Food and Nutrition Sciences, 2015, 6, 1095-1102
Published Online September 2015 in SciRes. http://www.scirp.org/journal/fns
http://dx.doi.org/10.4236/fns.2015.612114

Fatigue Alleviation Mechanism of Citric Acid Determined by Gene Expression Analysis in the Mouse Liver

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Received 25 July 2015; accepted 4 September 2015; published 7 September 2015

Abstract

Citric acid has been recognized for its ability to alleviate fatigue; however, the mechanism contributing to this effect has not yet been elucidated. Our previous study suggested that the alleviation of fatigue by citric acid intake might be induced by the acceleration of gluconeogenesis. In this study, we tested this hypothesis and evaluated whether the intake of citric acid accelerates gluconeogenesis in the mouse liver through microarray analysis. Because the microarray of focus includes 200 metabolic genes, it could be used to provide information on genes related to glucose metabolism as well as other pathways such as inflammation. Eight-week-old male C57/BL6J mice were divided into the distilled water and citric acid groups (n = 6), and were administered distilled water or a citric acid solution (20 mg/30g body weight) intragastrically with a probe, respectively. Forty minutes after administration, the mice were sacrificed by decapitation. The blood glucose level of the citric acid group was significantly higher (P = 0.001) than that of the distilled water group. The expression levels of genes related to glucose metabolism (*Gck*, *Pfkl*, *Pklr*, *Pck*1, *G6pc*) were not changed by the intake of citric acid. On the other hand, some transcriptional differences were observed in several other genes, including those related to inflammation, blood pressure, the electron transport system, uncoupling proteins, and anti-oxidation. The expression levels of genes related to inflammation were reduced in the citric acid group; e.g., the Il6 expression level was 0.78 times lower in the citric acid group than that in the distilled water group. In conclusion, the results of this study suggest that citric acid intake could accelerate gluconeogenesis and suppresses inflammation. Therefore, the fatigue-alleviation effects of citric acid might be induced by an additive effect between the maintenance of blood glucose levels and the suppression of inflammation.

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How to cite this paper: Hara, Y. and Watanabe, N. (2015) Fatigue Alleviation Mechanism of Citric Acid Determined by Gene Expression Analysis in the Mouse Liver. *Food and Nutrition Sciences*, **6**, 1095-1102.
http://dx.doi.org/10.4236/fns.2015.612114

Keywords

Citric Acid, Fatigue, Gluconeogenesis, Inflammation, Fatigue-Alleviation Mechanism

1. Introduction

Citric acid, one of the organic acids contained in citrus fruits such as lemon, has been recognized for its ability to alleviate fatigue. Thus, citric acid has been used as a nutritional supplement for athletes. For instance, consumption of 0.5 g/kg body mass of sodium citrate was found to improve anaerobic exercise performance in humans [1]. Moreover, oral citric acid (2700 mg/day) administration was shown to attenuate physical fatigue in humans, as assessed on a visual analogue scale [2].

However, the mechanism contributing to the observed benefits of citric acid against physical fatigue has not yet been elucidated. One hypothesis has been put forward that these benefits could be induced by the promotion of ATP production followed by the incorporation of dietary citric acid into the tricarboxylic acid (TCA) cycle [2]; however, there is no evidence that dietary citric acid can be incorporated into the mitochondria, *i.e.*, the TCA cycle. Therefore, the specific mechanism of the benefits of citric acid on physical fatigue has not yet been established.

In our previous study, we validated the effects of citric acid intake on energy metabolism and gene expression levels in fasted mice, using biochemical analyses of the blood and DNA microarrays of the skeletal muscles [3]. This study showed that there was no change in the expression levels of any of the genes related to the TCA cycle following intake of citric acid. On the other hand, the expression of the phosphoenolpyruvate carboxykinase 1 gene (*Pck*1), which encodes a major regulatory enzyme of gluconeogenesis, is 11 times higher in the skeletal muscle of mice administered a citric acid solution than in mice administered distilled water. In addition, the blood glucose level in mice administered the citric acid solution was significantly higher than that in mice administered distilled water. Therefore, the results of this study demonstrated the potential of citric acid to accelerate gluconeogenesis in mice. Accordingly, we inferred that citric acid might alleviate fatigue by inducing the acceleration of gluconeogenesis. However, this previous study was performed using the skeletal muscle, whereas gluconeogenesis is generally believed to occur in the liver, and there is no credible evidence that gluconeogenesis can occur in the skeletal muscle [4].

