Oxidation of fatty acid may be enhanced by a combination of pomegranate fruit phytochemicals and acetic acid in HepG2 cells

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Abstract
We investigated whether the combination of phytochemicals and acetic acid in the form of fruit vinegar provides an additive effect on changes of mRNA levels related to fatty acid oxidation in human hepatocyte (HepG2). Among the seven fruit vinegars (Rubus coreanus, Opuntia, blueberry, cherry, red ginseng, mulberry, and pomegranate) studied, treatment of HepG2 with pomegranate vinegar (PV) at concentrations containing 1 mM acetic acid showed the highest in vitro potentiating effect on the mRNA expression levels of peroxisome proliferator-activated receptor γ, carnitinepalmitoyl transferase-1, and acyl-CoA oxidase compared to the control group (P < 0.05). Reversed-phase liquid chromatography in combination with quadrupole time-of-flight mass spectrometry analysis revealed four potential compounds (punicaglin B, ellagic acid, and two unidentified compounds) responsible for altered gene expression in HepG2 cells treated with PV as compared with the others. Further investigations are warranted to determine if drinking PV beverages may help to maintain a healthy body weight in overweight subjects.

Key Words: Vinegar beverage, acetic acid, pomegranate, fatty acid oxidation, HepG2 cells

Introduction
Vinegar is a useful liquid that results from the fermentation of ethanol. Historically, it has been used not only as a seasoning and preservative but also as a traditional folk medicine. The key component of vinegar is acetic acid, which is present at a concentration of 3-9% for consumer use [1]. The liver is the principal organ responsible for the metabolism of absorbed acetic acid after oral administration. It is metabolized via acetyl-CoA in the tricarboxylic acid cycle, resulting in an elevation of the AMP/ATP ratio and subsequent phosphorylation of AMP kinase (AMPK) [2]. Activated AMPK leads to concomitant inhibition of fatty acid synthesis and activation of fatty acid oxidation. Gene expressions of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and the transcription factors sterol regulatory element binding protein (SREBP-1) and peroxisome proliferator-activated receptor γ (PPARγ) are downregulated, whereas gene expressions of acyl-CoA oxidase (ACO), carnitinepalmitoyl transferase-1 (CPT-1), uncoupling protein-2 (UCP-2), and transcription factor PPARα are upregulated through activating AMPK [3]. In fact, Fushimi et al. [4] have reported that the expression levels of lipogenesis-related proteins such as ACC, FAS, and SREBP-1 are reduced in the rat liver due to acetic acid administration. More recently, Kondo et al. [3] investigated the effect of acetic acid on the activation of fatty acid oxidation in mice fed a high-fat diet. They reported that acetic acid upregulates genes involving AMPK-mediated fatty acid oxidation and thermogenic proteins, such as ACO, CPT-1, and UCP-2, in the liver. They also demonstrated the effect of vinegar intake on the reduction of body fat mass in obese subjects [5].

As public interest in the potential health benefits of vinegar has increased over the past few years, many different types of vinegars are being introduced into the market with a range of diverse uses. In Asia, the popularity of vinegar beverages containing various kinds of fruit extracts has been increasing. Fruits are the richest source of phytochemicals, which are capable of performing a number of functions. Recent studies have shown that some fruit phytochemicals such as rutin (rich in citrus fruits), quercetin (rich in lovage and apples), and resveratrol (rich in grapes) are able to increase AMPK phosphorylation and thus reduce lipid accumulation in hepatic cells [6-7]. In addition, cranberry extract administration improved the lipid profile and reduced visceral fat mass mediated by the activation of the adiponectin/AMPK pathway in obese mice [8]. However, all
phytochemicals do not necessarily have the same traits. Moreover, it is necessary to figure out a strategy to determine which phytochemicals, in combination with acetic acid, are in fact providing the additive health benefits of fatty acid oxidation.

Based on previous studies mainly focusing on fat oxidation, we investigated whether the combination of phytochemicals and acetic acid in seven fruit vinegar beverages provides an additive effect in a model cell system. Then, we studied the phytochemical composition of fruit vinegars by using liquid chromatography in combination with quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). This combined approach led to the identification of candidate phytochemicals responsible for the additive effect on fat oxidation.

