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Inhibitory effect of fermented milk on delayed-onset muscle damage after exercise

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Abstract

Milk fermented with a starter containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae* is drunk on a daily basis by many people in Japan and has several beneficial effects. We studied the influence of this fermented milk product on muscle damage after prolonged exercise in rats. Wistar rats were divided into four groups: rested controls, rested rats given fermented milk diet, exercised rats and exercised rats given fermented milk diet. After 3 weeks of acclimatization, both exercise groups were made to run on a treadmill at 26 m/min for 60 min. Exercise increased the serum creatine kinase level, as well as myeloperoxidase activity and the level of thiobarbituric-acid-reactive substances in the gastrocnemius muscle after 24 h. These changes were ameliorated by intake of fermented milk. An increase of CINC-1 was also ameliorated by fermented milk. Furthermore, milk diet increased the mRNA and protein levels of protective proteins such as antioxidants and chaperone proteins. These results indicate that fermented milk can ameliorate delayed-onset muscle damage after prolonged exercise, which is associated with an increased antioxidant capacity of muscles.

Keywords: Fermented milk; Exercise; Muscle damage; Antioxidant; Reactive oxygen species

1. Introduction

The fermentation of skim milk with a starter culture containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae* produces a drink that is marketed in Japan. It has been reported that several beneficial effects, such as prolonged lifespan [1], antihypertensive effect [2] and antitumorigenic effect [1,3], are obtained by the ingestion of fermented milk.

Fermented milk has been traditionally used as a drink for daily consumption. It is also often taken by athletes during or after exercise. However, the effect of this fermented milk drink on physiological changes that occur during exercise is not yet well-known. Therefore, we investigated the effect of fermented milk on muscle damage after exercise.

Delayed-onset muscle damage that becomes manifest after exercise has several underlying mechanisms, including mechanical stress [4–6] and calcium overload [6–8]. In addition, we recently reported that muscle damage after prolonged exercise is associated with a redox-sensitive inflammatory cascade that is regulated by oxidative stress [9,10]. The redox-sensitive inflammatory cascade is activated by reactive oxygen species (ROS) that are generated in the mitochondria, peroxisomes and endothelium during exercise, and it leads to the invasion of phagocytes into muscles after exercise. We have also demonstrated that antioxidants can inhibit muscle damage by scavenging ROS [9,10]. Therefore, the present study was performed to investigate the influence of fermented milk on delayed-onset muscle damage after exercise in relation to its antioxidant activity. We found that intake of fermented milk was able to ameliorate muscle damage after exercise.

2. Materials and methods

2.1. Preparation of fermented milk

Fermented milk product was prepared at Calpis Co., Ltd. (Tokyo, Japan), as reported previously [11]. Reconstituted

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9% (wt/wt) skim milk powder (Yotsuba, Hokkaido, Japan) was fermented with a starter culture containing *L. helveticus* and *S. cerevisiae* at 37°C for 24 h. Then the fermented milk was converted to powder by freeze-drying method after mixing with an equal amount of dextrin on a dry matter basis.

2.2. Animals

The present study complied with the principles and guidelines of the Japanese Council on Animal Care, and it was also approved by the Committee for Animal Research of the Kyoto Prefectural University of Medicine (permission no. M13-91). Forty-eight male Wistar rats (5 weeks old; Clea Japan, Inc., Osaka, Japan) were acclimatized for 1 week to an air-conditioned (22±2°C) room with a 12-h light/dark cycle (lights on from 0730 to 1930 h). The rats were allotted into four groups of 12 animals each, consisting of a rested control group (C), a rested control group given fermented milk (M), an acute exercise group (RC) and an acute exercise group given fermented milk (RM). All animals were trained to run on a motorized treadmill designed for rats, beginning at 8 m/min for 10 min, 3 days/week. Over a 3-week period, the intensity of exercise was gradually increased to 26 m/min, while a duration of 10 min was maintained. On the day of the experiment, RC and RM rats performed treadmill run for 60 min at 26 m/min. None of the rats ceased exercise prematurely due to injury. Rats were fed a control diet containing 10% dextrin or a fermented milk diet containing 10% fermented milk and 10% dextrin. There were no significant differences in weight gain and daily food intake among all groups. At 24 h after exercise, the gastrocnemius muscle was rapidly harvested from each rat, and blood was simultaneously collected from the abdominal aorta for the measurement of serum creatine phosphokinase (CPK).