Therefore, in this study, we evaluated whether the intake of citric acid could accelerate gluconeogenesis in the liver of mice using a combination of biochemical analyses and determinations of the expression levels of genes related to glucose metabolism. Because the gene expression levels were measured with a focused microarray including 200 metabolic genes, it could reveal information on the genes related to not only glucose metabolism but also other pathways such as inflammation. The results of this study could contribute toward explaining the mechanism underlying citric acid's fatigue-alleviation effects.

2. Materials and Methods

2.1. Animals and Experimental Design

Eight-week-old male C57/BL6J mice were purchased from Charles River Laboratories Inc. (Yokohama, Japan). The mice were kept in individual plastic cages at 23˚C ± 2˚C with a 12-hour light-dark cycle (light from 8 a.m. to 8 p.m.). Prior to performing experiments, mice were maintained for 1 week on commercial, non-purified chow diet (CRF-1: Charles River Laboratories Inc., Yokohama, Japan), and divided into distilled water (control) and a citric acid treatment group (n = 6). The mean weights of the distilled water and citric acid groups were equal. Prior to collection of blood and liver samples, mice were fasted for 20 hours and then deprived of water for 2 hours, after which distilled water or a citric acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution (20 mg/30g body weight) was administered intragastrically with a probe. Forty minutes after administration, the mice were sacrificed by decapitation and samples of their blood and liver were collected. The blood samples were used to measure blood glucose and lactic acid, and an aliquot was centrifuged (10,000 × g, 5 min) to obtain plasma. The plasma was frozen and stored at −30˚C until it was analyzed to measure the citric acid concentration. Mouse livers were dissected into two sections, with one section (5 × 5 mm) immediately im-

mersed in RNAlater (QIAGEN N.V., Hilden, Germany) and submitted for microarray analysis, while the other section (approximately 100 mg) was used to measure glycogen levels. All animal studies were performed according to the approved animal research protocol of Showa Women's University.

2.2. Measurement of Plasma Citric Acid Levels

Plasma citric acid levels were measured as previously described [5] [6]. Briefly, 500 µL of 6% perchloric acid (Kishida CHEMICAL Co., Ltd., Osaka, Japan) was added to 250 µL plasma, incubated at room temperature (23˚C ± 2˚C) for 5 minutes, then centrifuged (10,000 × g, 5 min) to obtain a deproteinized supernatant. Next, 500 µL of this supernatant was neutralized with 150 µL of 2N KOH (Wako Pure Chemical Industries, Ltd., Osaka, Japan), mixed with 350 µL of 1.2 M KCl (Wako Pure Chemical Industries, Ltd., Osaka, Japan)/1.0 M Tris (Wako Pure Chemical Industries, Ltd., Osaka, Japan) HCl (Wako Pure Chemical Industries, Ltd., Osaka, Japan) buffer (pH 8.6), and then centrifuged (10,000 × g, 5 min). Then, 500 µL of this supernatant was collected, mixed with 500 µL of reagent (1.0 M TrisHCl buffer (pH 8.6) 0.025 mL/sample, 0.4 mM $ZnSO_4$ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 0.25 mL/sample, β-NADH (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 0.2 mL/sample, malate dehydrogenase (Roche Diagnostics Co., Ltd., Mannheim, Germany) 0.025 mL/sample) and incubated for 5 minutes at room temperature. Samples were then divided into 2 equal fractions, with 20 µL of solvent (0.15 M TrisHCl buffer, pH 8.6) added to one, and 20 µL of citrate lyase (citrate lyase (Roche Diagnostics Co., Ltd., Mannheim, Germany) 0.5 mg/0.15 M TrisHCl buffer, pH 8.6) added to the other. The resulting solutions were incubated at room temperature for 20 minutes, before the absorbance (340 nm) was measured with a spectrophotometer. NADH consumption was determined from the difference in absorbance between the two samples and used to calculate the plasma citric acid level.

