructus Choerospondiatis)](image)

**Table 1 Effect of TFFC on the growth of cardiac fibroblasts (cells, x ± s)**

<table>
<thead>
<tr>
<th>Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFFC 0 mg/mL</td>
<td>4</td>
<td>4.1±0.3</td>
<td>5.6±0.7</td>
</tr>
<tr>
<td>TFFC 25 mg/mL</td>
<td>4</td>
<td>3.6±0.4</td>
<td>4.5±0.4</td>
</tr>
<tr>
<td>TFFC 50 mg/mL</td>
<td>4</td>
<td>2.8±0.5</td>
<td>3.6±0.5</td>
</tr>
<tr>
<td>TFFC 100 mg/mL</td>
<td>4</td>
<td>2.7±0.6</td>
<td>3.1±0.5</td>
</tr>
</tbody>
</table>

Notes: growth of cardiac fibroblasts growth was analyzed using a hemocytometer. Proliferation of cardiac fibroblasts was monitored 24, 48, and 72 h. After pretreatment with TFFC (25, 50, 100 mg/mL), cell growth was inhibited in a dose- and time-dependent manner. Treatment values are the mean ± standard deviation (n = 4). TFFC: total flavonoids of Guangzao (Fructus Choerospondiatis).
TFFC and ODQ on cardiac fibroblasts proliferation induced by Ang Ⅱ

To determine whether the inhibitory action of TFFC was affected by ODQ, we incubated cells with 100 nM Ang Ⅱ, followed by exposure to TFFC and ODQ for 72 h. As shown in Table 3, pretreatment with Ang Ⅱ and ODQ stimulated cardiac fibroblasts proliferation compared with Ang Ⅱ group (P < 0.01). TFFC (25-100 mg) decreased in MTT value. These results indicated that the inhibitory effect of TFFC was partly blocked by 10 μm ODQ.

Effect of TFFC on LDH release

To confirm that the inhibitory effects of TFFC on the proliferation of cardiac fibroblasts were not a result of cytotoxicity, the activity of LDH (a biochemical marker of cellular damage) in the culture medium of TFFC- and Ang Ⅱ-stimulated cardiac fibroblasts was assayed. TFFC (25-100 mg) had no effect on LDH release for ≤ 72 h (Table 4). However, the effect of longer exposure to, and higher concentrations of, TFFC were not tested.

To determine whether the inhibitory action of TFFC on cardiac fibroblasts proliferation at concentrations of 25, 50, and 100 mg/L in a dose- and time-dependent manner. At the doses tested, TFFC did not significantly affect cell detachment, and only a few dead cells were detected in the trypan blue exclusion test (data not shown). To verify that the effects of TFFC on cardiac fibroblasts were not a consequence of cytotoxicity, we assayed LDH activity (a parameter of cytotoxicity) in the supernatants of TFFC- (25-100 mg/L) and Ang Ⅱ-treated cells. LDH activity in the supernatants of treated and untreated cells remained unchanged. This finding was consistent with observations under light microscopy. Therefore, the anti-proliferative effects were due mainly to inhibition of cell proliferation and not due to cytotoxicity.

DISCUSSION

The anti-proliferative effects of TFFC on cardiac fibroblasts treated with Ang Ⅱ were investigated. Based on our results, the mechanism of TFFC action may correlate with activation of the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) signaling pathway. We found that TFFC inhibited Ang Ⅱ-induced proliferation of cardiac fibroblasts at concentrations of 25, 50, and 100 mg/L in a dose- and time-dependent manner. At the doses tested, TFFC did not significantly affect cell detachment, and only a few dead cells were detected in the trypan blue exclusion test (data not shown). To verify that the effects of TFFC on cardiac fibroblasts were not a consequence of cytotoxicity, we assayed LDH activity (a parameter of cytotoxicity) in the supernatants of TFFC- (25-100 mg/L) and Ang Ⅱ-treated cells. LDH activity in the supernatants of treated and untreated cells remained unchanged. This finding was consistent with observations under light microscopy. Therefore, the anti-proliferative effects were due mainly to inhibition of cell proliferation and not due to cytotoxicity.