2.3. GENE chip analysis

Total RNA was extracted from frozen gastrocnemius muscle with an RNeasy mini kit (Qiagen, Hataworth, CA, USA). Preparation of cRNA and target hybridization were performed according to the Affymetrix GeneChip Technical Protocol. In brief, double-stranded cDNA was synthesized from 5 μg of total RNA using a Life Technologies SuperScript Choice system (Life Technologies, Inc., Gaithersburg, MD, USA) and an oligo-(dT)24-anchored T7 primer. Biotinylated RNA was synthesized from double-stranded cDNA by in vitro transcription using 3′ amplification reagents for the IVT Labeling kit (Affymetrix, Santa Clara, CA, USA). Transcription products were purified on a Qiagen RNeasy column (Qiagen). After biotinylation, in vitro transcription products were fragmented for 35 min at 94°C in a buffer composed of 200 mM Tris–acetate (pH 8.1), 500 mM potassium acetate and 150 mM magnesium acetate. Affymetrix GeneChip arrays (Rat Toxicology array; Affymetrix) were hybridized with biotinylated products (0.05 μg/ml/chip) for 16 h at 45°C using the manufacturer's hybridization buffer. After washing the arrays, hybridized RNA was detected by staining with streptavidin–phycoerythrin (6× SSPE, 0.01% Tween-20 pH 7.6, 2 mg/ml acetylated bovine serum albumin and 10 μg/ml streptavidin–phycoerythrin; Molecular Probes). Then DNA chips were scanned using a special confocal scanner (GeneChip Scanner 3000; Affymetrix).

Microarray data were analyzed and clustered using GeneSpring ver. 6.1 software (Silicongenetics, Redwood City, CA, USA). Briefly, array measurements for all samples were normalized using the samples obtained from rested controls. After normalization, detectable expressed genes were defined using P-calls, M-calls or A-calls (according to Affymetrix algorithm) and gene intensity, and then filtered according to two steps: a minimum of one P-call or M-call and a minimum of >100 in four groups. Fold changes (compared to normalized controls) and hierarchical clustering (gene tree) on protection-related proteins were performed based on filtered genes.

2.4. Real-time reverse transcription–polymerase chain reaction (RT-PCR)

First-strand cDNA were synthesized from total RNA (50 ng/μl) using the TaKaRa RNA PCR kit (TaKaRa Bio, Inc., Kusatsu, Shiga, Japan) and 2.5 μM random hexamer primers. Real-time RT-PCR was performed with an ABI Gene Amp 5700 (PE Applied Biosystem, Foster City, CA, USA) using the DNA-binding dye SYBR Green for the detection of PCR products. The following PCR primers were used in the present study: superoxide dismutase 1 (SOD1), forward (5′-GCAAGCGGTGAACCAGTTGT) and reverse (5′-CAGCCCTTGTGTATTGTCCCCA); SOD2, forward (5′-CACAAGCACAGCCTCCCT) and reverse (5′-GCGTTAATGTGCGGCTCC); SOD3, forward (5′-GGTGGCCTTCTTGTTCTGCA) and reverse (5′-GCCGGTCTGCTAAGTCGACA); catalase, forward (5′-TGCAATTCACACCTACGTACAGG) and reverse (CCCGTGCTTTACAGGTTAGCTT); glutathione-*S*-transferase α1 (GSTα1), forward (5′-GAGTTTGATGCCAGCCTTCTG) and reverse (5′-GGCTGCTGATTCTGCTCTTGA); heat shock protein 70 (HSP70), forward (5′-CAAGAATGCGCTCGAGTCCT) and reverse (5′-GCTGATCTTGCCCTTGAGACC); p21-activated kinase 2 (PAK2), forward (5′-ACGGGAGAGTTCACTGGCAT) and reverse (5′-TTGGTAATGTTGGAGGTCTGCA); β-actin, forward (5′-GCCAGGATAGAGCCACCAATC) and reverse (5′-ACTGCCCTGGCTCCTAGCA). The ratio of different signals to β-actin signal was calculated for every sample.