Materials and Methods

Fruit vinegars

Seven kinds of fruit vinegars (Rubus coreanus, Opuntia, blueberry, cherry, red ginseng, mulberry, and pomegranate) were obtained from Daesang Corp. (Seoul, Korea). Fruit vinegar, the main ingredient of fruit vinegar beverages, was prepared by two stage fermentation. Briefly, each aqueous fruit extract was added after the alcohol fermentation and then acetic acid fermentation was continued. The content of acetic acid in each fruit vinegar was analyzed using high performance liquid chromatography (HPLC, Agilent Technologies 1200, Santa Clara, CA, USA). HPLC separation was achieved using an Aminex HPX-87H column (300 × 7.8 mm i.d., 9 μm particle size; Bio-Rad, Hercules, CA, USA). The mobile phase consisted of 0.05 N sulfuric acid, and the flow rate was 0.5 mL/min. Total acids were detected at 210 nm with a UV detector, and they were quantified as the sum of oxalic acid, citric acid, malic acid, succinic acid, lactic acid, and acetic acid. All organic acid standards were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The percentages of total acid, acetic acid, and fruit extract in fruit vinegars are presented in Table 1.

Cell culture

Human hepatocytes (HepG2; ATCC No. HB-8065) were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in high glucose Dulbecco’s Modified Eagles Medium (DMEM; Gibco, Grand Island, NY, USA), 10% fetal bovine serum (Gibco), 100,000 U/L penicillin (Gibco), and 100 mg/L streptomycin (Gibco) at 37 °C in a humidified atmosphere with 5% CO₂.

For experiment, HepG2 cells were culture in a 6-well plate and serum-starved for 24 h and subsequently cultured in serum-free DMEM containing fruit vinegars for an additional 3 h. Sterile filtered fruit vinegars were added to each well at concentrations from 0.25 to 5 mM as acetic acid, and their viability was measured by the tetrazolium dye (MTT) based assay. While cell viability was reduced to 82 and 60% at 2.5 mM and 5 mM, respectively, no significant toxic effect as well as no changes in pH were observed at concentration less than 1 mM (data not shown) regardless of the kinds of fruit vinegars treated; hence, all studies were performed at acetic acid concentrations less than 1 mM. Acetic acid-free DMEM was used as the control.

RNA preparation

RNA was then extracted using TRIZol (Invitrogen, Carlsbad, CA, USA) as directed by the manufacturer’s instructions. RNA was treated with DNase I (Quagen, Crawley, UK) to avoid DNA contamination. The quality and purity of total RNA was measured by a BioSpec-nano (Shimadzu Corp., Kyoto, Japan). cDNA was constructed using high capacity RNA with a cDNA kit (Applied Biosystems, Foster City, CA, USA).

Quantitative Taqman Reverse Transcription-Polymerase Reaction (RT-PCR)

PCR was performed with a Step-One-Plus RT-PCR System (Applied Biosystems) in 96-well microtiter plates using a final volume of 20 μL. Optimum reaction conditions were obtained with 10 μL of Universal Master Mix (Applied Biosystems) containing dNTPs, MgCl₂, reaction buffer, AmpliTaq Gold, and fluorescence-labeled Taqman probe. Template cDNA (2 μg) was added to the reaction mixture. Amplifications were performed starting with a 10 min template denaturation step at 95 °C, followed by 40 cycles at 95 °C for 1 s and holding at 60 °C for 1 min. The primer sets used targeted the following human genes: PPARα [PPARα; Hs00231882_m1], ACO [ACOX1; Hs00

Table 1. The percentages of total acid, acetic acid and fruit extract found in the fruit vinegars used in this study

<table>
<thead>
<tr>
<th>Fruit vinegar</th>
<th>Scientific name</th>
<th>Rubuscoreanus vinegar</th>
<th>Opuntia Bung</th>
<th>Opuntia ficus-India</th>
<th>Vaccinium uliginosum</th>
<th>Prunus pauciflora</th>
<th>Panax ginseng</th>
<th>Morus bombycis</th>
<th>Punica granatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total acid (%) w/v</td>
<td></td>
<td>6.00 ± 0.3</td>
<td>6.76 ± 0.3</td>
<td>5.08 ± 0.3</td>
<td>4.30 ± 0.2</td>
<td>4.93 ± 0.3</td>
<td>4.30 ± 0.3</td>
<td>4.69 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Acetic acid (%) w/v</td>
<td>41.19</td>
<td>52.69</td>
<td>59.14</td>
<td>52.35</td>
<td>50.98</td>
<td>92.39</td>
<td>38.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit extract (%) w/v</td>
<td>4.2</td>
<td>11.3</td>
<td>5.6</td>
<td>8.4</td>
<td>0.2</td>
<td>5.2</td>
<td>24.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brix</td>
<td>21.1 ± 2.0</td>
<td>28.9 ± 2.0</td>
<td>18.6 ± 2.0</td>
<td>21.0 ± 2.0</td>
<td>18.4 ± 2.0</td>
<td>21.5 ± 2.0</td>
<td>29.3 ± 2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1/*% of total acid