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**Table 2** Cell proliferation of groups (x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFFC 0 mg/mL</td>
<td>4</td>
<td>0.48±0.07</td>
<td>0.62±0.07</td>
<td>0.78±0.07</td>
</tr>
<tr>
<td>Ang Ⅱ</td>
<td>4</td>
<td>0.85±0.04</td>
<td>1.01±0.06</td>
<td>1.14±0.07</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 25 mg/mL</td>
<td>4</td>
<td>0.70±0.07</td>
<td>0.85±0.04</td>
<td>0.96±0.04</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 50 mg/mL</td>
<td>4</td>
<td>0.62±0.05</td>
<td>0.74±0.07</td>
<td>0.88±0.06</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 100 mg/mL</td>
<td>4</td>
<td>0.52±0.05</td>
<td>0.69±0.09</td>
<td>0.80±0.07</td>
</tr>
<tr>
<td>Ang Ⅱ + valsartan 10 μmol/L</td>
<td>4</td>
<td>0.50±0.07</td>
<td>0.62±0.09</td>
<td>0.78±0.07</td>
</tr>
</tbody>
</table>

Notes: final concentration of Ang Ⅱ was 100 nM. The frequency of proliferation was monitored after 24, 48, and 72 h. TFFC: total flavonoids of Guangzao (Fructus Choerospondiatis).

**Table 3** Effects of TFFC and L-NAME, ODQ and TFFC on cardiac fibroblasts proliferation (x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TFFC and L-NAME</th>
<th>TFFC and ODQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFFC 0 mg/mL</td>
<td>4</td>
<td>0.76±0.05</td>
<td>0.80±0.05</td>
</tr>
<tr>
<td>Ang Ⅱ</td>
<td>4</td>
<td>1.16±0.05</td>
<td>1.02±0.09</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 25 mg/mL</td>
<td>4</td>
<td>1.33±0.06</td>
<td>1.34±0.07</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 50 mg/mL</td>
<td>4</td>
<td>1.24±0.07</td>
<td>1.24±0.05</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 100 mg/mL</td>
<td>4</td>
<td>1.09±0.06</td>
<td>1.01±0.07</td>
</tr>
<tr>
<td>Ang Ⅱ + valsartan 10 μmol/L</td>
<td>4</td>
<td>0.94±0.07</td>
<td>0.92±0.07</td>
</tr>
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</table>

Notes: both Ang Ⅱ and L-NAME stimulated cardiac fibroblasts proliferation. The inhibitory effect of TFFC was partly blocked by 100 μM L-NAME. Both Ang Ⅱ and ODQ stimulated cardiac fibroblasts proliferation. The inhibitory effect of TFFC was partly blocked by 10 μM ODQ. TFFC: total flavonoids of Guangzao (Fructus Choerospondiatis); L-NAME: NG-nitro-L-arginine methyl ester; ODQ: 1H-[1, 2, 4] oxadiazolo [4,3-a] quinoxalin-1-one.

**Table 4** LDH activity in the culture medium of cardiac fibroblasts (U/L, x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFFC 0 mg/mL</td>
<td>4</td>
<td>23.0±0.7</td>
<td>23.6±0.8</td>
<td>24.7±0.6</td>
</tr>
<tr>
<td>Ang Ⅱ</td>
<td>4</td>
<td>23.1±0.7</td>
<td>23.7±0.7</td>
<td>24.8±0.9</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 25 mg/mL</td>
<td>4</td>
<td>23.2±1.1</td>
<td>23.8±1.2</td>
<td>24.9±0.9</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 50 mg/mL</td>
<td>4</td>
<td>23.6±0.7</td>
<td>24.4±0.9</td>
<td>25.1±0.6</td>
</tr>
<tr>
<td>Ang Ⅱ + TFFC 100 mg/mL</td>
<td>4</td>
<td>24.1±0.8</td>
<td>24.7±1.4</td>
<td>26.0±0.8</td>
</tr>
</tbody>
</table>

Notes: LDH activity was assessed using a colorimetric method. aP > 0.05; vs control group at same time points. TFFC (25-100 mg) and 100-nM Ang Ⅱ had no effect on LDH release at 24 h to 72 h. Ang Ⅱ: angiotensin Ⅱ; TFFC: total flavonoids of Guangzao (Fructus Choerospondiatis); LDH: lactate dehydrogenase.
not due to direct cytotoxicity. Pretreatment with L-NAME (an inhibitor of nitric oxide synthase (NOS)) or ODQ (an inhibitor of soluble guanylate cyclase (sGC)) blocked the inhibitory effects of TFFC. Our previous report showed that TFFC increased levels of NO and NOS in the culture medium and increased intracellular levels of cGMP in cardiac fibroblasts. Therefore, we speculate that TFFC may exert its inhibitory effects through activation of the NO-cGMP signaling pathway.

This study had one main limitation. We did not investigate whether TFFC affected the production of NO and cGMP. Further studies are needed to elucidate the mechanism of TFFC activity in vivo.

We demonstrated that TFFC extracted from guangzao directly inhibited Ang II-induced proliferation of cardiac fibroblasts. Our findings demonstrate that TFFC: (a) may have important cardioprotective effects; (b) administration to cardiac fibroblasts may serve as a novel approach for the treatment of cardiac fibrosis.

REFERENCES