DOCTORAL DISSERTATION

# The effect of fermented milk and milk casein hydrolysate on muscle damage after exercise

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# Chapter 1. General introduction

In this chapter, it was described the background from previous studies and the purpose of this study.

## 1-1. Exercise-induced muscle damage

Unaccustomed and strenuous exercise causes muscle damage that clinically presents as muscular pain and involves protein degradation and ultrastructural changes. Muscle damage usually manifests with a delay after exercise and peaks after approximately 24–48 h, a condition known as delayed-onset muscle damage. Muscle functions, such as energy metabolism and power output, are difficult to maintain in damaged muscles. Depending on the magnitude of muscle damage, muscle force at isometric and dynamic testing conditions is impaired after exercise [1–4]. Soluble muscle enzymes, such as creatine kinase (CK), are released, indicating a disruption of the sarcomere architecture [1, 5] and surface membrane damage [6, 7]. Muscle-damaging exercise results in phagocytic infiltration into the damaged muscle, causing an inflammatory response that induces delayed-onset muscle damage [8]. Other important symptoms of muscle damage are stiffness and swelling of the muscle [9–11]. Furthermore, in a previous study [12], central arterial stiffness transiently increased in a damaged muscle after exercise, which may have acute unfavorable cardiovascular effects.

Previous studies have shown that delayed-onset muscle damage is primarily induced by mechanical stress, particularly eccentric muscle contractions [13–16]. Concentric exercise shortens contracting muscles; by contrast, eccentric exercise forcibly lengthens a contracting muscle. During stepping down a slope, the contracting quadriceps muscle controls the rate of knee flexion against the force of gravity. In this process, the muscle undergoes eccentric contraction with each step. In fact, compared to uphill running (i.e., concentric exercise), downhill running (i.e., eccentric contraction) causes greater muscle damage [17], moreover, in our unpublished study, which even occurs during the low-intensity physical activity of walking. The disturbance of calcium homeostasis also causes muscle damage by protease activation [18]. A muscle contraction requires a transient increase in intracellular calcium, which is released from the sarcoplasmic reticulum into the cytosol through the excitation–contraction coupling system. An overload of intracellular calcium activates calpain (a calcium-dependent protease), which destroys muscle protein [19]. In addition, phagocyte infiltration into muscles occurs after strenuous exercise [8, 20], and an inflammatory response appears to be strongly involved in delayed-onset muscle damage.

Several reports [21–23] about the mechanism of inflammation have demonstrated that oxidative stress directly causes damage via oxidation of cell components such as lipids, proteins, and deoxyribonucleic acid (DNA) and also acts as a regulator of

inflammation. Namely, oxidative stress promotes the translocation of certain redox-sensitive transcription factors that regulate inflammatory mediators, such as cytokines, chemokines, and adhesion molecules, to the nucleus. In response, phagocytes infiltrate the tissues expressing these mediators, causing proteolysis, ultrastructural damage, and oxidative injury. Prolonged exercise results in reactive oxygen species (ROS) generation by the mitochondrial electron transport chain in muscle cells through an increase in oxygen consumption [24, 25]. Also, xanthine oxidase is activated via the ischemia-reperfusion process during exercise, resulting in ROS generation by the capillary endothelium in contracting muscles [26, 27]. Therefore, oxidative stress due to exercise is involved in delayed-onset muscle damage associated with phagocyte infiltration, which is secondary to the increased expression of inflammatory mediators.

## 1-2. Impaired insulin sensitivity after muscle-damaging exercise

Previous reports showed that downhill running impairs the insulin-stimulated activity of insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase (PI3-K), and Akt in damaged muscle tissues from healthy humans [26] and animals [27]. In addition, glucose transporter 4 (GLUT4) protein level was decreased in damaged muscles 1 and 2 days after either downhill running [27] or eccentric resistant exercise [28]. It is well known that pro-inflammatory cytokines impair glucose transport by inhibiting insulin signal transduction in skeletal muscles. A representative cytokine, tumor necrosis factor-α (TNF-α), prevents insulin-induced activation of the insulin receptor, PI3-K, and Akt in muscle tissue [29–31]. In contrast, inhibition of TNF-α induced signaling prevents this impairment of insulin-mediated Akt phosphorylation and glucose uptake [32]. In exercise-induced muscle damage, endotoxin-induced production of TNF-α by mononuclear cells is increased and positively correlates with reduced insulin-stimulated activity of PI3-K [26], which presumably decreases insulin-mediated glucose uptake in skeletal muscles. Other studies also reported that at 24–48 h after muscle-damaging exercise in humans and animals, levels of interleukin (IL)-lβ, IL-6, monocyte chemoattractant protein-1, and IL-8 are increased in muscle tissues [33–35]. These inflammatory factors may be partly associated with the initiation and progression of impaired muscle damage-induced insulin signaling.

Elevated generation of ROS in delayed-onset muscle damage can also impair insulin-stimulated glucose uptake. In the past decade, a close relationship has been suggested between oxidative stress and insulin effects [36, 37]. In muscle cells, stimulation by oxidants such as hydrogen peroxide blocks insulin-induced glucose uptake and GLUT4 translocation by impairing upstream signaling [38, 39]. Oxidative stress has shown to increase IRS-1 degradation along with the activity of the ubiquitin-dependent proteolytic pathway in muscle cells. In addition, stress-mediated activation of serine kinases, with subsequent modulation of IRS-1 protein levels and functionality is associated with the effects of oxidative stress on insulin action. It has been shown that hydrogen peroxide induces the activity of various serine kinases, including p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, IκB kinaseβ, and ERK1/2, with subsequent phosphorylation of IRS-1 on Ser307 and selective degradation of IRS-1, ultimately leading to insulin resistance [40–43]. In fact, previous studies have reported elevated levels of oxidative products in the muscles of patients with type 2 diabetes [44, 45] and diabetic mice [46]. In contrast, several antioxidants improve the insulin signaling of muscle cells for glucose uptake and reduce the production of oxidative products [38, 47, 48], which supports a role for oxidative stress in the development of insulin resistance.

### 1-3. Fermented milk and milk casein hydrolysate

Milk fermented with lactobacillus is recognized as a pleasant sour-tasting drink, which has superior storage properties, making it beneficial for human health. In recent years, through some studies [49–52], fermented milk has been shown to have useful functions including prolonged lifespan, antihypertensive, antitumorigenic effects, and immune system regulation. In addition, some types of fermented milk possess anti-inflammatory and antioxidant properties [53–56].

Fermented milk is manufactured by fermenting skimmed milk with a starter culture. During this process, proteins contained in the milk casein are digested and converted into small peptides, which are absorbed more in amount from the intestines, compared to amino acids or large oligopeptides [57]. It has been suggested that fermented milk contains several particular peptides and amino acids that could influence multiple physiological effects.

It has been shown that the fermentation of skim milk with a starter culture containing *Lactobacillus helveticus* (*L. helveticus*) has several beneficial effects including anti-inflammation and contains specific peptides, such as Val-Pro-Pro and Ile-Pro-Pro. During the processing of these peptides, the importance of specific proteolytic enzymes in *L. helveticus* became apparent [58, 59]. However, *L. helveticus*-fermented milk still contains much unhydrolysed casein, and the productivity of peptides by milk

fermentation is limited. A new enzymatic method for manufacturing these peptides from casein was recently developed using an *Aspergillus oryzae* (*A. oryzae*) protease [60]. The peptide material produced by the *A. oryzae* protease has advantages over *L. helveticus*-fermented milk in that the production cost is lower and the applications are more versatile. Milk casein hydrolysate (MCH) prepared from *A. oryzae* demonstrated significant antihypertensive effects in animals [60] and humans [61] as was observed with *L. helveticus*-fermented milk. Thus, MCH is a possibility even with same effects as well as *L. helveticus*-fermented milk.

## 1-4. The purpose of this study

Muscle soreness, fatigue, and reduction of glucose metabolism that occurs with muscle damage are obstacles to the competitive ability and daily life after exercise. These features may lead to a decrease in performance and/or exercise motivation. Therefore, it is necessary to develop an approach to inhibit or reduce those events associated with muscle damage in both sports person and health enthusiasts.

Previously, Aoi et al. showed that *L. helveticus*-fermented milk prevents muscle damage induced by acute exercise via activation of antioxidative enzymes of skeletal muscle in animals [62]. However, the effect of *L. helveticus*-fermented milk on muscle damage in human is unclear. Moreover, *L. helveticus*-fermented milk and MCH include common peptides [60, 63], suggesting the possibility that the same effects will be acquired.

The purpose of this study was to clarify: (1) the effect of *L. helveticus*-fermented milk supplementation on glucose metabolism in damaged muscle after acute resistance exercise in young healthy men; (2) the effect of MCH supplementation on muscle soreness and fatigue in damaged muscle after acute exercise in middle-aged to elderly men.

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# Chapter 2. The effect of fermented milk on muscle damage after exercise in young men

## 2-1. Introduction

Growing evidence has shown that impaired insulin sensitivity occurs physiologically with muscle damage, in contrast to the improvement in insulin sensitivity after non muscle-damaging exercise. Oxidative stress and certain inflammatory cytokines impair glucose uptake via inactivation of insulin signaling pathways in muscle cells [1–3]. Infiltration of phagocytes into the damaged muscle is observed after strenuous exercise, and an inflammatory response is implicated in the development of delayed-onset muscle damage [4, 5]. In addition, elevation of the levels of oxidative damage in cellular components is also observed in damaged muscle [4, 6]. Thus, inflammatory cytokines and oxidative stress can decrease insulin-dependent glucose uptake in exercise-induced damaged muscles [7]. Therefore, it was speculated that the decrease of glucose metabolism associated with muscle damage can be prevented by suppression of inflammation and oxidative stress.