2.3. Measurement of Blood Glucose and Lactic Acid Levels

Blood glucose and lactic acid levels were measured at the time of collection. Medisafe Chips (Terumo Corp., Tokyo, Japan) were used to measure blood glucose levels, and Lactate Pro (Arkray Inc., Kyoto, Japan) was used to measure lactic acid levels. All measurements were performed in duplicate.

2.4. Measurement of Liver Glycogen Levels

Liver glycogen levels were measured according to the phenol-sulfuric acid method [7]. Briefly, each section of liver (approximately 100 mg) was homogenized in 0.8 mL of 10% trichloroacetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and then centrifuged (1900 × g, 5 min) to obtain a deproteinized supernatant. 0.4 mL of this supernatant was then mixed with 0.8 mL of 95% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and centrifuged (1900 × g, 5 min) to precipitate glycogen. Next, the supernatant was removed and the precipitated glycogen pellet was re-suspended in 0.5 mL of distilled water. The re-suspended glycogen solution was then mixed with 0.5 mL of 5% phenol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2.5 mL of concentrated sulfuric acid (KANTO CHEMICAL Co., INC., Tokyo, Japan) and incubated at room temperature for 20 minutes, before the absorbance (490 nm) was measured with a spectrophotometer. Standard curve was obtained by 40 mg/L glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution.

2.5. Microarray Analysis

Total RNA was isolated from liver samples of distilled water and citric acid groups (n = 4) using the RNeasy Mini Kit (QIAGEN N.V., Hilden, Germany), according to manufacturer's instructions. Metabolic Chip microarray's (Mitsubishi Rayon Co., Tokyo, Japan) [8], which included 200 metabolic genes, were used to analyze transcriptional differences in the liver between the distilled water and citric acid groups. Microarray analysis was conducted Mitsubishi Rayon Co., Tokyo, Japan and transcriptional changes were expressed as a ratio between citric acid group/distilled water groups (CA/DW). Transcriptional differences were reported if either the ratio of citric acid group/distilled water group (CA/DW) was either ≤ 0.8, or ≥ 1.2 or $P < 0.05$.

2.6. Statistical Analysis

All data was expressed as mean ± standard error (SE). Student's t-tests were used to examine differences between the two groups. Results were considered significant at $P < 0.05$.

3. Results

3.1. Plasma Citric Acid, Blood Glucose, and Lactic Acid Levels

The concentrations of plasma citric acid, blood glucose, and lactic acid were measured 40 minutes after intragastric administration of distilled water or citric acid. Plasma citric acid levels were significantly higher in the citric acid group than in the distilled water group (8.3 ± 0.9 and 4.2 ± 0.1 mg/dL, respectively) (**Figure 1(a)**). Blood glucose levels were also significantly elevated in the citric acid group relative to that in the distilled water group (153 ± 4 and 125 ± 4 mg/dL, respectively) (**Figure 1(b)**). In contrast, blood lactic acid levels did not differ between citric acid group and distilled water group (2.4 ± 0.2 and 2.2 ± 0.1 mM, respectively) (**Figure 1(c)**).

3.2. Transcriptional Analysis of Metabolic Genes: DNA Microarray Analysis

A focused DNA microarray, which included 200 metabolic genes, was used to analyze gene expression changes in the liver between the distilled water and citric acid groups. No differences were observed for the genes *Gck*, *Pfkl*, *Pklr*, *Pck*1, and *G6pc*, which are related to glucose metabolism (**Table 1**).

In contrast, transcriptional changes were observed in several genes related to inflammation, blood pressure regulation, electron transport system, uncoupling proteins, and anti-oxidation (**Table 2**). Expression of the inflammation related gene, *Il*6, was 0.78 times lower in the citric acid group than in the distilled water group. The expression of genes related to blood pressure regulation—*Ren*1, *Agtr*1*a*, and *Agtr*2—was 0.80, 0.89, and 0.80 times lower in the citric acid group than in the distilled water group. The expression of all genes related to the electron transport system, including Ndufa4, was lower in the citric acid group than in the distilled water group. In contrast, the expression of genes related to uncoupling proteins—*Ucp*1, *Ucp*2, and *Ucp*3—was 0.80, 0.81, and 0.85 times lower in the citric acid group than in the distilled water group. Interestingly, the expression of genes related to anti-oxidation—*Gsta*2, *Gsta*4, and *Gpx*1—was increased, whereas the expression of *Gpx*2 and *Gpx*3 was decreased following citric acid intake.