2.5. Thiobarbituric-acid-reactive substances (TBARS) and myeloperoxide (MPO)

The gastrocnemius muscle was harvested and homogenized with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl using a Polytron homogenizer. Then TBARS concentration and MPO activity in muscle

Table 1
Muscle damage parameter

	C	M	RC	RM
CPK (U/L)	158±20.3	131±14.4	566±68.1*	390±36.7*,†
TBARS (mU/mg protein)	0.67±0.03	0.70±0.02	1.22±0.07*	1.07±0.03*,†
CINC-1 (pg/μg protein $\times 10^{(-2)}$)	713±100	621±64	1159±132*	879±49*,†
MCP-1 (pg/μg protein $\times 10^{(-2)}$)	263±43	317±55	485±63*	419±62*

Values are represented as mean±S.E. for 8–12 rats.
* Statistically significant differences from the sedentary group at $P<.05$.
† Statistically significant differences from the normal-diet-fed exercise group (RC) at $P<.05$.

tissues were measured as reported previously [10,12,13]. The TBARS level in muscle homogenate was expressed as nanomoles of malondialdehyde per milligram of protein using 1,1,3,3-tetramethoxypropane as standard. MPO activity was assessed by continuously monitoring the H_2O_2-dependent oxidation of 3,3′ ,5,5′ -tetramethylbenzidine, and 1 U of activity was defined as the amount of MPO that caused a 1.0-min^{-1} change in absorbance at 655 nm (expressed as units per gram of wet tissue).

2.6. Western blot analysis

We homogenized the gastrocnemius muscle in a lysis buffer (CelLytic-MT Lysis/Extraction Reagent; Sigma, St. Louis, MO, USA) and shook it for 60 min at 4°C. We spun the homogenate at 14,000 rpm for 20 min at 4°C and collected the supernatant fluid. Samples of 40-μg proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred from the gel to nitrocellulose membranes. The blots were incubated with primary antibodies against proteins [anti-MnSOD (Upstate, Charlottesville, VA, USA) and anti-β-actin (Sigma)], which were visualized using horseradish-peroxidase-conjugated secondary antibodies (Amersham, Buckinghamshire, UK) and enhanced chemiluminescence (Amersham). Band densities were calculated with NIH Image software (NIH, Research Service Branch) and were normalized by the protein content of β-actin.

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISA for CINC-1 and MCP-1 was performed with a commercially available kit developed by IBL (Gunma, Japan) and BioSource International (Camarillo, CA, USA), respectively, according to the manufacturer's instructions. Absorbance was measured with a microplate reader (MPR-A4I; Tosho, Tokyo, Japan), and the concentrations of CINC-1 and MCP-1 were calculated by comparison with a calibration curve.

2.8. Statistical analysis

Data are represented as mean±S.E. Differences between groups were evaluated by two-way analysis of variance (ANOVA). If ANOVA indicated a significant difference, Fisher's PLSD test was performed to determine the significant difference between mean values. $P<.05$ was considered statistically significant.

3. Results

3.1. Muscle damage parameters

We measured several parameters to assess the extent of muscle damage. Serum CPK increased 24 h after exercise, while the ingestion of fermented milk suppressed such increase (Table 1). TBARS was also significantly increased by exercise, but this increase was significantly suppressed by fermented milk (Table 1). CINC-1, a redox-sensitive chemokine, was significantly increased by exercise, but this increase was significantly ameliorated by the intake of fermented milk (Table 1).

