Eccentric exercise-induced delayed-onset muscle soreness and changes in markers of muscle damage and inflammation

Kazue Kanda[1], Kaoru Sugama[1], Harumi Hayashida[2], Jun Sakuma[3], Yasuo Kawakami[3], Shigeki Miura[4], Hiroshi Yoshioka[4], Yuichi Mori[4], Katsuhiko Suzuki[1,3]

[1] Cooperative Major in Advanced Health Science, Tokyo University of Agriculture and Technology/ Waseda University, Japan.
[2] Institute for Sport Sciences, Waseda University, Japan.
[3] Faculty of Sport Sciences, Waseda University, Japan.
[4] Mebiol Inc., Japan.

ABSTRACT

The purpose of this study was to determine the relationships among delayed-onset muscle soreness (DOMS), muscle damage and inflammatory responses to eccentric exercise and investigate the underlying mechanisms. Nine healthy males performed one-leg calf-raise exercise with their right leg on a force plate. They performed 10 sets of 40 repetitions of exercise at 0.5 Hz by the load corresponding to the half of their body weight, with a rest for 3 min between sets. DOMS was evaluated by a visual analogue scale (VAS). Blood and urine samples were collected before and 2, 4, 24, 48, 72 and 96 h post-exercise. Blood samples were analyzed for leucocyte differential counts and neutrophil functions (migratory activity and oxidative burst activity). We also determined a serum marker of muscle damage, myoglobin (Mb), and plasma and urinary prostaglandin E_2 as an algesic substance. As for the inflammatory mediators, plasma and urine were analyzed for cytokines (interleukin (IL)-1β, IL-1 receptor antagonist, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, tumour necrosis factor-α, interferon-γ, monocyte chemotactic protein-1, granulocyte colony-stimulating factor, macrophage colony-stimulating factor, and granulocyte macrophage colony-stimulating factor), leucocyte activation markers (calprotectin and myeloperoxidase), and neutrophil chemotactic factor complement 5a. All subjects reported muscle soreness on subsequent days and VAS peaked at 72 h after exercise. Serum Mb concentration significantly increased ($p<0.05$) at 72 h after exercise as compared with the pre-exercise values which was correlated with the increases in VAS at 72 h ($r=0.73$, $p<0.05$). Circulating neutrophil count and migratory activity increased significantly ($p<0.01$, and $p<0.05$, respectively) at 4 h after exercise, whereas there

Address for correspondence:
Katsuhiko Suzuki, MD, PhD, Faculty of Sport Sciences, Waseda University,
2-579-15, Mikajima, Tokorozawa, Saitama 359-1192, Japan.
Tel: +81-4-2947-6898, Fax: +81-4-2947-6898, Email: katsu.suzu@waseda.jp

were no significant changes in the other plasma and urinary inflammatory mediators. These results suggest that neutrophils can be mobilized into the circulation and migrate to the muscle tissue several hours after the eccentric exercise. There were also positive correlations between the exercise-induced increases in neutrophil migratory activity at 4 h and the increases in Mb at 48 h ($r=0.67$, $p<0.05$). These findings suggest that neutrophil mobilization and migration after exercise may be involved in the muscle damage and inflammatory processes.

Key words: delayed-onset muscle soreness, eccentric exercise, exercise-induced muscle damage, inflammatory mediators, neutrophils

INTRODUCTION

Unaccustomed exercise with eccentric muscle contraction and exhaustive exercise cause muscle damage, inflammation, leakage of muscle proteins into the circulation and soreness on and several days after, which is called delayed-onset muscle soreness (DOMS) (14, 21, 24, 25). Although a large number of researchers have investigated the effects of exercise on muscle damage and DOMS in humans, there were various results in inflammatory responses (13, 17, 30), which may depend on exercise mode, intensity, and duration, as well as an individual's sex and age.

