Effects of combination of concentrated Kurozu supplementation and endurance training on mitochondrial enzyme activity and energy metabolism in mice

Suguru NAKANO 1, Kohei SEIKE 2, Mai BANJO 1, Yumiko TAKAHASHI 1, Kenya TAKAHASHI 1, Yoshiyuki MATSUMOTO3, Hideo HATTA 1 *

1Department of Sports Sciences, The University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 1538902, Japan; 2 Department of Sports Science, Kyushu Kyoritsu University, 1-8 Jiyugaoka, Yahatanishi-ku, Kitakyushu-city, Fukuoka 8078585, Japan; 3 Egao Health Laboratory, 4-10-1 Higashi-machi, Kumamoto 8620901, Japan

*Corresponding author: Hideo Hatta, Department of Sports Sciences, The University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 1538902, Japan. E-mail: hatta@idaten.c.u-tokyo.ac.jp

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Running title: Effects of combination of concentrated Kurozu and training
Abstract

We examined the effects of endurance training with chronic pre-exercise concentrated Kurozu supplementation on mitochondrial enzyme activity and energy metabolism in Institute of Cancer Research (ICR) mice. Mice were divided into a control group, an endurance training group, and an endurance training + concentrated Kurozu supplementation group. Mice were orally supplemented with water or concentrated Kurozu solution (500 mg/kg body weight/day) for 3 weeks. The mice in the training group were subjected to exercise on a treadmill (20–25 m/min × 30 min, five times/week) starting 30 min after the supplementation. The maximal activity of citrate synthase in the plantaris muscle in the endurance training + concentrated Kurozu supplementation group was significantly higher than that in the control group (p < 0.01). The maximal activity of ß-hydroxyacyl coenzyme dehydrogenase (β-HAD) in the soleus muscle in the endurance training + concentrated Kurozu supplementation group was significantly higher than that in the other two groups (p < 0.05 for both). In the final week, significant negative correlation between blood lactate concentration after exercise and soleus β-HAD activity was observed. These findings suggest that endurance training with concentrated Kurozu supplementation increases mitochondrial enzyme activity and might enhance lipid metabolism during exercise.

Key words: endurance training, mitochondria adaptation, skeletal muscle, concentrated Kurozu
黒酢摂取と持久的トレーニングの併用がミトコンドリア生合成及びエネルギー代謝に与える影響

中野卓1、清家空併2、萬城麻衣1、高橋祐美子1、高橋謙也1、松本祥幸3、八田秀雄1

1 東京大学身体運動科学研究室 1538902 目黒区駒場 3-8-1
2 九州共立大学スポーツ学部 8078585 八幡西区自由が丘 1-8
3 えがお健康研究所 8620901 熊本市東町 4-10-1

要旨

ICR マウスを用いて、黒酢摂取と持久的トレーニングの併用がミトコンドリア酵素活性やエネルギー代謝に与える影響について検証した。マウスはコントロール群(Con)、持久的トレーニング群(Tr)、黒酢投与+持久的トレーニング群(Tr + KZ)に分けた。マウスには 3 週間、500 mg/kg 体重/日の水、あるいは黒酢を投与した。トレーニングを実施する群では、トレッドミルを用いて、水あるいは黒酢投与 30 分後に持久的トレーニング(20-25 m/min×30 分、5 回/週)を実施させた。
その結果、Tr + KZ 群の足底筋のクエン酸合成酵素(CS)活性は Con 群よりも有意に高かった (p < 0.01)。また Tr + KZ 群のヒラメ筋の β-ヒドロキシアシル CoA 脱水素酵素(β-HAD)活性は Con 群、Tr 群よりも有意に高かった (p < 0.05)。最終週において、トレーニング直後の血中乳酸濃度とヒラメ筋 β-HAD 活性との間には負の相関関係が見られた (p<0.05)。これらのことから、黒酢摂取と持久的トレーニングの併用により、ミトコンドリア酵素活性が増加し、運動中の脂質代謝が高まる可能性が考えられた。
Introduction

Mitochondria play a key role in producing energy, and there is a close relationship between mitochondrial-related enzyme activity and exercise performance\(^1\). Furthermore, mitochondrial content is related to both exercise performance and lifestyle-related disease. For example, previous studies have been reported that lower mitochondrial content is involved in type-2 diabetes and muscle atrophy\(^2\)\(^3\). Therefore, increasing mitochondrial content may be important for enhancing exercise performance and lifestyle quality.