3.2. Infiltration of neutrophils

Neutrophils infiltrated tissues after being attracted by chemokines, and the neutrophil enzyme MPO was measured in gastrocnemius muscle samples as a marker of cell infiltration. As a result, MPO activity was significantly increased after exercise, while the ingestion of fermented milk significantly ameliorated this increase of MPO (Fig. 1).

3.3. Measurement of mRNA and protein

Microarray analysis was employed to compare the expression of 850 genes between the four groups of rats. Four genes were up-regulated and 22 were down-regulated at 24 h after exercise, compared with rested animals that received a normal diet. In addition, 32 genes were up-regulated and 3 were down-regulated by the intake of fermented milk, compared with a normal diet in the animals that performed exercise. Fig. 2 shows a hierarchical clustering display in the mRNA expression profile of proteins with a protective function in relation to exercise or a fermented milk diet. DNA microarray data indicated that fermented milk caused an increase of mRNA for

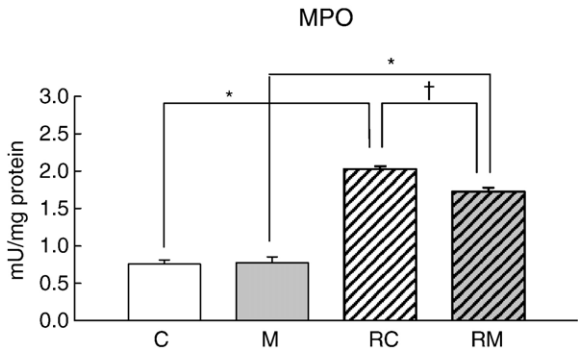

Fig. 1. MPO activity in gastrocnemius muscle samples obtained from eight rats sacrificed at 24 h after exercise. Values are represented as mean±S.E. *Significant difference from the sedentary group: $P<.05$. †Significant difference from the normal-diet group: $P<.05$.

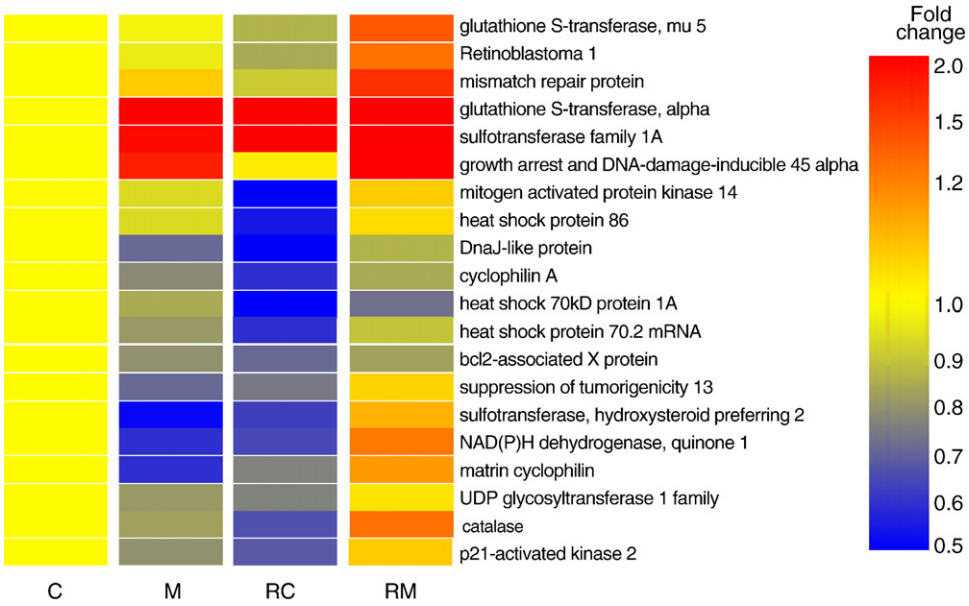

Fig. 2. Hierarchical clustering display of data on protection-related proteins. Clustering display was performed using GeneSpring data analysis software. Each gene is represented by a single row of colored bars. Red indicates the up-regulation of gene expressions, and blue denotes the down-regulation of gene expressions, compared to controls.