Fermented milk has several salutary effects, including anti-inflammatory and antioxidant effects [8–11]. Previously, it has been shown that *L. helveticus*-fermented milk prevents muscle damage induced by acute exercise via activation of antioxidative enzymes of skeletal muscle in an animal study [12], suggesting that fermented milk may

prevent the impairment of glucose metabolism associated with muscle damage. Additionally, in the previous study, the effect of long-term administration of *L. helveticus*-fermented milk was observed [12], but the effect of a short-term administration, e.g. drinking in pre- and post-exercise, is not known.

The purpose of this study was to investigate the effect of *L. helveticus*-fermented milk of short-term supplementation on glucose metabolism in damaged muscle after acute resistance exercise in young healthy men.

## 2-2. Methods

*Participants*

Eighteen healthy young men with no regular exercise regimen habits were recruited to participate in this study. The mean ± S.E. characteristics of the participants were as follows: age, 21.6 ± 0.8 years; height, 171.1 ± 1.5 cm; body weight, 59.9 ± 1.5 kg; body mass index (BMI), 20.5 ± 0.4 kg/m$^2$; and body fat percentage, 16.2 ± 0.8%. All participants were free of the signs, symptoms, and history of any overt chronic disease. None of the participants had a history of smoking and none were currently taking any medications or dietary supplements. This study was approved by the Ethics Committee of Kyoto Prefectural University (2008, No. 22), and all of the participants signed an informed consent form after reading about the design and protocol of the study.

*Study design*

All of the participants attended the three trials included in the study, rest with placebo intake (rest), exercise with placebo intake (placebo), and exercise with fermented milk intake (fermented milk) in a repeated-measures experimental design. These trials were performed in a random order using a counter-balanced design and were separated by at least six weeks for any individual participants in order to avoid biasing of the muscle damage. The participants were also asked to refrain from caffeine

and alcohol ingestion 24 h before each trial and were asked not to eat or drink anything except for water from 22:00 on the night before the trial to the next morning. Dietary records on the day of the trial were performed to avoid significant differences of food intakes between rest, placebo, and fermented milk trials. In the first trial, an example of the recording method was shown to the participants beforehand, and they recorded dietary contents according to it. And, the recorded contents were repeated in the second and third trials.

*Examination beverage*

*L. helveticus*-fermented milk (Amiel S®, Calpis Co., Ltd., Kanagawa, Japan) was used in the fermented milk trial. A placebo beverage was unfermented milk, with adjusted contents of protein, fat, carbohydrate, and pH to be equivalent with that of fermented milk (Table 2-1). Each of the participants consumed 200 mL of each beverage once before and twice after exercise using a double-blinded method; therefore, they totally consumed 600 mL.

*Experiment schedule*

On the first experiment day of each trial, the participants came to laboratory at 9:00, where they sat on a chair until the beginning of the test. Subsequently, the test beverage

(placebo or *L. helveticus*-fermented milk) were consumed. In the rest trial, participants refrained from exertional activity, and were maintained in a state of rest. In the placebo and fermented milk trials, resistance exercise was performed from 30 min after consumption of the test beverage. Immediately after the exercise, the blood lactate was measured. Afterwards, the participants consumed the test beverage again at 1 h and 3 h after the end of the exercise. The participants were asked to not to eat or drink anything except for water from 22:00 to the measurement of the next morning.

On the second day of the study, the participants collected a sample from the first urine flow in the morning (urine accumulated overnight) and returned to the laboratory at 9:00. Subsequently, the body composition was measured and blood sample was collected from the antecubital vein. Glucose solution containing 75 g glucose (Trelan®-G75, Ajinomoto Pharmaceuticals Co. Ltd., Tokyo, Japan) was orally consumed at 9:30, and the expiration gas was measured from 10:00 for 30 min in the supine position. Blood glucose was measured before, 30 min after, and 60 min after oral glucose administration, by a fingerstick prick. Next, the degree of subjective muscle soreness was evaluated. A schematic illustration of the experimental schedule was shown in Figure 2-1.

Table 2-1. Nutritional information of test beverage

| | Placebo | Fermented milk |
|---|---|---|
| Energy (kcal) | 102 | 102 |
| Protein (g) | 6.6 | 6.6 |
| Val-Pro-Pro, Ile-Pro-Pro (mg) | 0.0 | 10.2 |
| Fat (g) | 0.0 | 0.0 |
| Carbohydrate (g) | 21.6 | 21.6 |
| pH | 3.75 | 3.75 |

Values are represented as composition included in 600 mL.

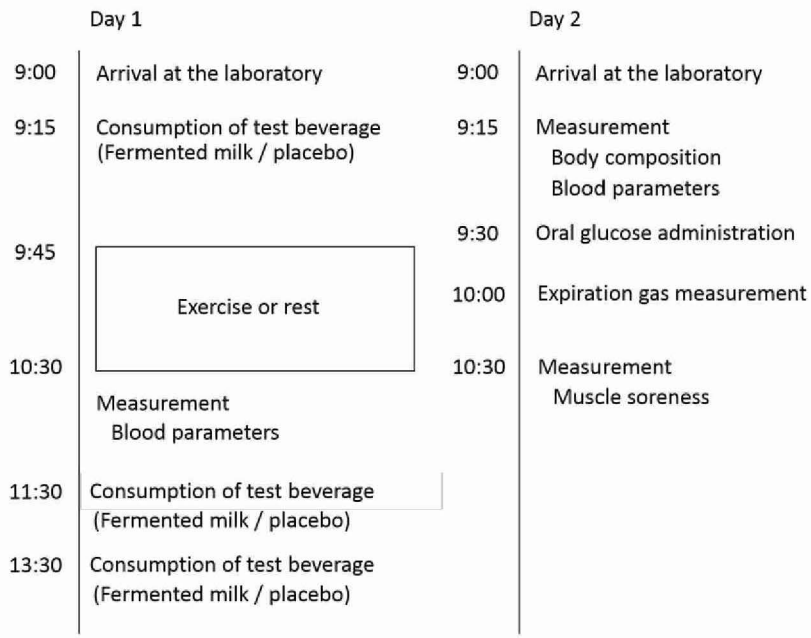


Figure 2-1. Schematic illustration of experimental schedule.

*Exercise procedure*

After warm-up with a bicycle ergometer for 5 min and stretching, the participants performed resistance exercise for 45 min. The resistance exercise was composed of leg and bench presses using a compound-type resistance training machine (Senoh Ltd., Chiba, Japan). Five sets of leg and bench presses were performed at a strength of 70–100% with a 12-repetition maximum (RM: maximum number of occurrence). This strength was determined using the methods of Drummond et al [13]. All participants performed 10 repetitions at the load of 100% 12 RM in 1–3 sets and then the load of 70% 12 RM in 4–5 sets. The exercises were repeated at a pace of one repetition every 3 sec, with a 2 min interval between sets.

*Indirect metabolic performance*

Oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$) were measured using a breath-by-breath respiromonitor system (MetaMax 3B, Cortex, Leipzig, Germany). The respiratory quotient (RQ) and substrate utilization were calculated from the level of $VO_2$ and $VCO_2$, as described previously [14].

*Muscle soreness*

Subjective muscle soreness in the pectoralis major, quadriceps, and gluteus maximus muscles was evaluated by palpation and movement (bending and stretching) using the visual analogue scale (VAS). The participants were asked to indicate the intensity of perceived soreness for each muscle part on a 100-mm horizontal line. The left side stated "having no soreness", while the right side stated "having max soreness". The total soreness value was calculated by adding the soreness values for the 3 different muscles.

*Blood and urine parameters*

Blood samples were collected from the antecubital vein. The separated serum was stored at –80° until measurement. The blood lactate and glucose were measured using simple measuring instruments (blood lactate: Lactate Pro, Arkray, Inc., Kyoto, Japan; glucose: Glu Test, Sanwa Kagaku Kenkyusho Co., Ltd., Aichi, Japan). The analysis of triglyceride, cholesterols (LDL-cholesterol, HDL-cholesterol, and total cholesterol), free fatty acid, high sensitivity C-reactive protein (hs CRP), and creatine phosphokinase (CPK) in the serum was entrusted to FALCO Biosystems Ltd. (Kyoto, Japan). TNF-α and carbonyl protein levels in the serum were measured by using an enzyme-linked immunosorbent assay (ELISA) kit (TNF-α: Human TNF-alpha Quantikine ELISA Kit, R&D Systems, MN, USA;

carbonyl protein: Biocell™ Protein Carbonyl ELISA Assay, BioCell, Auckland, NZ). Oxygen radical absorbance capacity (ORAC), a marker that reports antioxidant capacity, was measured by using the methods of Watanabe et al [15]. The concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of DNA oxidative damage, was measured using an ELISA kit (ELISA kit for 8-OHdG, Japan Institute for the Control of Ageing, Shizuoka, Japan) on the gathered urine, and the total amount of 8-OHdG was calculated by the volume of urine. Moreover, measurement of the creatinine was requested (FALCO Biosystems Ltd.) and used to correct the amount of 8-OHdG.