3.3. Liver Glycogen Levels

Liver glycogen levels were higher (P = 0.19) in the citric acid group than in the distilled water group (50.6 ± 7.0 and 38.9 ± 4.0 mg/100g wet weight, respectively) (**Figure 2**).

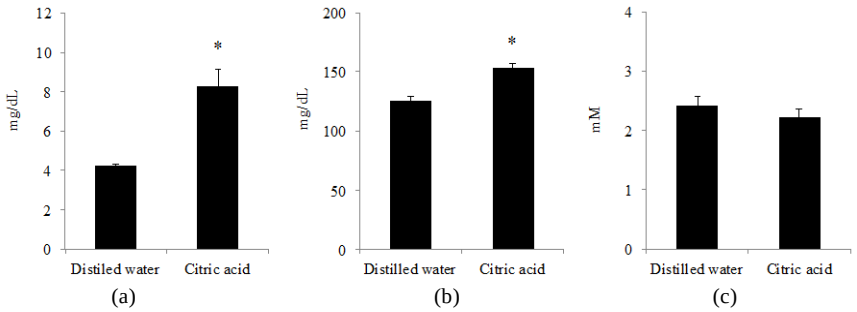

Figure 1. (a) Plasma citric acid levels; (b) Blood glucose levels; (c) Blood lactic acid levels. Data are means ± SE (n = 6). *P < 0.05.

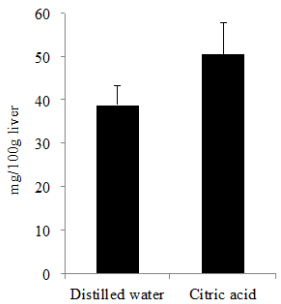

Figure 2. Liver glycogen levels. Data are mean ± SE (n = 6).

Table 1. mRNA expression ratios of citric acid group/distilled water group (CA/DW) for genes related to glucose metabolism.

Accession No.	Gene Symbol	Gene name	CA/DW	P value
NM_010292	Gck	Glucokinase	1.08	0.58
NM_008826	Pfkl	Phosphofructokinase, liver, B-type	0.83	0.17
NM_013631	Pklr	Pyruvate kinase liver and red blood cell, nuclear gene encoding mitochondrial protein, transcript variant 1	1.16	0.36
NM_011044	Pck1	Phosphoenolpyruvatecarboxykinase 1, cytosolic	0.99	0.92
NM_008061	G6pc	Glucose-6-phosphatase, catalytic	0.88	0.36

4. Discussion

Our results demonstrate that the intake of citric acid accelerates gluconeogenesis in mice. The main evidence confirming this finding was that the blood glucose level of the citric acid group was significantly higher than that of the distilled water group. Moreover, in our previous study, administration of citric acid significantly increased the blood glucose level [3]. Nevertheless, the expression levels of genes related to gluconeogenesis, e.g., *Pck*1 and *G*6*pc*, were not changed in this study.

The liver and kidney contribute to glucose production via gluconeogenesis; in particular, the liver has been reported to play a predominant role in gluconeogenesis [4]. On the other hand, in the kidney, the activity of PCK1 which is a key enzyme of gluconeogenesis, was increased in 24-hour-fasted rats [9]. In this study, the mice were fasted for 20 hours, and consequently, gluconeogenesis in the kidney might have been accelerated by the increase in citric acid. This suggests that shortening the fasting time could potentially increase the *Pck*1 gene expression level in the liver. Although the mechanism by which citric acid alleviates fatigue has not yet been elucidated conclusively, our results suggest that it could be related to the acceleration of gluconeogenesis.