protection-related proteins such as antioxidants and chaperone proteins, or inhibited a decrease due to exercise. We further measured the expression of some antioxidant and chaperone proteins by RT-PCR and Western blot analysis. Although SOD1 was not changed by either exercise or fermented milk, SOD2 was increased by the intake of fermented milk plus exercise (Fig. 3). The level of SOD2 protein (MnSOD) after exercise was also significantly increased by the intake of fermented milk (Fig. 4). SOD3 mRNA was decreased by exercise, but ingestion of fermented milk reversed the decrease (Fig. 3). Similar to microarray results, other antioxidants (catalase and GST) were also significantly increased after exercise by fermented milk, compared with a normal diet (Fig. 3). HSP70, a major chaperone protein, was increased by fermented milk during exercise loading (Fig. 3), as shown by DNA microarray analysis. In addition, PAK2, a protein relating apoptosis and the immune system, was elevated by fermented milk plus exercise, but was unchanged by exercise plus normal diet (Fig. 3).

4. Discussion

The present study revealed the following: (a) the intake of fermented milk could ameliorate delayed-onset muscle damage after exercise, and (b) it also increased the

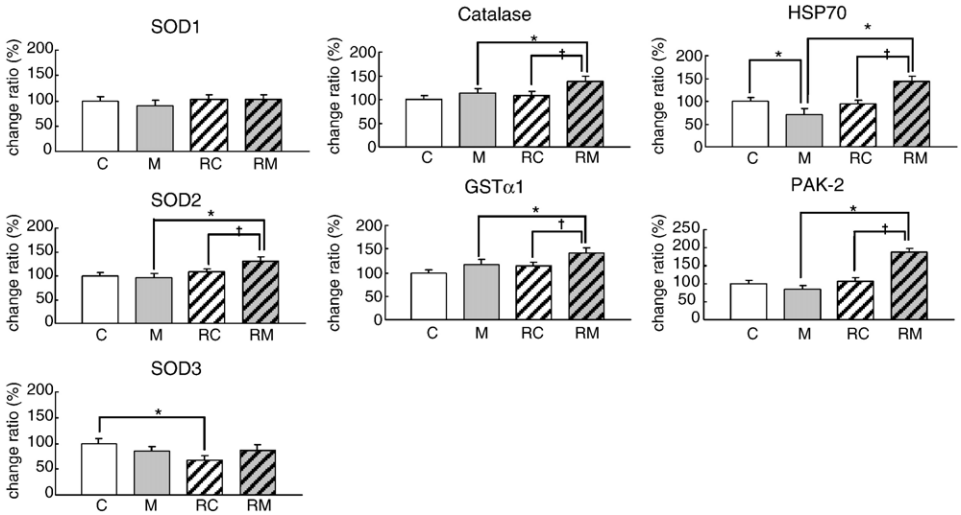

Fig. 3. mRNA levels in gastrocnemius muscle samples obtained from four to six rats sacrificed at 24 h after exercise. Values are represented as mean±S.E. *Significant difference from the sedentary group: $P<.05$. †Significant difference from the normal-diet group: $P<.05$.