*Statistical analysis*

All of the data were shown as the mean ± standard error. The repeated-measures analysis of variance (ANOVA) was used to compare the date the 3 trials. In the index of 3 collection points per trial, such as blood glucose, 2-way repeated-measures ANOVA was used. If ANOVA indicated a significance difference, Tukey-Kramer test was used to determine the significance of the differences between mean values. Paired t-tests was used for data on blood lactate and muscle soreness with a normal distribution to compare between 2 trials. The significance level was assumed to be 5%.

## 2-3. Results

*Muscle damage parameters and blood lactate*

Immediately after exercise, the blood lactate value was markedly increased immediately after exercise, but it was significantly suppressed in the fermented milk trial, compared with placebo trial (placebo, 10.8 ± 0.9 mmol/L vs. fermented milk, 8.6 ± 0.5 mmol/L, $P = 0.04$) (Figure 2-2).

On the next day of the exercise, serum CPK was significantly elevated in the placebo and fermented milk trials compared with rest trial (rest, 96 ± 7 IU/L vs. placebo, 193 ± 27 IU/L, $P = 0.001$; rest, 96 ± 7 IU/L vs. fermented milk, 152 ± 15 IU/L, $P = 0.02$), although serum CPK of the fermented milk trial showed a tendency to decrease compared with placebo trial (placebo, 193 ± 27 IU/L vs. fermented milk, 152 ± 15 IU/L, $P = 0.06$) (Figure 2-3). Muscle soreness of pectoralis major muscle was significantly suppressed by the consumption of fermented milk compared with that of placebo in the evaluation by palpation ($P = 0.01$) (Table 2-2). Muscle soreness of quadriceps and gluteus maximus muscles was no significant difference (quadriceps muscle: $P = 0.48$, gluteus maximus muscle: $P = 0.50$) (Table 2-2). The total score of muscle soreness in the three parts was significantly suppressed by the consumption of fermented milk compared with that of placebo ($P = 0.02$) (Table 2-2). In the evaluation by movements, the level of muscle soreness was also significantly suppressed (date not shown).

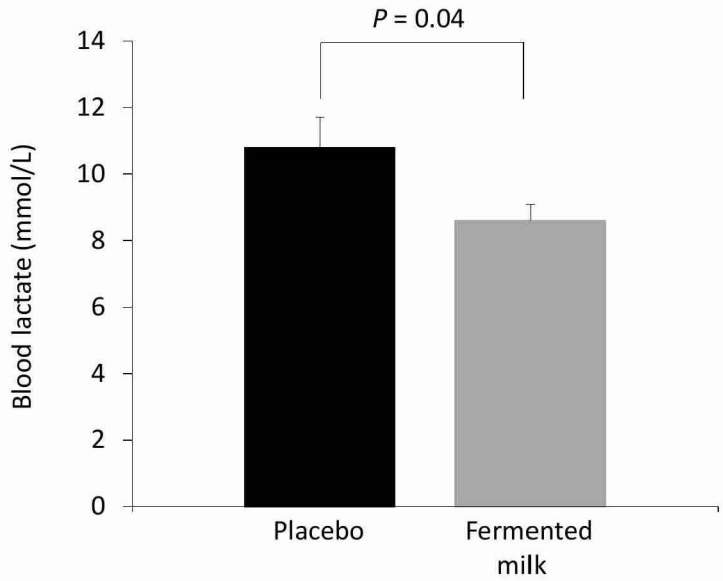


Figure 2-2. Comparison of blood lactate among placebo and fermented milk trials immediately after exercise.

Values are represented as mean ± S.E. for 18 participants. The trials analyzed include: Placebo, exercise with placebo intake; Fermented milk, exercise with fermented milk intake. Paired t-test.

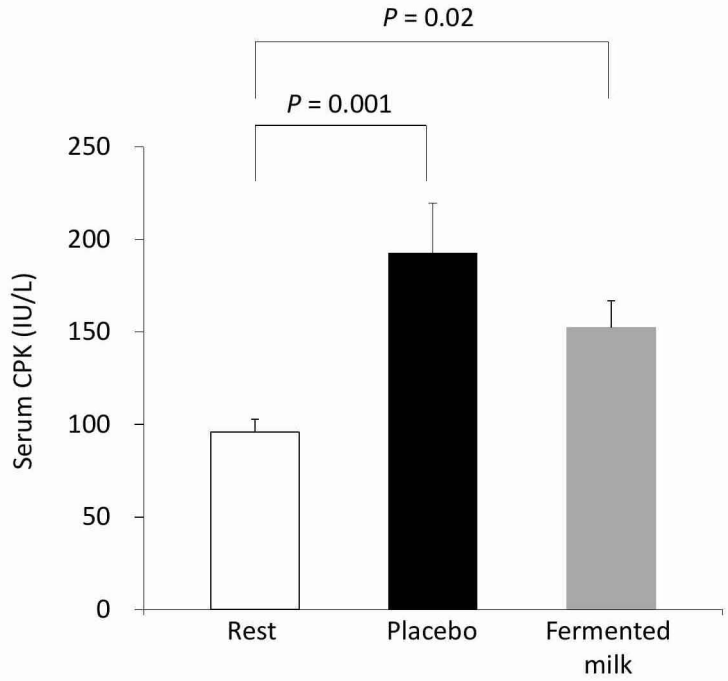


Figure 2-3. Comparison of serum CPK among rest, placebo, and fermented milk trials on the next day of exercise.

Values are represented as mean ± S.E. for 18 participants. The trials analyzed include: Rest, rest with placebo intake; Placebo, exercise with placebo intake; Fermented milk, exercise with fermented milk intake. One-way ANOVA (Tukey-Kramer). CPK, creatine phosphokinase.

Table 2-2. Comparison of muscle soreness among rest, placebo, and fermented milk trials on the next day of exercise

| | Rest | Placebo | Fermented milk | *P*-value |
|---|---|---|---|---|
| Pectoralis major muscle (score) | N.D. | 5.2 ± 0.5 | 4.3 ± 0.5 | 0.01 |
| Quadriceps muscle (score) | N.D. | 3.3 ± 0.5 | 3.0 ± 0.5 | 0.48 |
| Gluteus maximus muscle (score) | N.D. | 5.6 ± 0.5 | 5.3 ± 0.5 | 0.50 |
| Total score (score) | N.D. | 14.2 ± 1.2 | 12.6 ± 1.1 | 0.02 |

Values are represented as mean ± S.E. for 18 participants. The trials analyzed include: Rest, rest with placebo intake; Placebo, exercise with placebo intake; Fermented milk, exercise with fermented milk intake. The total score was calculated by adding the soreness values for the 3 different muscles. Paired t-test. N.D., not detected.

*Indirect metabolic performance*

The RQ and carbohydrate oxidation were compared among the mean values of the three trials for 30 min after glucose administration on the next day of the exercise. The RQ was significantly decreased in the placebo trial compared with the rest trial (rest, 0.88 ± 0.01 vs. placebo, 0.84 ± 0.02, $P$ = 0.03), although this decrease was negated fermented milk trial (placebo, 0.84 ± 0.02 vs. fermented milk, 0.88 ± 0.01, $P$ = 0.02) (Figure 2-4A). Carbohydrate oxidation was significantly decreased in the placebo trial compared with the rest trial (rest, 2.78 ± 0.02 mg/kg/min vs. placebo, 2.10 ± 0.31 mg/kg/min, $P$ = 0.02), although carbohydrate oxidation of the fermented milk trial showed a tendency to decrease compared with rest trial (rest, 2.78 ± 0.02 mg/kg/min vs. fermented milk, 2.41 ± 0.26 mg/kg/min, $P$ = 0.08) (Figure 2-4B). On the other hand, fat oxidation and $VO_2$ did not significantly differ among the trials of the study (data not shown).