Mitochondrial content is increased by endurance training\(^4\). However, not all people perform sufficient training, which can be due to body weight, injury, and lifestyle factors. Therefore, it is important to induce mitochondrial biogenesis in short, low-intensity daily exercises. Many studies have examined the benefits of nutrient supplements before endurance training on training-induced mitochondrial adaptations.

Kurozu (black vinegar) is traditionally brewed from grain and contains various amino acids, organic acids such as acetic acid, and polyphenols such as catechin and chlorogenic acid\(^5\). Many studies have examined the physiological effects of acetic acid, the main ingredient of Kurozu, and we also confirmed that acetate supplementation improved carbohydrate metabolism in a high fat diet condition\(^6\). However, although concentrated Kurozu supplement contains various amino acids, there is almost no acetic acid. Thus, the amino acids contained in Kurozu may also provide beneficial effects on training-induced metabolic adaptations.

We previously reported that ingestion of amino acid-rich supplements such as royal
jelly and casein peptide enhances training-induced mitochondrial adaptations in muscles mainly dominated by slow-twitch fibers\(^7\)\(^8\). Concentrated Kurozu supplementation also has beneficial health effects including decreased adipocyte size\(^9\), body weight\(^10\), and the incidence of hepatic steatosis\(^11\). However, it remains unclear whether concentrated Kurozu supplementation enhances training-induced mitochondrial adaptations. Therefore, the aim of the present study was to determine the effects of concentrated Kurozu supplementation with endurance training on muscular mitochondria-related factors in Institute of Cancer Research (ICR) mice.

**Material and Methods**

**Ethical approval**

All procedures performed in this study involving animals were in accordance with the ethical standards of the Committee on Animal Care and Use, The University of Tokyo. All the protocols of research on animals were approved by this committee (Approval number: 24-4).

**Animals**

Male 4-week-old ICR mice were purchased commercially (CLEA Japan Inc., Tokyo, Japan). All mice were housed individually in a temperature-controlled room at approximately 21–23°C with alternating 12-h periods of dark and light. Before the experiments, the animals had *ad libitum* access to water and a standard chow (MF; 3.6 kcal/g, 60% kcal from carbohydrate, 13% kcal from fat, 27% kcal from protein;
Oriental Yeast Co., Ltd., Tokyo, Japan). Mice were adapted to these environments for 1 week before experimentation.

Following the 1-week acclimation period, mice were randomly divided into three groups: control (Con, n=7), training (Tr, n=7), and training with concentrated Kurozu supplementation (Tr + KZ, n=5). Kurozu to be administered to mice was prepared by diluting 0.5 mL of concentrated Kurozu (Egao Inc., Kumamoto, Japan) with 9.5 mL of water. All mice were provided with MF chow. Mice received oral administration of either water or concentrated Kurozu solution diluted with distilled water (500 mg/kg body weight/day, 10 µL/g body weight/day) using a stomach tube, and then received endurance training using a motor-driven treadmill (20–25 m/min, 30 min, five times/week for 3 weeks) from 30 min after supplementation. The nutritional information of the concentrated Kurozu is shown (Table 1).

Blood lactate concentration were measured with an auto-analyzer (Lactate Pro 2; Arkray Inc., Kyoto, Japan) from the tail vein at immediately after the last treadmill running of the week. Food consumption and body weight were recorded daily. At 24–28 h after the final supplementation, the tissues were harvested. Organ samples included the plantaris and soleus muscles were taken under anesthesia. All samples were rapidly frozen in liquid nitrogen and stored at −80°C until further analysis.

Animal characteristics

Mice were weighed at the same time every day (11:00-12:00 AM) using an electronic balance. Food intake was also measured using an electronic balance at the same time
every day (11:00-12:00 AM). Body weight gain was calculated as the difference between the weight on the first day and the last day. Food efficiency was calculated by dividing the total food intake during the experimental period (excluding the adaptation period) by the body weight gain.

