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Received January 6, 2012; Accepted February 13, 2012; Online Publication, May 7, 2012
[doi:10.1271/bbb.111006]

3,4-Dihydroxycinnamic acid (3,4-DA) is a natural compound with high antioxidant potential found in various foods. This study found that animals administered with 3,4-DA had higher exercise tolerance, reduced blood lactate, and markers of hepatic oxidation. Blood glucose and antioxidant enzymes were not affected by this treatment. 3,4-DA may have applicability in reducing the fatigue associated with exercise.

Key words: dihydroxycinnamic acid; exercise; fatigue; oxidative stress

3,4-Dihydroxycinnamic acid (3,4-DA) is a metabolite produced by the hydrolysis of chlorogenic acid, a major phenolic phytochemical in various foods, including fruits, honeybee propolis, and coffee.1,2 It has been stated that consumption of about 400 mL of coffee results in the ingestion of 0.5–1.0 g of chlorogenic acid and 250–500 mg of 3,4-DA per day.2,3 It is recognized that 3,4-DA has several pharmacological properties, including antioxidant,3,4 anti-inflammatory,5 and immunomodulatory6 activities. At a concentration of 10 μM, 3,4-DA completely blocks the production of reactive oxygen species (ROS) in human neutrophils and the xanthine oxidase system.4

It has been found that increased oxidative stress due to increased ROS production is an important mechanism that leads to the anticipation of fatigue and exercise intolerance in health and disease conditions.6,7 During in vivo screening for endurance capacity, coffee was found to have a marked stimulatory effect on exercise tolerance in humans3,4 and animal models.5,8 Although the ergogenic activity of coffee is attributed mainly to caffeine, coffee is also one of the main natural sources of 3,4-DA, a compound that has not been tested in relation to its effect on exercise tolerance.

In this study, which was designed to investigate the fatigue-attenuating effect of 3,4-DA treatment together with changes in the oxidative status of liver and blood metabolic markers, rats submitted to an aerobic running test to the point of fatigue were used to determine the relevant factors for fatigue and antioxidation.

Thirty-two 8-week-old male Wistar rats (365 ± 16 g body weight, b.w.) were obtained from the main animal laboratory of the Federal University of Viçosa (Brazil) and were kept under controlled temperature conditions (21 ± 2 °C) at relative air humidity of 60 to 70% and 12:12-h light-dark cycle. The animals had free access to rat chow and water. All the experiments were conducted in accordance with internationally accepted laboratory animal use and care standards in the guidelines and rules of the Ethics Committee of the Federal University of Viçosa (approval protocol 064/2010). The animals were randomized into four groups with eight animals per group according to the treatment administered: vehicle-ve (negative control): 0.5 mL of 10% ethanol solution (vehicle); DA5: 3,4-DA (5 mg/kg b.w.); DA25: 3,4-DA (25 mg/kg b.w.); and vit C (positive control): vitamin C (25 mg/kg). The acid and vitamin C (purchased from Sigma-Aldrich, St. Louis, MO) were diluted in 0.5 mL of 10% ethanol solution and administered by gavage.

The fatigue test was conducted using an incremental running protocol on a motor-driven treadmill (Insight Instruments®, Ribeirão Preto, Brazil) at a constant slope of 5% with a starting speed of 10 m/min−1.9 The treadmill velocity was increased by 1 m/min−1 every 3 min and, each rat was run until fatigued. Fatigue was defined as the point at which the animals were no longer able to keep pace with the treadmill and remained in contact with it rear edge for more than 10 s. Time to fatigue (min), speed (m/min), distance traveled (m), and workload (kg/m) were used as exercise tolerance indexes.9 Immediately before and after this test blood levels of lactate and glucose were measured (Accutrend, Roche, Basel, Switzerland), and also triglycerides (Bioclin Laboratories, Belo Horizonte, Brazil). The blood used in these analyses was collected by tail puncture. The animals of all groups were subjected to two fatigue tests 144 (test 1) and 72 (test 2) h before the administration of each treatment. The purpose of these preliminary tests was to adapt the animals to the test protocol9 and to compare initial physical performance and metabolic parameters among the groups. In these tests the animals in all groups showed no statistical
Protocol of Treadmill Running to the Point of Fatigue