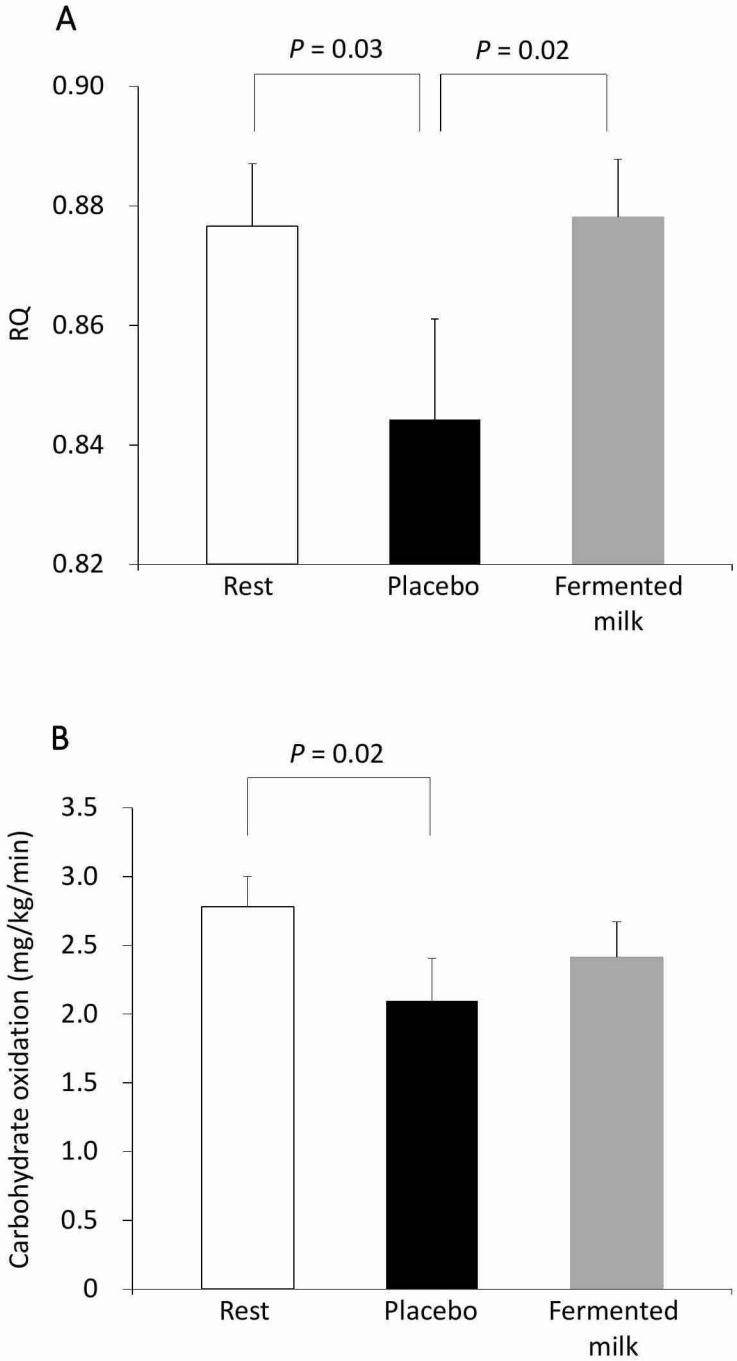


Figure 2-4. Comparison of respiratory metabolic performance among rest, placebo, and fermented milk trials on the next day of exercise.

RQ (A) and carbohydrate oxidation (B) were calculated using oxygen consumption and carbon dioxide production and were compared among the mean values of the three trials for 30 min after glucose administration. Values are represented as mean ± S.E. for 18 participants. The trials analyzed include: Rest, rest with placebo intake; Placebo, exercise with placebo intake; Fermented milk, exercise with fermented milk intake. One-way ANOVA (Tukey-Kramer). RQ, respiratory quotient.

*Blood glucose and serum lipids*

On the next day of the exercise, blood glucose levels were increased after oral glucose administration, although the change was not deemed to be statistically significant among the three trials at fasting, 30 min, and 60 min (fasting: $P = 0.34$, 30 min: $P = 0.32$, 60 min: $P = 0.20$) (Table 2-3). Levels of LDL-cholesterol, HDL-cholesterol, total cholesterol, triglyceride, and free fatty acids were not significantly changed among three trials (LDL-cholesterol: $P = 0.19$, HDL-cholesterol: $P = 0.46$, total cholesterol: $P = 0.35$, triglyceride: $P = 0.24$, free fatty acids: $P = 0.23$) (Table 2-4).

Table 2-3. Comparison of blood glucose level among rest, placebo, and fermented milk trials after oral glucose administration

| | Rest | Placebo | Fermented milk | *P*-value |
|---|---|---|---|---|
| Fasting (mg/dL) | 89 ± 2 | 87 ± 2 | 90 ± 2 | 0.34 |
| 30 min (mg/dL) | 148 ± 6 | 148 ± 6 | 157 ± 5 | 0.32 |
| 60 min (mg/dL) | 121 ± 6 | 122 ± 6 | 132 ± 7 | 0.20 |

Values are represented as mean ± S.E. for 18 participants. The trials analyzed include: Rest, rest with placebo intake; Placebo, exercise with placebo intake; Fermented milk, exercise with fermented milk intake. Two-way ANOVA.

Table 2-4. Comparison of serum lipids among rest, placebo, and fermented milk trials on the next day of exercise

| | Rest | Placebo | Fermented milk | *P*-value |
|---|---|---|---|---|
| LDL cholesterol (mg/dL) | 84 ± 5 | 88 ± 5 | 91 ± 6 | 0.19 |
| HDL cholesterol (mg/dL) | 57 ± 3 | 59 ± 3 | 59 ± 3 | 0.46 |
| Total cholesterol (mg/dL) | 158 ± 7 | 163 ± 5 | 164 ± 7 | 0.35 |
| Triglycerides (mg/dL) | 85 ± 12 | 80 ± 14 | 67 ± 7 | 0.24 |
| Free fatty acids (mEq/L) | 0.38 ± 0.04 | 0.47 ± 0.06 | 0.38 ± 0.05 | 0.23 |

Values are represented as mean ± S.E. for 18 participants. The trials analyzed include: Rest, rest with placebo intake; Placebo, exercise with placebo intake; Fermented milk, exercise with fermented milk intake. One-way ANOVA.

*Inflammation and oxidant stress parameters*

On the next day of the exercise, serum hs CRP was not significantly changed among the trials of the study, although it showed a tendency of increasing in the placebo trial, but not in the fermented milk trial ($P$ = 0.32) (Table 2-5). Serum TNF-α and carbonyl protein levels were not significant changed among the trials of the study, nor were urine 8-OHdG levels (serum TNF-α: $P$ = 0.13, serum carbonyl protein: $P$ = 0.06, urine 8-OHdG: $P$ = 0.71) (Table 2-5). However, the exercise trial reported significantly decreased levels of serum ORAC compared with the rest trial (rest, 6.9 ± 0.4 μmol trolox equivalent (TE)/g vs. placebo, 6.0 ± 0.3 μmol TE/g, $P$ = 0.01), although this decrease was not observed in the fermented milk trial (rest, 6.9 ± 0.4 μmol TE vs. fermented milk, 6.2 ± 0.3 μmol TE/g, $P$ = 0.16) (Figure 2-5).

Table 2-5. Comparison of inflammatory factors and oxidant stress marker among rest, placebo, and fermented milk trials on the next day of exercise

| | Rest | Placebo | Fermented milk | *P*-value |
|---|---|---|---|---|
| Serum hs CRP (ng/mL) | 89 ± 12 | 114 ± 22 | 79 ± 11 | 0.32 |
| Serum TNF-α (pg/mL) | 0.161 ± 0.002 | 0.166 ± 0.001 | 0.170 ± 0.004 | 0.13 |
| Serum carbonyl protein (nmol/mg) | 0.086 ± 0.003 | 0.089 ± 0.004 | 0.096 ± 0.003 | 0.06 |
| Urine 8-OHdG (μg) | 1.82 ± 0.16 | 1.70 ± 0.16 | 1.72 ± 0.19 | 0.71 |

Values are represented as mean ± S.E. for 12 participants (hs CRP and TNF-α), 18 participants (carbonyl protein), and 13 participants (8-OHdG). The trials analyzed include: Rest, rest with placebo intake; Placebo, exercise with placebo intake; Fermented milk, exercise with fermented milk intake. One-way ANOVA. hs CRP, high sensitivity C-reactive protein; TNF-α, tumor necrosis factor-α; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

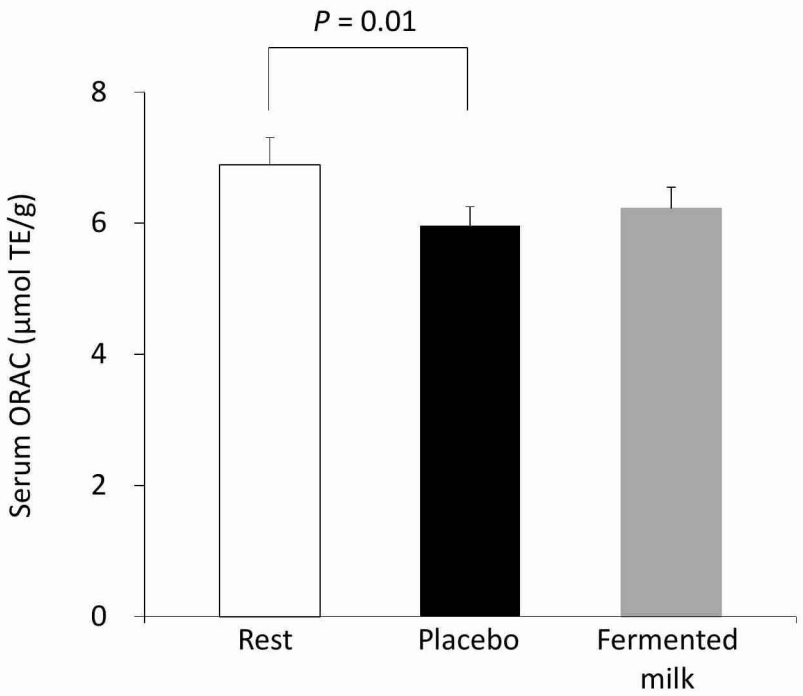


Figure 2-5. Comparison of serum ORAC among rest, placebo, and fermented milk trials on the next day of exercise.

Values are represented as mean ± S.E. for 18 participants. The trials analyzed include: Rest, rest with placebo intake; Placebo, exercise with placebo intake; Fermented milk, exercise with fermented milk intake. One-way ANOVA (Tukey-Kramer). ORAC, oxygen radical absorbance capacity; TE, trolox equivalent.