**Mitochondrial enzyme activity analysis**

We examined mitochondrial adaptation, including the activity of mitochondrial enzymes (e.g., citrate synthase [CS] and β-hydroxyacyl CoA dehydrogenase [β-HAD]). CS catalyzes the rate-limiting step of the tri-carboxylic acid cycle, while β-HAD catalyzes the rate-limiting step of fatty acid β-oxidation. Specifically, whole plantaris and soleus muscle samples were homogenized in 100 (vol/wt) of 100 mM potassium phosphate buffer. The maximal CS activity was determined via addition of oxaloacetate to a buffer solution containing the muscle homogenates, 5,50-dithiobis (2-nitrobenzoic acid), and acetyl coenzyme A (CoA) in 100 mM Tris-HCl buffer (pH = 8.3)\(^\text{12}\)). The rate change in absorbance (412 nm) was monitored over 180 s with readings every 20 s. CS produces citric acid and acetyl coenzyme A (CoASH). Reaction of CoASH with 5,50-dithiobis yields 5-Mercapto-2-nitrobenzoic acid. 5-Mercapto-2-nitrobenzoic acid is measured by absorbance at 412 nm.

The maximal activity of β-HAD was determined via addition of acetoacetyl CoA to a buffer solution containing the muscle homogenates, nicotinamide adenine dinucleotide, and ethylenediaminetetraacetic acid (EDTA) in 50 mM Tris-HCl buffer (pH = 7.0)\(^\text{13}\)). The rate change in absorbance (340 nm) was monitored over 180 s with readings every
20 s. In this method, NADH produced by the β-HAD enzyme is measured at an absorbance of 340 nm.

**Statistical analysis**

Data are presented as mean ± standard error of the mean. A one-way analysis of variance was used for all analyses except blood lactate concentration. When significant overall differences were found, group comparisons were made using the Tukey–Kramer post hoc test. A two-way analysis of variance was used for blood lactate concentration. Correlation analysis was used to examine the relationship between lactate and mitochondrial enzyme activities.

**Results**

**Animal characteristics**

There was no difference in final body weight, body weight gain, food intake, or food efficiency at the end of training period between the groups (Table 2).

**Mitochondrial adaptation**

The maximal CS activity in the plantaris muscle was significantly higher in the Tr + KZ group compared with the Con group (p < 0.01; Fig. 1). The maximal β-HAD activity in the soleus muscle was significantly higher in the Tr + KZ group compared with the Con and Tr groups (p < 0.05, for both; Fig. 2). There were no differences in maximal CS activity in the soleus muscle or β-HAD activity in the plantaris muscle
between the groups.

**Blood lactate concentrations after the treadmill running**

There was a significant main effect of training period on blood lactate levels ($p < 0.01$). No significant main effect of Kurozu was observed. No significant interaction between Kurozu and training period was observed (Fig. 3).

**Correlation between blood lactate concentration after the final training and mitochondrial enzyme activity**

There was a significant negative correlation between blood lactate concentration at immediately after the final training and the maximal $\beta$-HAD activity in soleus muscle ($r = 0.61$, $p < 0.05$; Fig. 4B). No significant positive and negative correlation was found between blood lactate concentration after the final training and the maximal CS activity in the soleus and plantaris muscle, and the maximal $\beta$-HAD activity in the plantaris muscle (Fig. 4A, 4C, 4D).

**Discussion**

The main finding of the present study was that maximal CS activity was significantly higher in the Tr + KZ (i.e., training with concentrated Kurozu supplementation) group than the Con (i.e., sedentary) group in the plantaris muscle, which is mainly composed
of fast-twitch muscle fibers. Furthermore, the maximal β-HAD activity in the soleus muscle, which is mainly composed of slow-twitch muscle fibers, was significantly higher in the Tr + KZ group than the Con and Tr (i.e., training without concentrated Kurozu supplementation) groups. Finally, the blood lactate concentration immediately after the last training was significantly correlated with the maximal β-HAD activity in the soleus muscle. Collectively, these data suggest that a combination of endurance training and concentrated Kurozu supplementation can increase mitochondrial enzyme activity, and promote lipid metabolism during exercise, particularly in slow-twitch muscle fibers.