Exercise disrupts skeletal muscle ultrastructurally, resulting in leucocyte infiltration and release of myocellular proteins such as myoglobin (Mb) into the circulation (3, 24, 28). Systemically, marked neutrophilia with a left shift (6, 23), and enhanced capacity of neutrophils to produce reactive oxygen species (ROS) have been documented after endurance exercise (12, 23). Peripheral leucocyte count has also been shown to increase several hours after eccentric exercise (4). Histological examination of muscle biopsy in humans demonstrates leucocyte accumulation in muscle tissues (10, 29). After muscle and connective tissue damage following exercise, neutrophils are rapidly mobilized into the circulation, and soon migrate and infiltrate into the damaged tissue and produce ROS. If neutrophil functions, especially ROS production are over activated, tissue damage may occur. Within 24 h, neutrophils are replaced by macrophages which are active inflammatory cells that produce several pro-inflammatory cytokines, and promote removal of debris and remodeling of muscle tissue (9, 29). Indeed, several researchers investigated the inflammatory responses to eccentric exercise in humans (15, 19, 32). Peake *et al.* reported that changes in circulating leucocyte count after eccentric exercise are dependent on the muscle groups, or the amount of muscle mass recruited during eccentric exercise (16). Concerning changes in leucocyte receptor expression and oxidative burst activity, there were no clear effects of different types of eccentric exercise, but they might be due to differences in the type of eccentric exercise (16).

Cytokines are proteins which regulate immune and inflammatory responses. They are classified into pro-inflammatory cytokines which promote inflammation, anti-inflammatory cytokines which inhibit inflammation, immunomodulatory cytokines which control inflammation, multifunctional cytokines, chemokines

and colony-stimulating factors. Some of these substances are induced remarkably in plasma and urine following exhaustive endurance exercise (26). Other substances, involved in inflammatory responses, are neutrophil chemotactic factor complement 5a (C5a) and prostaglandin E_2 (PGE_2). Although numerous studies have investigated the effects of eccentric exercise on exercise-induced muscle damage, changes in plasma cytokines and neutrophil activation, there are several contradictory findings, partly due to 1) differences in the mode and intensity of exercise, 2) a limited range of variables measured, and 3) unreliable methods of neutrophil activity measurement.

Migration of neutrophils to the tissue microenvironment is the first step to evoke local inflammation. The production of ROS, not only of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), but also of MPO-dependent HOCl production of neutrophils, can be measured by luminol-dependent chemiluminescence (LmCL) (7, 23, 24, 27). Various neutrophil functions, such as migration and ROS production, could work as a dual-edged sword on both sides of host defence and tissue injury (23, 27). Thus, the balance between beneficial and harmful effects of neutrophil functions should be properly assessed. The use of hydrogel made it possible to mimic the *in vivo* microenvironment of neutrophil infiltration into tissues and LmCL can be detected through the transmissive gel (27). In this method, neutrophil migratory activity and ROS producing activity can be measured without the alteration of neutrophil functions by separating process in the conventional methods (7).

The aims of this study were at first to clarify relationships among muscle soreness, muscle damage markers, circulating leucocyte dynamics and changes in inflammatory mediators in blood and urine of which we tried to detect any possible changes. Especially, we applied a newly-developed measurement system of neutrophil migratory activity and ROS-producing activity by use of *ex vivo* hydrogel methodology with extracellular matrix to the investigation of the mechanisms of muscle damage (27).

METHODS

Subjects

Nine untrained healthy males participated in this study. Their mean (±SD) characteristics were as follows: age (24.8±1.3 yrs), body mass (62.3±6.3 kg), and height (1.72±0.05 m). At the time of the study, the subjects had not been involved in any hard exercise or resistance training for at least two weeks before the exercise bout, and were not taking any supplements, or participating in recovery strategies such as massage, stretching, or cryotherapy. The subjects were instructed to maintain their usual daily schedule during the experiment. The study protocol was approved by the ethics committee of Waseda University, Japan, and the subjects provided their informed consent.

Experimental design

Subjects performed a calf-raise exercise, including repetitive eccentric muscle contractions with their right leg on a force plate. The range of motion of the ankle

joint during the exercise was regulated from -20° (dorsiflexion position) to 15° (planter flexion position) using a goniometer (SG 110/A, Biometrics, Newport, UK) with its ends attached onto the skin over the tibia and calcaneus. They performed 10 sets of 40 repetitions of exercise at 0.5 Hz by the load corresponding to half of their body weight, with a rest for 3 min between sets. DOMS was rated using a visual analogue scale (VAS) that had a 100-mm line with "no pain" on one end and "extremely sore" on the other. Blood and urine samples were collected before and 2, 4, 24, 48, 72 and 96 h after the exercise.