## 2-4. Discussion

The present study revealed the following main findings: (1) parameters of muscle damage were elevated on the next day after acute resistance exercise; (2) the decrease of carbohydrate oxidation along with RQ was observed with exercise; and (3) the muscle soreness and metabolic changes were mitigated by the consumption of *L. helveticus*-fermented milk in pre- and post-exercise. Previously, it had been unclear whether dietary intervention can improve metabolic impairment after muscle-damaging exercise. Collectively, these observations demonstrate that dietary fermented milk improved the impairment of glucose metabolism associated with exercise-induced muscle damage in humans. Moreover, this study showed that even the supplementation of *L. helveticus*-fermented milk on the day of exercise suppressed muscle damage, although in a previous study [12], *L. helveticus*-fermented milk prevented muscle damage by 3 weeks of continuous ingestion. Therefore, this study for the first time identified the effect of a shot-term administration of the *L. helveticus*-fermented milk in humans.

In recent years, certain methods of reducing muscle damage following exercise have been studied, such as the ingestion of milk. Cockburn et al. [16] suggested that consumption of unfermented milk attenuates the decreases in isokinetic muscle performance and the increases in CK following muscle damaging exercise. Furthermore, consumption of milk immediately after muscle-damaging exercise may limit the

decreases in one-off sprinting performance and likely reduces the increases in agility time and the ability to perform repeated sprints during physiological simulation of field-based team sports [17]. A possible mechanism for milk-inhibited muscle damage is the ingestion of proteins and carbohydrates. Therefore, in this study, the placebo trial that used unfermented milk may have also suppressed muscle damage to some extent. However, these results showed that fermented milk is more effective than unfermented milk in parameters such as muscle damage and glucose metabolism.

Generally, it is well-known that a single bout of exercise elevates glucose uptake for a period of time post-exercise [18–20]. However, this study have shown that insulin-mediated glucose uptake in muscle is decreased by muscle-damaging exercises, but not by non-muscle-damaging exercises [21]. Therefore, the decreases of carbohydrate oxidation and RQ in the present study are suspected to be caused by insulin-dependent glucose uptake in damaged muscle. This decrease of glucose uptake is presumably due to a reduction of GLUT4 translocation via the insulin signaling pathway, which is the rate-limiting step in glucose metabolism. It has been reported that some inflammatory cytokines and chemokines, such as TNF-α and IL-1, attenuate the activity of insulin-mediated signaling in muscle cells [22, 23]. In addition, oxidative stress can also decrease glucose uptake by reducing the activity of insulin-mediated signaling [3, 24, 25]. In the damaged muscle on the next day of exercise, these cytokines and oxidative components

are elevated [26–28], which could lead to the impairment of insulin-dependent glucose uptake. However, because it did not find any significant changes of inflammatory markers and oxidative products in serum and urine, the response is considered to be limited to muscle tissue, rather than the whole body, as suggested in the preceding study [21].

Previously, Aoi et al. demonstrated that *L. helveticus*-fermented milk attenuates delayed-onset muscle damage after acute exercise in rats [12]. In this study, phagocyte infiltration and inflammatory cytokines expression, markers of inflammation in damaged muscle, were markedly reduced by the consumption of fermented milk. In addition, lipid peroxide levels were elevated after exercise, although the fermented milk uptake significantly reduced this elevation. Therefore, these observations suggest that fermented milk consumption mitigated inflammation and oxidative stress as well as muscle damage, which results in improvements in glucose metabolism by maintaining the insulin signaling pathway. In fact, the present study showed that ORAC, a marker of antioxidant capacity, was reduced in the placebo trial, but not in the fermented milk trial. Thus, the inhibitory effect of glucose metabolic impairment and muscle damage may be associated with elevated antioxidant levels caused by the consumption of fermented milk. Additionally, Aoi et al. demonstrated that, in the skeletal muscle of rats, fermented milk upregulates expression of antioxidant enzymes, such as superoxide dismutase-2,

catalase, and glutathione S-transferase α-1 [12]. In addition, heat shock protein 70, a chaperone protein that can function as an antioxidant and an anti-inflammatory agent, was also elevated by the consumption of fermented milk. These observations suggest that fermented milk improves glucose metabolism and muscle damage at least, in part, by controlling endogenous antioxidant and anti-inflammation factors, a hypothesis that is supported by the ORAC results in the present study.

Although the detailed mechanisms of the effects of fermented milk on mitigating muscle damage remain unclear, small peptides present in fermented milk may be the causable agent, because fermented milk is more effective than unfermented milk. *L. helveticus*-fermented milk is manufactured by fermenting skim milk with a starter culture containing *L. helveticus*. During this process, the proteins in skim milk are digested by *Lactobacillus* and converted into small peptides, which are absorbed more in amount from the intestines compared to amino acids or large oligopeptides. Such peptides may also have additional physiological benefits aside from their use as a source of protein. Several studies have reported that peptides from fermented milk have various salutary effects [29]. The present study suggests that small digested peptides in fermented milk may contribute to increasing the level of antioxidant capacity in muscle.

## 2-5. Conclusion

*L. helveticus*-fermented milk supplementation improved glucose metabolism and alleviated the effects of muscle soreness after high-intensity exercise in young men, possibly associated with the regulation of antioxidant capacity. Moreover, the present study demonstrated the beneficial effect by a short-term supplementation before and after exercise. *L. helveticus*-fermented milk is expected to contribute to the maintenance of an athlete's performance.

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# Chapter 3. The effect of milk casein hydrolysate on muscle damage after exercise in middle-aged to elderly men

## 3-1. Introduction

Physical inactivity is the fourth leading cause of death worldwide in life style [1], which corresponds to the "pandemic" state. In contrast, daily exercise prevents and improves metabolic syndrome, a pre-disease state; thus, it leads to the prevention of non-communicable diseases [2, 3]. Based on the background, the World Health Organization (WHO) announced global recommendation on physical activity for health, particularly for middle-aged to elderly (over 40 years) people [4, 5] who is higher the risk of metabolic diseases than younger people [6]. Walking is one of the major exercises recommended for middle-aged to elderly people, because it can be performed easily and safely. It has actually been reported that habitual walking prevented or improved cardiovascular diseases and overweight [7–9]. However, middle-aged to elderly people are more susceptible to muscle soreness and fatigue than young people [10, 11]; which could cause a decrease in their motivation for exercise.

In Chapter 2, it was determined that *L. helveticus*-fermented milk supplementation improved glucose metabolism and alleviated the effects of muscle soreness after high-intensity exercise in young healthy men. The active ingredient in *L. helveticus*-fermented milk was considered to be peptides produced by fermentation. These peptides can be

released via fermentation by *L. helveticus* or via enzymatic hydrolysis by *A. oryzae* protease [12] from casein, a major protein in milk. In fact, in our unpublished study, it has been found, as well as *L. helveticus*-fermented milk, that MCH produced by the *A. oryzae* protease improved muscle damage after high-intensity exercise in young men. However, the effect on populations other than young people, particularly middle-aged and elderly people is not clear. Moreover, the effect on regular common exercise, such as “walking exercise”, is also obscure.

The purpose of this study was to investigate the effect of short-term supplementation of MCH on muscle soreness and fatigue in damaged muscle after acute walking exercise in middle-aged to elderly men.

## 3-2. Methods

*Participants*

Fourteen healthy middle-aged to elderly men with no regular exercise regimen habits were recruited to participate in this study. The mean ± S.E. characteristics of the participants were as follows: age, 56.6 ± 2.8 years; height, 168.9 ± 1.4 cm; body weight, 68.4 ± 1.9 kg; BMI, 23.9 ± 0.5 kg/m$^2$; and body fat percentage, 22.6 ± 1.1%. All participants were free of the signs, symptoms, and history of any overt chronic diseases. None of the participants were currently taking any medications or dietary supplements. This study was approved by the Ethics Committee of Kyoto Prefectural University (2013, No. 45), and all of the participants signed an informed consent form after reading about the design and protocol of the study.

*Study design*

All of the participants attended the two trials included in the study, exercise with placebo intake (placebo) and exercise with MCH intake (MCH) in a repeated-measures experimental design. These trials were performed in a random order using a counter-balanced design and were separated by at least two weeks for any individual participants in order to avoid biasing of the muscle damage. The participants were also asked to refrain from caffeine and alcohol ingestion 24 h before each trial and were asked not to

eat or drink anything except for water from 22:00 on the night before the trial to the next morning. Dietary records on the day of the trial were performed to avoid significant differences of food intakes between placebo and MCH trials. In the first trial, an example of the recording method was shown to the participants beforehand, and they recorded dietary contents according to it. And, the recorded contents were repeated in the second trial.

*Examination tablets*

The MCH hydrolyzed casein was produced by proteolytic enzymes. MCH containing peptides were prepared according to a previously described procedure [12]. The powdered casein hydrolysate was punch-pressed into tablets after the addition of diluents, emulsifier, and lubricant (test sample). The amount of peptides in the test sample was measured by the liquid chromatography-mass spectrometry method as described in the previous report [13] with some modification. Val-Pro-Pro and Ile-Pro-Pro were quantified using the internal standard method with Val-Pro-Pro isotope (($^{13}C^5$) Val-($^{13}C^5$) Pro-Pro, m/z 324.2) and Ile-Pro-Pro isotope (Ile-($^{13}C^5$) Pro-Pro, m/z 332.2) obtained from SCRUM Inc. (Tokyo, Japan). The placebo employed was non-hydrolyzed casein, and it was also presented in the shape of a tablet (Table 3-1). Each of the

participants consumed 2 tablets with a cup of water before and after the exercise, using a double-blinded method.