The DNA microarray also revealed some unexpected results, including that the intake of citric acid increased the expression of genes related to inflammation, blood pressure, the electron transport system, uncoupling proteins, and anti-oxidation. In particular, the expression of *Il*6, an inflammation-related gene, was decreased by the intake of citric acid in the present study. In a previous study, the addition of citric acid to the medium decreased IL6 secretion, apoptosis, and necrosis in hyperglycemia-induced-inflamed endothelial cells [10]. In addition, it has been reported that the intake of citric acid decreased tumor necrosis factor-α production in the brain of lipopolysaccharide (LPS)-induced inflamed mice; LPS injection to mice or rats is widely used as an inflammation model in tissues or organs such as the brain [11]. Our result that citric acid intake could decrease inflammation is consistent with these reports. In addition, in general, IL6 levels have been shown to increase markedly during exercise [12]. Therefore, the intake of citric acid has potential to suppress exercise-induced inflammation, which might be the mechanism of its fatigue alleviation benefit.

Citric acid intake also decreased the expression of genes related to blood pressure, e.g., *Ren*1, *Agtr*1*a*, and *Agtr*2. Flavonoid glycosides in lemon peel have been reported to have a hypotensive effect. In particular, the administration of flavonoid glycosides such as 6,8-di-C-glucosyl apigenin and limocitrol-3*β*-d-glucoside have been found to suppress blood pressure in spontaneously hypertensive rats (SHR) [13] [14]. In another study, flavonoid glycosides had an inhibitory effect on angiotensin 1 converting enzyme in SHR rats [15]. However, there had been no report demonstrating the effect of citric acid on blood pressure; thus, this is the first study to suggest the potential for citric acid to decrease blood pressure.

Citric acid was also found to decrease the expression levels of genes related to the electron transport system, such as *Ndufa*4, *Ndufv*2, *Ndufs*8, *Uqcrb*, *Cox*4*i1*, and *Cox*5*a*, as well as those related to uncoupling proteins, such as *Ucp*1. At present, the reason for these effects is unknown, as there are no reports suggesting a relationship between citric acid and the electron transport system or uncoupling proteins.

The effect of citric acid on the expression of genes related to anti-oxidation is inconsistent. It has been reported that the dietary lemon flavonoids eriocitrin and hesperidin suppressed oxidative stress in streptozotocin-induced diabetic rats [16]. Therefore, further research is needed to reveal the nature of the relationship between citric acid and oxidative stress.

Table 2. mRNA expression ratios of citric acid group/distilled water group (CA/DW) for genes related to inflammation, blood pressure, energy production, anti-oxidation.

Functional classification	Accession No.	Gene symbol	Gene name	CA/DW	P value
Inflammation	NM_031168	Il6	Interleukin 6	0.78	0.05
Blood pressure	NM_031193	Ren1	Renin 1 structural	0.80	0.07
	NM_177322	Agtr1a	Angiotensin II receptor, type 1a	0.89	0.03
	NM_007429	Agtr2	Angiotensin II receptor, type 2	0.80	0.06
Energy production	NM_010886	Ndufa4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, nuclear gene encoding mitochondrial protein	0.73	0.01
	NM_133666	Ndufv1	NADH dehydrogenase (ubiquinone) flavoprotein 1, nuclear gene encoding mitochondrial protein	0.87	0.05
	NM_028388	Ndufv2	NADH dehydrogenase (ubiquinone) flavoprotein 2, nuclear gene encoding mitochondrial protein	0.89	0.04
	NM_145518	Ndufs1	NADH dehydrogenese (ubiquinone) Fe-S protein 1, nuclear gene encording mitochondrial protein	0.81	0.09
	NM_153064	Ndufs2	NADH dehydrogenese (ubiquinone) Fe-S protein 2	0.91	0.27
	NM_144870	Ndufs8	NADH dehydrogenese (ubiquinone) Fe-S protein 8	0.88	0.01
	NM_026219	Uqcrb	Ubiquinol-cytochrome c reductase binding protein, nuclear gene encoding mitochondrial protein	0.88	0.00
	NM_025650	Uqcr	Ubiquinol-cytochrome c reductase (6.4kD) subunit	0.91	0.11
	NM_025710	Uqcrfs1	Ubiquinol-cytchrome c reductase, Rieske iron-sulfur polypeptide 1	0.76	0.03
	COX3-315	mt-COX3	COX3 cytochrome c oxidase III, mitochondrial	0.71	0.12
	NM_009941	Cox4i1	Cytochrome c oxidase subunit IV isoform I	0.80	0.04
	NM_007747	Cox5a	Cytochrome c oxidase, subunit V a, nucleargene encoding mitochondrial protein	0.85	0.03
	NM_009942	Cox5b	Cytochrome c oxidase, subunit V b	0.84	0.10
	NM_007748	Cox6a1	Cytochrome c oxidase, subunit VI a, polypeptide 1, nuclear gene encoding mitochondrial protein	0.89	0.13
	NM_009943	Cox6a2	Cytochrome c oxidase, subunit VI a, polypeptide 2, nuclear gene encoding mitochondrial protein	0.77	0.07
	NM_007807	Cybb	Cytochrome b-245, beta polypeptide	0.80	0.05
	NM_016774	Atp5b	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit, nuclear gene encoding mitochondrial protein	0.83	0.14
	NM_020615	Atp5c1	ATP synthase, H+ transporting, itochondrial F1 complex, gamma poly-peptide 1, nuclear gene encoding mitochondrial protein, transcriptvariant 1	0.76	0.06
	NM_016920	Atp6v0a1	ATPase, H+ transporting, lysosomal V0 subunit A1	0.85	0.05