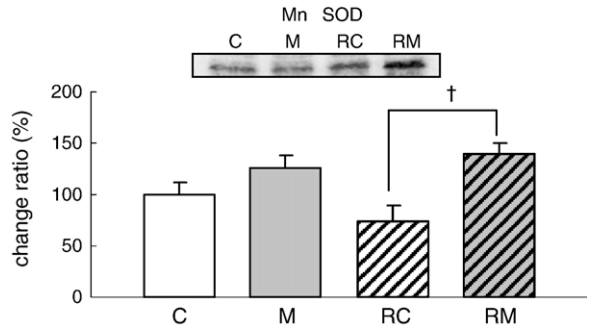

Fig. 4. MnSOD protein content in gastrocnemius muscle samples obtained from four rats sacrificed at 24 h after exercise. Values are represented as mean±S.E. †Significant difference from the normal-diet group: $P<.05$.

antioxidant capacity of skeletal muscles. Muscle damage is induced by exercise via various mechanisms. It has been reported that mechanical stress and disturbance of intracellular calcium homeostasis both increase protease activity and thus cause muscle damage [4–8]. In addition, we recently reported that ROS could indirectly provoke muscle damage via an inflammatory reaction secondary to phagocyte infiltration [10], in contrast to the occurrence of direct oxidative damage. Namely, ROS generated by the mitochondria and endothelium during exercise activate redox-sensitive transcription factors, resulting in elevated expression of inflammatory cytokines, chemokines and adhesion molecules, which leads to the infiltration of phagocytes into muscle tissues. Redox-sensitive inflammation initiated by ROS has also been demonstrated in other tissues by several experimental models [14–16]. In the present study, fermented milk ameliorated the elevation of serum CPK induced by acute exercise, along with a decrease of MPO activity, suggesting that the milk lessened muscle damage by inhibition of neutrophil infiltration. In addition, expression of the chemokine CINC-1, which promotes phagocyte infiltration, was increased by exercise and was reduced again by the intake of fermented milk. CINC-1 expression is mainly regulated by redox-sensitive transcription factors [17–19], although the regulatory mechanism involved has not been fully determined. Thus, the decrease of CINC-1 would suggest less intracellular oxidization in muscle tissues due to elevation of antioxidant activity. These findings indicate that fermented milk can inhibit delayed-onset muscle damage after exercise by diminishing the redox-sensitive inflammatory cascade by ROS scavenging. The finding that the muscle level of TBARS (a marker of lipid peroxidation) was decreased by the fermented milk diet also supports this assumption.

Fermented milk has several beneficial effects [1–3], but the mechanism by which milk exerts these effects is unclear. It is also unknown whether fermented milk has an antioxidant effect. The present study demonstrated that the postexercise expression of antioxidant and chaperone proteins, such as SOD, catalase and HSP70, was increased by a fermented milk diet. These results suggest that fermented milk elevates the expression of endogenous antioxidant enzymes, resulting in an antioxidant effect on skeletal muscles, which would be one of the mechanisms causing a decrease of muscle damage.

Fermented milk is made by fermenting skim milk with a starter culture containing *L. helveticus* and *S. cerevisiae*. During this process, the proteins in skim milk are digested by *Lactobacillus* and converted into small peptides, which, compared to amino acids or large oligopeptides, are more easily absorbed by the intestines. Such peptides may also have several physiological effects and may not merely be used as a source of protein. Nakamura et al. [20,21] found that peptides from fermented milk have an antihypertensive effect via the inhibition of angiotensin I-converting enzyme in spontaneously hypertensive rats. The present study suggested that small digested peptides in fermented milk may contribute to increasing the muscle level of antioxidants. There was no reduction of muscle damage by unfermented milk (data not shown), unlike fermented milk — a finding that would support the above hypothesis. In addition, another possibility that some metabolites generated in the body may affect the increase of antioxidative capacity is also considered. In future studies, we need to detect specific small peptides or its metabolites in the muscles after the intake of fermented milk, and we need to confirm that ingestion of these peptides leads to elevation in antioxidant capacity.

In conclusion, we studied the influence of fermented milk on muscle damage after prolonged exercise. We found that the milk attenuated muscle damage, along with a decrease of redox-sensitive inflammation. The intake of fermented milk also elevated the expression of antioxidant enzymes in skeletal muscles. These results indicate that fermented milk decreases delayed-onset muscle damage after exercise, which is associated with an increase in antioxidant capacity.

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