standard deviation (\( \sigma \)) on Parameters of Exercise Tolerance of Rats during an Incremental protocol of treadmill running to the point of fatigue. All values were expressed as mean and standard deviation (\( \sigma \)). The data on glucose, lactate, and triglycerides were compared by two-way analysis of variance, and the additional data were compared by one-way analysis of variance. Tukey’s post hoc test was applied in both analyses.

The treatments were administrated 1 h before the last fatigue test (test 3) and the same exercise tolerance indexes and metabolic parameters were determined. Fifteen min after this test, the animals were euthanized under anesthesia (ketamine 10 mg/kg and xylazine 2 mg/kg, i.p.) to determine the proportion of glycogen cytoplasmic inclusions in the hepatocytes,10) malondialdehyde,11) the protein carbonyl content,12) total protein levels,13) catalase,14) and superoxide dismutase activity 15) in the liver tissue. All values were expressed as mean and standard deviation (\( \sigma \)). The data on glucose, lactate, and triglycerides were compared by two-way analysis of variance, and the additional data were compared by one-way analysis of variance. Tukey’s post hoc test was applied in both analyses. \( p < 0.05 \) was regarded as significant.

The animals treated with 3,4-DA presented significant attenuation of fatigue and increased exercise tolerance, and the best results being seen in the DA25 group (Table 1). In this group the animals supported a high workload, a parameter highly relevant in physical assessment, since it is determined by the relationships among body weight, speed, and running angle.9)

There was no significant difference in blood glucose level before and after exercise in either group, indicating similar glucose consumption during exercise (Fig. 1A). On the other hand, the proportion of liver glycogen was significantly higher in the groups treated with 3,4-DA, suggesting lesser liver glycogen mobilization during exercise than in the VE and VIT C groups (Table 2). Lactate and triglycerides levels at rest were similar as between the groups. However, at the fatigue point, lactate levels were significantly lower in the groups treated with 3,4-DA than in the VE and VIT C groups, and the best results were observed in the DA25 group (Fig. 1B). At the fatigue point, triglycerides levels were similar between the groups (Fig. 1C).

Taking together the exercise tolerance indexes and metabolic data, it is evident that the animals treated with 3,4-DA showed a higher metabolic efficiency, suggesting a dose-dependent effect. These animals remained in the fatigue test longer and consumed similar levels of glucose during the test, but produced less lactate at the fatigue point, indicating a more active metabolic aerobic component16) as compared to the VE and VIT C groups.

### Table 1. Effect of 3,4-Dihydroxycinnamic Acid (DA, 5 and 25 mg/kg) on Parameters of Exercise Tolerance of Rats during an Incremental Protocol of Treadmill Running to the Point of Fatigue

<table>
<thead>
<tr>
<th>Parameters</th>
<th>VE</th>
<th>VIT C</th>
<th>DA5</th>
<th>DA25</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTF (min)</td>
<td>30 ± 6</td>
<td>32 ± 6</td>
<td>42 ± 4*</td>
<td>51 ± 4**</td>
</tr>
<tr>
<td>Speed (m/min)</td>
<td>20 ± 2</td>
<td>20 ± 2</td>
<td>23 ± 1*</td>
<td>26 ± 1**</td>
</tr>
<tr>
<td>Distance (m)</td>
<td>427 ± 125</td>
<td>477 ± 114</td>
<td>680 ± 101*</td>
<td>903 ± 84**</td>
</tr>
<tr>
<td>Workload (kg m)</td>
<td>19 ± 6</td>
<td>21 ± 6</td>
<td>31 ± 5*</td>
<td>42 ± 5**</td>
</tr>
</tbody>
</table>

VE, vehicle; VIT C, vitamin C; TTF, time to fatigue. Data are represented as mean ± SD. *\( p < 0.05 \) vs. VE; **\( p < 0.01 \) vs. VE; 1* vs. VIT C; 1** vs. DA5.