We found that the combination of concentrated Kurozu supplementation and endurance training effectively increased the maximal CS activity in the plantaris muscle. A previous study reported that maximal CS activity was strongly correlated with the mitochondrial content. A potential mechanism of the increased maximal mitochondrial enzyme activity with concentrated Kurozu supplementation involves the effects of amino acids. Some components of Kurozu, including amino acids and proteins (particularly leucine), have been reported to activate AMP-activated protein kinase (AMPK), a major mediator of mitochondrial biogenesis, and increase maximal mitochondrial enzyme activity. Thus, one possible mechanism by which plantaris CS activity was increased is amino acids in Kurozu. In the present study, the maximal CS activity in the plantaris muscle was increased by endurance training with Kurozu supplementation, but there was no effect on the soleus muscle. By contrast, supplementation with casein peptide or royal jelly (both of which contain various amino
acids, as for Kurozu) was reported to increase CS activity in the soleus muscle induced by endurance training, but have no effect in the plantaris muscle. These contrasting findings may relate to differences in the nutritional composition of the various supplements. For example, catechin, which is contained in Kurozu, was reported to increase CS activity in the quadriceps (contains predominantly fast-twitch fibers), but not in the soleus muscle, after ingestion with endurance training. Thus, differences in nutritional composition and content may cause differential responses in skeletal muscles depending on their fiber composition. Further studies are required to determine the main active compounds in Kurozu.

We also found that β-HAD activity, which is related to lipid metabolism, was increased in the soleus muscle in the Tr + KZ group, suggesting that concentrated Kurozu supplementation promotes lipid metabolism in slow-twitch fibers. Kurozu supplementation was reported to activate skeletal muscle peroxisome proliferator-activated receptor-α (PPARα) protein expression, which increases lipid metabolism-related enzyme expression. Kurozu contains palmitic acid, stearic acid, oleic acid, and linoleic acid. These fatty acids act as ligands for PPARα. Therefore, PPARα is thought to be one of possible mechanisms by which Kurozu enhances lipid metabolisms in soleus muscle. In plantaris muscle, maximal β-HAD activity is not significantly different between the Con and Tr and Tr + KZ groups. The reason why maximal β-HAD activity was increased in soleus muscle, but not in plantaris muscle is unknown. From this point of view, further study is needed.

In the present study, no main effect of Kurozu was observed, and no interaction
between Kurozu and training period was observed. However, there was a strong negative correlation between soleus maximal β-HAD activity and blood lactate concentration immediately after the final training. An increase in blood lactate concentration during exercise is widely used as an indicator of activated glycolysis/glycogenolysis. It is also generally accepted that people with a higher fat oxidation capacity show a greater lactate threshold intensity, whereby blood lactate concentrations increase concomitantly with increased exercise intensity. Previous study using fibroblasts showed that knocking out the β-HAD gene increased the formation of lactate\(^\text{19}\), suggesting that β-HAD activity affects lactate production. Also, it is known that there is an inverse relationship between blood lactate concentration and lipid metabolism during exercise\(^\text{20}\). Therefore, increased β-HAD activity in skeletal muscle may stimulate lipid oxidation during exercise, resulting in attenuated blood lactate concentration elevation. Although it is thought that blood lactate concentration reflects lipid metabolism during exercise, it is not a direct indicator of how much lipids have been oxidized. In the future, it is necessary to directly evaluate the effect of Kurozu on lipid metabolism during exercise.

**Conclusion**

We found that concentrated Kurozu supplementation can further stimulate an endurance exercise-induced increase in maximal CS activity in the plantaris muscle (which is mainly composed of fast-twitch fibers) and β-HAD activity in the soleus muscle (which is mainly composed of slow-twitch fibers). In the final week, significant
negative correlation between blood lactate concentration after exercise and soleus β-HAD activity was observed. These results suggest that endurance training with Kurozu supplementation can increase mitochondrial enzyme activity and promote lipid metabolism during exercise.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interests.