Continued

Energy production	NM_033617	Atp6v0b	ATPase, H+ transporting, lysosomal V0 subunit B	0.84	0.04
	NM_007509	Atp6v1b2	ATPase, H+ transporting, lysosomal V1 subunit B2	0.82	0.09
	NM_013477	Atp6v0d1	ATPase, H+ transporting, lysosomal V0 subunit D1	0.87	0.24
	NM_007510	Atp6v1e1	ATPase, H+ transporting, lysosomal V1 subunit E1	0.87	0.06
	NM_025381	Atp6v1f	ATPase, H+ transporting, lysosomal V1 subunit F	0.84	0.07
	NM_133826	Atp6v1h	ATPase, H+ transporting, lysosomal V1 subunit H	0.89	0.31
	NM_009463	Ucp1	Uncoupling protein 1 (mitochondrial, proton carrier), nuclear gene encoding mitochondrial protein	0.80	0.04
	NM_011671	Ucp2	Uncoupling protein 2 (mitochondrial, proton carrier), nuclear gene encoding mitochondrial protein	0.81	0.08
	NM_009464	Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier), nuclear gene encoding mitochondrial protein	0.85	0.11
Anti-oxidation	NM_008182	Gsta2	Glutathione S-transferase, alpha 2	1.28	0.01
	NM_010357	Gsta4	Glutathione S-transferase, alpha 4	1.11	0.26
	NM_008160	Gpx1	Glutathione peroxidase 1	1.30	0.08
	NM_030677	Gpx2	Glutathione peroxidase 2	0.80	0.08
	NM_008161	Gpx3	Glutathione peroxidase 3	0.88	0.09
Others	NM_007489	Arntl	Aryl hydrocarbon receptor nuclear translocator-like	0.80	0.07
	NM_011850	Nr0b2	Nuclear receptor subfamily 0, group B, member 2	1.36	0.18
	NM_010936	Nr1i2	Nuclear receptor subfamily 1, group I, member 2, transcript variant 1	0.84	0.06
	NM_011145	Ppard	Peroxisome proliferator activator receptor delta	0.80	0.10
	NM_008719	Npas2	Neuronal PAS domain protein 2	0.77	0.04
	NM_009931	Col4a1	Collagen, type IV alpha 1	0.83	0.03
	NM_008288	Hsd11b1	Hydroxysteroid 11-beta dehydrogenase 1, transcript variant 1	0.85	0.01
	NM_011185	Psmb1	Proteasome (proteasome, macropain) subunit, beta type 1	0.81	0.00
	NM_011186	Psmb5	Proteasome (proteasome, macropain) subunit, beta type 5	0.87	0.05

5. Conclusion

This study suggests that citric acid intake could accelerate gluconeogenesis and suppresses inflammation. Therefore, the fatigue-alleviation effects of citric acid might be induced by an additive effect between the maintenance of blood glucose levels and the suppression of inflammation during exercise.

Acknowledgements

This study is supported by the Research Fellow of Japan Society for the Promotion of Science (JSPS Research

Fellow).

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