*Experiment schedule*

On the first experiment day of each trial, the participants ate breakfast at 7:30 and came to laboratory at 9:00, where they sat on a chair until the beginning of the test. Subsequently, the test tablets (placebo or MCH) were consumed. Walking exercise was performed from 30 min after consumption of the test tablets. During the exercise, the heart rate and the rating of perceived exertion (RPE)-the Borg 15 points (6–20) scale, were measured. Immediately after the exercise, the blood lactate, blood glucose, and fatigue grade were measured. Afterwards, the participants consumed the test tablets again at 30 min after the end of the exercise. The participants were asked to not to eat or drink anything except for water from 22:00 to the measurement of the next morning.

On the second day of the study, the participants ate breakfast (steamed rice, 200 g) at 7:30 and returned to the laboratory at 9:00. Subsequently, the body composition was measured and blood sample was collected from capillary using a fingerstick test method. Then, blood pressure, heart rate, skin blood flow, and pulse wave velocity (PWV) were measured. Next, the degree of subjective muscle soreness was evaluated. A schematic illustration of the experimental schedule was shown in Figure 3-1.

Table 3-1. Nutritional information of test tablet

| | Placebo | MCH |
|---|---|---|
| Energy (kcal) | 5.4 | 5.4 |
| Protein (g) | 0.4 | 0.3 |
| Free amino acid (mg) | 0.0 | 182 |
| Peptide (mg) | 0.0 | 154 |
| Val-Pro-Pro (mg) | 0.0 | 1.4 |
| Ile-Pro-Pro (mg) | 0.0 | 2.0 |
| Fat (g) | 0.04 | 0.04 |
| Carbohydrate (g) | 0.9 | 0.9 |
| Sodium (mg) | 5.0 | 5.0 |

Values are represented as composition included in 4 tablets.

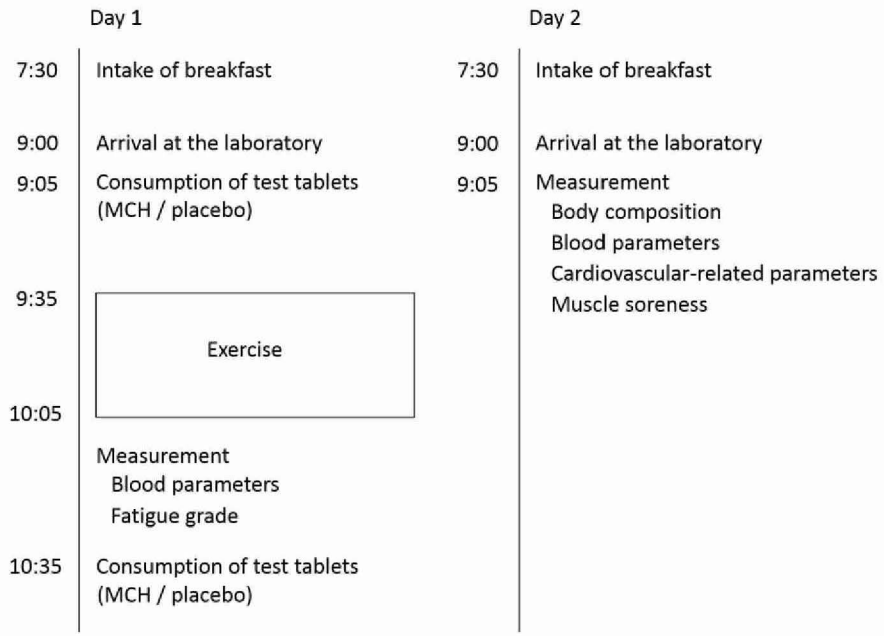


Figure 3-1. Schematic illustration of experimental schedule.

*Exercise procedure*

After stretching, the participants performed walking exercise for 30 min. The walking exercise was performed on a downhill treadmill (My Mountain 5050, Tobeone Co., Ltd., Gyeonggi-do, Republic of Korea) at a 5% decline grade. Walking speed was increased to 5 km/h after a warming-up period at 3 km/h for 2 min, and it was maintained at that speed until the end of exercise. Heart rate and RPE were measured every 3 min during the exercise.

*Fatigue grade*

Two questionnaires using a VAS and a profile of mood state (POMS) were used to evaluate the fatigue grade immediately after the exercise. Using the VAS, the participants were asked to indicate the intensity of perceived fatigue on a 100-mm horizontal line. The left side stated “having no fatigue”, while the right side stated “having max fatigue”. The POMS questionnaire consists of 65 items, providing answers ranging from 0 (not at all) to 4 (extremely). That can be consolidated into six mood scales: "tension-anxiety", "depression-dejection", "anger-hostility", "vigor", "fatigue", and "confusion".

*Muscle soreness*

Subjective muscle soreness in the femoral, crural, and gluteus maximus muscles was evaluated by palpation and movement (bending and stretching) using the VAS. The participants were asked to indicate the intensity of perceived soreness for each muscle part on a 100-mm horizontal line. The left side stated "having no soreness", while the right side stated "having max soreness". The total soreness value was calculated by adding the soreness values for the 3 different muscles.

*Blood parameters*

Blood samples were collected from capillary using a fingerstick test method. The separated plasma was stored at –80° until measurement. The blood lactate and glucose were measured using simple measuring instruments (blood lactate: Lactate Pro, Arkray, Inc., Kyoto, Japan; glucose: Glu Test, Sanwa Kagaku Kenkyusho Co., Ltd., Aichi, Japan). CK and the insulin level in the plasma were measured by using an ELISA kit (CK: MaxDiscovery™ Creatin kinase Enzymatic Assay kit, Bioo Scientific Co., TX, USA; insulin: Mercodia Ultrasensitive Human Insulin ELISA, MercodiaAB, Uppsala, Sweden).

*Cardiovascular-related parameters*

The blood pressure and heart rate were monitored with a humerus sphygmomanometer (EW3100, Panasonic Electric Works Co., Ltd., Osaka, Japan). The skin blood flow was measured with a laser doppler blood perfusion imager (PeriScan PIM 3 System, Integral Co., Tokyo, Japan). The PWV was calculated using a blood-pressure pulse wave inspection apparatus (FORM BP-203PRE, Omron Colin Co., Ltd., Tokyo, Japan) in the supine decubitus position.

*Statistical analysis*

All of the data were shown as the mean ± standard error. Wilcoxon signed-ranks test was used for data on blood lactate, plasma insulin, and muscle soreness with a non-normal distribution. Paired t-test was used for data on other parameters with a normal distribution. The significance level was assumed to be 5%.

## 3-3. Results

*Heart rate and RPE*

Heart rate gradually increased over time during the exercise, and reached 87.1 ± 2.5 beats/min (placebo) and 86.4 ± 2.9 beats/min (MCH) at the end of the exercise, when RPE showed scores of 11.5 ± 0.7 (placebo) and 11.4 ± 0.5 (MCH). No significant differences were found between the placebo and MCH trials in either the heart rate ($P$ = 0.32) or the RPE ($P$ = 0.45) throughout the exercise, indicating that the same exercise load was applied to both conditions.

*Fatigue grade*

In the examination using VAS, the fatigue grade immediately after exercise showed a significantly low value for the MCH trial, compared with the placebo trial (placebo, 3.4 ± 0.5 score vs. MCH, 2.5 ± 0.4 score, $P$ = 0.03) (Figure 3-2A). Moreover, in the examination using POMS, the score of the fatigue-related item immediately after exercise was also significantly lower for the MCH trial, compared with the placebo trial (placebo, 6.3 ± 1.1 score vs. MCH, 5.0 ± 1.0 score, $P$ = 0.04) (Figure 3-2B). None of the other items in the POMS showed any significant change between the placebo and MCH trials (data not shown).