### Table 2. Effects of 3,4-Dihydroxycinnamic Acid (DA, 5 and 25 mg/kg) on Hepatic Glycogen, Markers of Hepatic Oxidation and Antioxidant Enzymes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>VE</th>
<th>VIT C</th>
<th>DA5</th>
<th>DA25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen (%)</td>
<td>5 ± 3</td>
<td>8 ± 2</td>
<td>13 ± 2**</td>
<td>19 ± 3**</td>
</tr>
<tr>
<td>P. carbonyl (nmol/mg)</td>
<td>28 ± 4</td>
<td>27 ± 2</td>
<td>19 ± 3**</td>
<td>12 ± 3**</td>
</tr>
<tr>
<td>MDA (nmol/mg)</td>
<td>3 ± 0.2</td>
<td>3 ± 0.4</td>
<td>1 ± 0.2*</td>
<td>1 ± 0.1*</td>
</tr>
<tr>
<td>CAT (U/mg)</td>
<td>8 ± 1</td>
<td>8 ± 2</td>
<td>8 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>SOD (U/mg)</td>
<td>15 ± 5</td>
<td>16 ± 5</td>
<td>16 ± 5</td>
<td>17 ± 6</td>
</tr>
</tbody>
</table>

VE, vehicle; VIT C, vitamin C; CAT, catalase; MDA, malondialdehyde; P. carbonyl, protein carbonyl; SOD, superoxide dismutase. Data are represented as mean ± SD. *\( p < 0.05 \) vs. VE; **\( p < 0.01 \) vs. VE; 1* vs. VIT C; 1** vs. DA5.

Fig. 1. Effects of 3,4-Dihydroxycinnamic Acid (DA, 5 and 25 mg/kg) on Metabolic Variables of Rats during an Incremental Protocol of Treadmill Running to the Point of Fatigue.

VE, vehicle; VIT C, vitamin C. Data are represented as mean ± SD. *\( p < 0.01 \) vs. VE; 1* vs. VIT C; 1** vs. DA5.
There was no significant difference in the hepatic activity of SOD and CAT among the groups. However, both groups receiving 3,4-DA showed significantly reduced levels of MDA and protein carbonyls as compared to the VE and VIT C groups, with the best result being observed in the DA25 group (Table 2).

These data suggest a possible antioxidant dose-dependent activity of 3,4-DA, which is perhaps not mediated by any influence of this acid on these enzymes, but by influence on other metabolic pathways. This requires further investigation. Altogether, the results indicate that 3,4-DA might have radical scavenging activity able to act together with CAT and SOD in the antioxidant defense process, reducing the depletion of these enzymes by excessive production of ROS in strenuous exercise.

Previous studies indicate that high production of ROS during exercise can lead to dysfunction of energetic cellular metabolism due to uncoupling of several enzymatic complexes that integrate the electron transport chain and hence negatively influence the aerobic process of energy production. Thus a reduction in ROS production, and the more oxidative and less glycolytic glucose metabolism in the groups receiving 3,4-DA is consistent with a reduced production of lactate and with the attenuation of fatigue during the progressive exercise protocol used in this study.

Vitamin C was used in this study as positive control. Although 3,4-DA and vitamin C have recognized antioxidant activity, only 3,4-DA supplementation was effective in alleviating fatigue and exercise-induced hepatic oxidation. It has been suggested that vitamin C neutralizes some of the ROS produced during exercise and thus improves physical performance. However, since current evidence in this area is very conflicting, an ergogenic effect cannot be attributed to vitamin C. Hence further studies are required to elucidate the effects of this vitamin during physical exercise.

Considering that ROS are important fatigue inducers, the use of natural antioxidants such as 3,4-DA may have applicability in reducing the levels of fatigue associated with acute and progressive physical exercise.

References