Author contributions

This study was conceived and designed by SN, MB, KS, and HH, and conducted by SN, MB, KS, KT, and YT. SN, MB, and KS analyzed the data, and SN, KS, YM, and HH interpreted the data. The paper was written by SN. All authors approved the final version of the manuscript.

References


### Tables

Table 1. Composition of Kurozu concentrate (100 g).

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<tr>
<th>Macronutrients</th>
<th>Amino acid</th>
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<tr>
<td>Water</td>
<td>2.4 g</td>
<td>Glutamic Acid</td>
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<tr>
<td>Protein</td>
<td>11.3 g</td>
<td>Alanine</td>
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<td>Lipid</td>
<td>0.9 g</td>
<td>Leucine</td>
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<td>Ash</td>
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<td>Aspartic acid</td>
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<tr>
<td>Carbohydrate</td>
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<td>Valine</td>
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<tr>
<td>Energy</td>
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<td>Glycine</td>
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<td></td>
<td></td>
<td>Proline</td>
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<tr>
<td></td>
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<td>Serine</td>
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<td></td>
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<td>Isoleucine</td>
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<td></td>
<td></td>
<td>Phenylalanine</td>
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<tr>
<td>Sodium</td>
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<td>Ion</td>
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<td>Calcium</td>
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<td>Potassium</td>
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<td>Magnesium</td>
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<td>Zinc</td>
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Table 2. Animal characteristics.

<table>
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<th></th>
<th>Con</th>
<th>Tr</th>
<th>Tr + KZ</th>
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<tr>
<td>Final Body Weight (g)</td>
<td>37.2 ± 1.1</td>
<td>35.5 ± 0.8</td>
<td>35.7 ± 0.5</td>
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<td>Body Weight gain (g)</td>
<td>6.0 ± 0.7</td>
<td>4.8 ± 0.8</td>
<td>5.0 ± 0.3</td>
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<tr>
<td>Food intake (g/day)</td>
<td>5.02 ± 0.11</td>
<td>4.79 ± 0.09</td>
<td>5.01 ± 0.04</td>
</tr>
<tr>
<td>Food efficiency</td>
<td>0.055 ± 0.005</td>
<td>0.046 ± 0.007</td>
<td>0.047 ± 0.003</td>
</tr>
</tbody>
</table>

Con: control group, Tr: endurance training with water treated group, Tr + KZ: endurance training with concentrated Kurozu treated group

**Figure legends**

Fig. 1. Maximal citrate synthase (CS) activity in the soleus and plantaris of mice after 3 weeks of endurance training and concentrated Kurozu supplementation (Tr + KZ group; black), endurance training and distilled water treatment (Tr group; gray), or distilled water treatment without training (Con group; white). Data are mean ± standard error (n = 5–7 per group). **, p < 0.01 between the Con group and the Tr + KZ group.

Fig. 2. Maximal β-hydroxyacyl CoA dehydrogenase (β-HAD) activity in the soleus and plantaris muscles of mice in the endurance training with concentrated Kurozu treated group (Tr + KZ; black), the endurance training with distilled water treated group (Tr; gray), and the no training with distilled water treated group (Con; white). Data are mean
± standard error (n = 5–7 per group). *, p < 0.05 between the Con group and the Tr + KZ group. #, p < 0.05 between the Tr group and the Tr + KZ group.

Fig. 3. Blood lactate concentration immediately after treadmill exercise from the first to third week in the endurance training with distilled water treated group (Tr) and the endurance training with concentrated Kurozu treated group (Tr + KZ). Data are mean ± standard error (n = 5–7 per group).

Fig. 4. Correlation between blood lactate concentration immediately after treadmill exercise and soleus maximal citrate synthase (CS) activity (A), soleus maximal β-hydroxyacyl CoA dehydrogenase (β -HAD) activity (B), plantaris maximal CS activity (C), and plantaris maximal β-HAD activity (D). There was a significant negative correlation between blood lactate concentration and soleus β-HAD activity (p < 0.05, r = 0.61) (B).
Figure 4

A. Lactate - Soleus CS

B. Lactate - Soleus β-HAD

C. Lactate - Plantaris CS

D. Lactate - Plantaris β-HAD

$r = 0.61$
$p < 0.05$