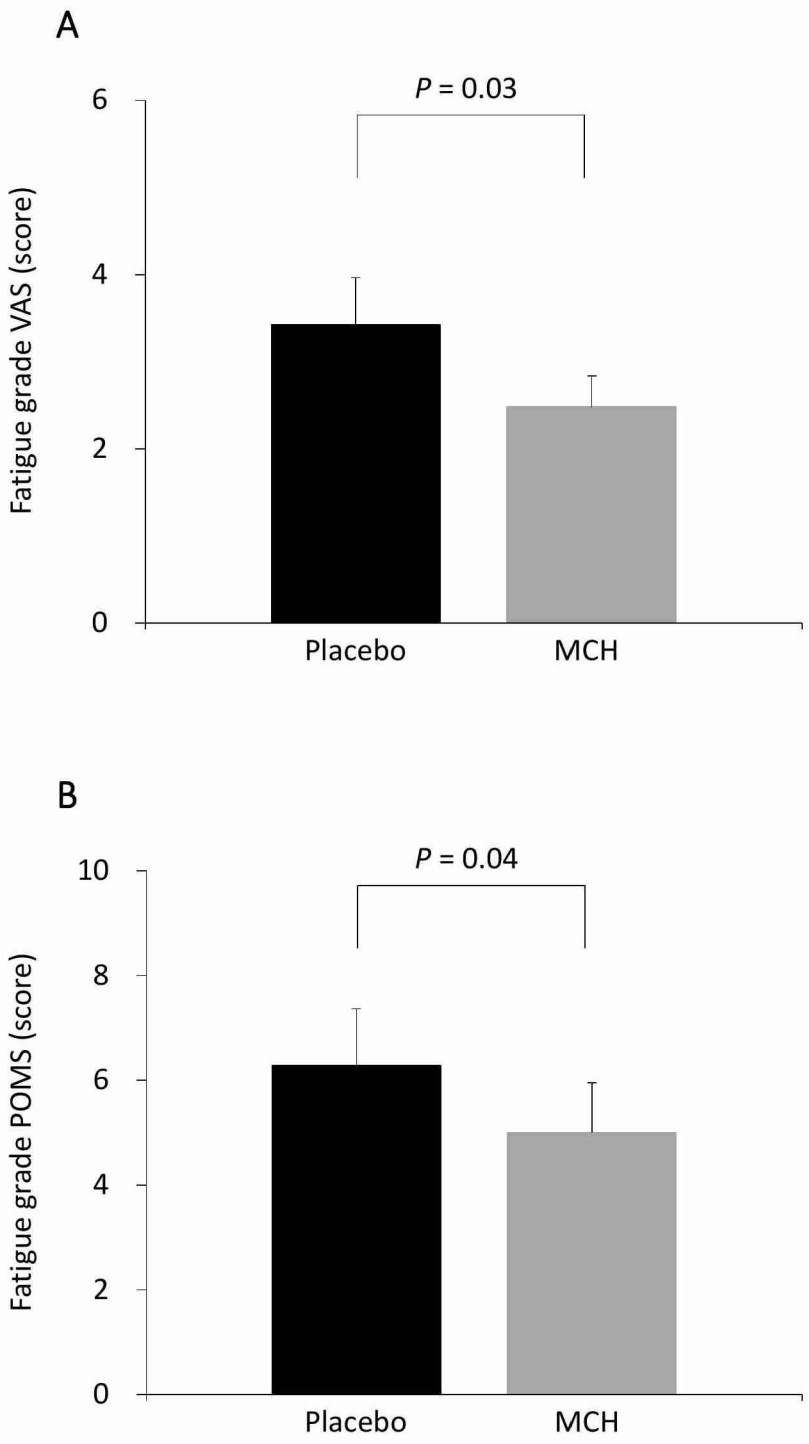


Figure 3-2. Comparison of fatigue grade among placebo and MCH trials immediately after exercise.

VAS (A) and POMS (B) were used to evaluate the fatigue grade immediately after the exercise. Values are represented as mean ± S.E. for 14 participants. The trials analyzed included: Placebo, exercise with placebo intake; MCH, exercise with MCH intake. Paired t-test. VAS, visual analog scale; POMS, profile of mood state.

*Muscle soreness*

On the next day of exercise, using the evaluation by palpation, the level of muscle soreness in the femoral and crural muscles was significantly suppressed in the MCH trial, compared with the placebo trial (femoral muscle: $P = 0.02$, crural muscle: $P = 0.04$) (Table 3-2). The significant effect was not found in the gluteus maximus muscle ($P = 0.24$) (Table 3-2). The total score for muscle soreness in the three muscles was significantly suppressed in the MCH trial, compared with the placebo trial ($P = 0.03$) (Table 3-2). In the evaluation by movements, the level of muscle soreness also showed a tendency to be suppressed in the MCH trial (date not shown). Significant difference in the level of muscle soreness was not observed between first and second trials, showing no repeat-bout effects.

Table 3-2. Comparison of muscle soreness among placebo and MCH trials on the next day of exercise

| | Placebo | MCH | *P*- value |
|---|---|---|---|
| Femoral muscle (score) | 1.8 ± 0.6 | 1.1 ± 0.4 | 0.02 |
| Crural muscle (score) | 1.7 ± 0.6 | 1.4 ± 0.6 | 0.04 |
| Gluteus maximus muscle (score) | 1.2 ± 0.5 | 0.9 ± 0.3 | 0.24 |
| Total score (score) | 4.7 ± 1.5 | 3.4 ± 1.3 | 0.03 |

Values are represented as mean ± S.E. for 14 participants. The trials analyzed included: Placebo, exercise with placebo intake; MCH, exercise with MCH intake. The total score was calculated by adding the soreness values for the 3 different muscles. Wilcoxon signed-ranks test.

*Blood parameters*

Immediately after the exercise, the blood lactate values showed a tendency for lower values in the MCH trial, compared with the placebo trial ($P = 0.08$) (Table 3-3). The blood glucose levels obtained immediately after exercise did not show any significant difference between the placebo and MCH trials ($P = 0.34$) (Table 3-3).

On the next day of exercise, no significant differences were found between the placebo and MCH trials for the blood glucose, plasma CK, or plasma insulin (blood glucose: $P = 0.29$, plasma CK: $P = 0.36$, plasma insulin: $P = 0.83$) (Table 3-3).

Table 3-3. Comparison of blood parameters among placebo and MCH trials after exercise

| | Placebo | MCH | *P*- value |
|---|---|---|---|
| Immediately after exercise | | | |
| Blood lactate (mmol/L) | 1.6 ± 0.2 | 1.2 ± 0.1 | 0.08 |
| Blood glucose (mg/dL) | 88 ± 4 | 90 ± 3 | 0.34 |
| The next day of exercise | | | |
| Blood glucose (mg/dL) | 131 ± 9 | 127 ± 7 | 0.29 |
| Plasma CK (IU/L) | 147 ± 16 | 153 ± 8 | 0.36 |
| Plasma insulin (μU/mL) | 16 ± 3 | 20 ± 5 | 0.83 |

Values are represented as mean ± S.E. for 14 participants. The trials analyzed included: Placebo, exercise with placebo intake; MCH, exercise with MCH intake. Blood lactate and plasma insulin: Wilcoxon signed-ranks test, other parameters: Paired t-test. CK, creatine kinase.

*Cardiovascular-related parameters*

On the next day of exercise, no significant difference were shown in the systolic blood pressure and diastolic blood pressure between the placebo and MCH trials (systolic blood pressure: $P = 0.68$, diastolic blood pressure: $P = 0.18$) (Table 3-4). The heart rate showed a tendency for lower value in the MCH trial, compared with placebo trial ($P = 0.08$) (Table 3-4). No significant changes were shown in the skin blood flow or PWV between the placebo and MCH trials (skin blood flow: $P = 0.44$, PWV: $P = 0.32$) (Table 3-4).

Table 3-4. Comparison of cardiovascular-related parameters among placebo and MCH trials on the next day of exercise

| | Placebo | MCH | *P*- value |
|---|---|---|---|
| Systolic blood pressure (mmHg) | 116 ± 4 | 115 ± 4 | 0.68 |
| Diastolic blood pressure (mmHg) | 80 ± 3 | 78 ± 2 | 0.18 |
| Heart rate (beats/min) | 69 ± 2 | 67 ± 2 | 0.08 |
| Blood skin flow (PU) | 106 ± 6 | 106 ± 6 | 0.44 |
| PWV (cm/s) | 1373 ± 53 | 1358 ± 56 | 0.32 |

Values are represented as mean ± S.E. for 14 participants. The trials analyzed included: Placebo, exercise with placebo intake; MCH, exercise with MCH intake. Paired t-test. PWV, pulse wave velocity.

### 3-4. Discussion

The present study revealed that the muscle soreness and fatigue parameters observed after downhill walking exercise, although these parameters were mitigated by the intake of MCH, with pre- and post-exercise. These observations primarily demonstrate that the effect of diet supplementation using milk-related peptides in middle-aged to elderly men. These results would help greatly to the improvement of the exercise habits of middle-aged to elderly men. Daily exercise is effective in the prevention and improvement of non-communicable diseases. Based on the research findings published by the WHO and Lee et al. [14], more than 150 min a week of moderate exercise, equivalent to brisk walking, is enough to ensure that one does not fall into the "physical inactivity" category, which accounts for 35.2% of the population worldwide. According to the statistics published by the WHO, physical inactivity is a major cause of death and development of non-communicable diseases, and it corresponds to 9.4% of total death risk. Accordingly, the WHO recommends that adults aged 18–64 years should do at least 150 min of moderate-intensity aerobic physical activity each week and increase that amount to 300 min if possible [5]. However, the half of participants drops out exercise within 6 months from the beginning, as shown in Dishman's report [15]. In contrast to barriers for younger adults, the major barrier for the elderly is related to health-related concerns [16], and it is difficult for over 80% of

elderly people to participate in exercise due to at least one physical or psychological barrier [17]. In addition, in an investigation of community-dwelling elderly people, health problems and pain were also suggested as the most common barrier to exercise [18]. Muscle soreness also continues after the next day of exercise, and this may interfere with activities in daily life. Moreover, muscle soreness after exercise could be one of the factors preventing the habituation of exercise [19]. In this study, MCH suppressed muscle soreness and fatigue in middle-aged to elderly men, which may contribute to the habituation of exercise.

In the present study, the subjective fatigue grade immediately after downhill walking exercise showed lower values in the MCH intake group. Although fatigue induced by exercise is caused by various peripheral and psychological factors [20], intracellular acidosis could be a key role for muscle fatigue. Even minimal decrease in muscle pH interferes with cross-bridge binding and ATPase activity due to competitive binding and reduced enzyme function [21]. Decreased intracellular pH may also impair oxidative enzyme activity and may adversely affect ryanodine receptor function [22]. Lactic acid, a major source of protons, is rapidly produced by muscle contraction, lowering the pH and inhibiting muscle contraction. Furthermore, the blood lactate after exercise tended to show lower values by ingesting MCH. Thus, these observations suggest that the MCH intake suppressed the subjective fatigue grade after exercise, which could be mediated

by improving muscular acidosis. Although detail mechanism is unclear, a possibility might be caused by improvement of peripheral blood flow after intake of MCH. In the previous animal study [23], milk-derived peptides increased concentrations of plasma nitrate and nitrite, which expands a diameter of an artery and increases a blood flow after oral administration. Therefore, MCH might elevate oxygen supply to muscle cells during exercise and induce a predominance of aerobic metabolism over anaerobic metabolism, which leads to the prevention of acidosis via suppression of lactic acid production.

It is well known that muscle soreness generally occurs as a part of delayed-onset muscle damage [24]. Therefore, MCH might reduce the degree of muscle soreness by suppressing the muscle damage. Delayed-onset muscle damage is caused by a variety of factors, but it is considered that oxidative stress and inflammatory cytokines are involved, at least to some extent. Indeed, oxidative stress and inflammatory cytokines also increase at the initiation and developmental stages of muscle damage [25, 26]. Therefore, it may be considered that muscle soreness will also be reduced by suppressing oxidative stress and inflammatory cytokines. MCH contains specific peptides, such as Val-Pro-Pro and Ile-Pro-Pro, which are equivalent to *L. helveticus*-fermented milk. As shown in the previous study [27] and Chapter 2, *L. helveticus*-fermented milk was found to inhibit the muscle damage by adjusting the antioxidant capacity. Therefore, these previous studies

support this study's concept that the consumption of MCH reduced muscle damage through regulating the antioxidant capacity induced by casein-derived peptides, which might also contribute the suppression of muscle soreness and fatigue induced by muscle damage. In the present study, CK, a muscle damage marker, was not changed between conditions, which might be caused by characteristics of subjects such as lower inflammatory response in middle-aged and elderly people than young people [28]. It has been documented that a decrease in muscle content in type II fibers linked with aging [29] and type II fibers are more susceptible to muscle damage than type I fibers [30]. Lower levels of muscle damage response in the middle-aged and elderly people may be explained by their lower muscle content of type II fibers.

The previous study [31] showed that MCH containing antihypertensive peptides such as Val-Pro-Pro and Ile-Pro-Pro were able to improve human blood pressure and PWV by continuous ingestion. However, the cardiovascular parameter was not changed by ingestion MCH in the present study, which might be affected by duration and amount of intake. These peptides increased concentrations of plasma nitrate and nitrite, which expands a diameter of an artery and increase a blood flow after a single oral administration to Wistar rats [23]. Therefore, this may occur in the present study during walking exercise, and affect a tendency for heart rate, although nitric oxide was not measured at that time.

The detailed mechanisms of the effects of MCH on mitigating muscle soreness and fatigue remain unclear. As discussed in Chapter 2, small peptides present in MCH as well as *L. helveticus*-fermented milk may be the causative agents. In addition to such peptides, amino acids are another possible ingredient contained in MCH that may be involved in the effect shown by the consumption of MCH, since the amount of amino acids in MCH is larger, compared with the placebo tablets. Moreover, despite having only a very small amount of peptides and amino acids, MCH exerted an inhibitory effect on muscle soreness and fatigue. In a previous study [32], the peptides from a milk beverage were absorbed intact into the circulation, which suggests that the peptides might have a direct function in peripheral tissues. Therefore, it is necessary to examine the direct effect of amino acids and peptides on skeletal muscle after absorption.

### 3-5. Conclusion

MCH supplementation alleviated muscle soreness and fatigue induced by downhill walking in middle-aged to elderly men. Moreover, the beneficial effect has been obtained by a short-term supplementation of MCH before and after exercise. MCH may be useful for middle-aged to elderly men who perform physical activity for health promotion.

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# Chapter 4. General discussion and conclusions

This study investigated the short-term effect of *L. helveticus*-fermented milk and MCH supplementation on muscle damage after exercise in humans. In the previous study [1], *L. helveticus*-fermented milk prevented muscle damage by continuous ingestion in rats. Moreover, in our unpublished recent study using mice, MCH alleviated muscle damage through a short-term administration.

In Chapter 2, it was determined that fermented milk supplementation improved glucose metabolism and alleviated the effects of muscle soreness after high-intensity exercise in young men, possibly associated with the regulation of antioxidant capacity. In this study, the effect was observed in the commercial *L. helveticus*-fermented milk (Amiel S®) but not in the extracted component or synthesized sample. Moreover, this study demonstrated an effect by a short-term supplementation of *L. helveticus*-fermented milk before and after exercise. Therefore, *L. helveticus*-fermented milk is considered to have a high utility value in daily exercise.

In the sports field, muscle damage and muscle fatigue caused by routine training and games are known to lead to performance degradation. In particular, muscle damage is caused by high-intensity training just before a game, when a decrease in energy production, endurance, and muscle strength occurs. Thus, it is believed that a maximum capacity cannot be exerted in a game. Therefore, considering the decline in performance

caused by muscle damage and the combination of exercise and a nutritionally balanced diet, it is necessary to improve physical performance and competitive ability. In this study, *L. helveticus*-fermented milk suppressed muscle damage and improved glucose metabolism, therefore, *L. helveticus*-fermented milk is expected to contribute to the maintenance of an athlete's performance.

In Chapter 3, it was demonstrated that MCH supplementation alleviated muscle soreness and fatigue induced by downhill walking in middle-aged to elderly men. In this study, as the exercise protocol mimicked movement in daily life, the “walking exercise” was performed, and an inhibitory effect on muscle soreness and fatigue was observed. Thus, MCH may be useful for persons who perform physical activity for health promotion. The powder containing MCH, for example, could be used by manufacture in capsule or tablet form. Moreover, MCH could be utilized by addition to a beverage, yogurt, jelly, or similar consumables. Since the described adaptations are possible, MCH could be effectively used in sports drinks, general foods, and dietary supplements. Therefore, since MCH was effective even with a short-term supplementation in daily exercise for health promotion, the utility value is likely to be very high.

The active ingredient of *L. helveticus*-fermented milk and MCH on mitigating muscle damage remain unclear. As discussed in Chapter 2 and 3, small peptides present in *L. helveticus*-fermented milk and MCH may be the causative agents. Moreover, despite

having only a very small amount of peptides and amino acids, *L. helveticus*-fermented milk and MCH exerted an inhibitory effect on muscle damage. In the previous study [2], the peptides from a milk beverage were absorbed intact into the circulation, which suggests that the peptides might have a direct function in peripheral tissues. In addition, growing evidence has been shown that the supplementation of lactic acid bacteria can affect the immune system through improving the intestinal environment, such as the intestinal flora, immunocompetent cells, and lactic acid bacteria [3]. Therefore, it is necessary to examine not only the direct effect of amino acids and peptides on skeletal muscle after absorption, but also any indirect effects, such as the regulation of the intestinal bacteria and brain-gut interaction.

Another limitation of this study is that the mechanism of muscle damage of *L. helveticus*-fermented milk and MCH is not elucidated. Similar to the previous study [1], this study suggests that antioxidant capacity obtained by their intake is associated with the improvement muscle damage. However, the response is considered to be limited to muscle tissue, rather than the whole body, as suggested in a previous study [4].

Further research is required to examine the detailed mechanisms of the effect of fermented milk and MCH in mitigating muscle damage, along with the benefit of fermented milk and MCH in different subjects and conditions.

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## Abbreviations

ANOVA: analysis of variance

*A. oryzae*: *Aspergillus oryzae*

BMI: body mass index

CK: creatine kinase

CPK: creatine phosphokinase

DNA: deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

GLUT4: glucose transporter 4

hs CRP: high sensitivity C-reactive protein

IL: interleukin

IRS-1: insulin receptor substrate-1

*L. helveticus*: *Lactobacillus helveticus*

MCH: milk casein hydrolysate

ORAC: oxygen radical absorbance capacity

PI3-K: phosphoinositide 3-kinase

POMS: profile of mood state

PWV: pulse wave velocity

RM: repetition maximum

ROS: reactive oxygen species

RPE: rating of perceived exertion

RQ: respiratory quotient

TNF-α: tumor necrosis factor-α

TE: trolox equivalent

$VCO_2$: carbon dioxide production

$VO_2$: oxygen consumption

VAS: visual analogue scale

WHO: world health organization

8-OHdG: 8-hydroxy-2'-deoxyguanosine

# Accomplishments

## Published papers

1. Iwasa M, Aoi W, Mune K, Yamauchi H, Furuta K, Sasaki S, Takeda K, Harada K, Wada S, Nakamura Y, Sato K, Higashi A: Fermented milk improves glucose metabolism in exercise-induced muscle damage in young healthy men. *Nutr J* 2013, 12: 83.
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