Blood and urine sampling and analyses

Approximately 12 ml of blood samples were drawn by a standard venipuncture technique from the antecubital vein. Blood samples were collected into serum separation tubes and vacutainers containing heparin and EDTA. A portion of whole blood was used to measure haemoglobin, haematocrit and complete blood cell counts using an automatic blood cell counter (PocH100i, Sysmex, Kobe, Japan). The serum separation tubes were left to clot at room temperature for 30 min, and the vacutainers containing EDTA for plasma separation were immediately centrifuged at 1000×G for 10 min. Serum and plasma samples were then removed and stored at –80°C for later analysis. Serum concentrations of creatinine (Cr) and Mb were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Urine samples were centrifuged immediately at 1000×G for 10 min to remove sediments, and the supernatants were stored at –80°C for later analyses. Urinary concentrations of Cr were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the plasma and urine concentrations of interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-6, IL-12p70, tumour necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) (R&D Systems; Minneapolis, MN, USA), IL-2, IL-4, IL-8, IL-10, IL-12p40, interferon-γ (IFN-γ), and C5a (Becton Dickinson Biosciences; San Diego, CA, USA), and calprotectin and myeloperoxidase (HyCult Biotechnology; Uden, the Netherland). ELISA measurements were performed according to the instructions for each ELISA kit using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). Plasma and urine concentrations of PGE_2 (ENZO Life Sciences Inc; Farminglale, NY, USA) were measured using a chemiluminescent microplate reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany).

Preparation of peptide-bound temperature-responsive polymer (G-TRP)

Collagen peptide (24 g; SCP-5000; Nitta Gelatin Co., Osaka, Japan) was dissolved in 96 ml of distilled water at 37°C and followed by reaction with 3.26 g of N-acryloylsuccinimide (Kokusan Kagaku, Tokyo, Japan) for 4 days at 37°C to obtain polymerizable collagen peptide. N-isopropylacrylamide (108.5 g; Kojin, Tokyo, Japan) and n-butylmethacrylate (4.26 g; Wako Chemical, Osaka, Japan) were dissolved in 600 ml of ethanol and then 123 g of the above aqueous solution of polymerizable collagen peptide was added. Under nitrogen atmosphere, 1 ml of N,N,N',N'-tetramethylethylenediamine (Wako Chemical, Osaka, Japan) and 10 ml of 10 wt% ammonium persulfate (Wako Chemical, Osaka, Japan) aqueous

solution were added to the mixed solution, and then reacted for 5 h at 4°C, maintaining the nitrogen atmosphere. After the reaction, 30 l of cold (4°C) distilled water were added and the mixture was concentrated to 3 l using an ultrafiltration membrane (molecular weight cut off 100,000) at 4°C. This dilution and concentration process was repeated 5 times in order to remove impurities and low molecular species. Lyophilization and sterilization of the final concentrated solution gave 105 g of peptide-bound temperature-responsive polymer (G-TRP).

Preparation of scaffold-thermoreversible gelation polymer (S-TGP) gel
Under a clean-air laminar hood workbench, 0.5 g of G-TRP and 0.5 g of TGP (Mebiol gel; Mebiol Inc, Kanagawa, Japan) were dissolved in 16.7 ml of Hank's balanced salt solution (HBSS) at 4ºC for overnight, yielding a viscous transparent S-TGP gel uniform liquid without any bubbles for use in the experiments (27). Mebiol gel is a pure synthesized biocompatible copolymer composed of thermoresponsive polymer blocks and hydrophilic polymer blocks, characterized by its temperature-dependent dynamic viscoelastic properties and used as a biocompatible scaffold for three-dimensional culture without any toxicity (22). S-TGP gel is a peptide-bound thermoreversible gel formed by mixing G-TRP with the Mebiol gel. It liquefies at low temperature, turns to gel immediately upon warming, and returns to liquid state again when cooled.

Neutrophil functions
Peripheral blood samples were drawn from subjects using 2 ml Na-heparin tubes (Venoject II, Terumo Co., Tokyo, Japan). The blood samples were mixed with 2.5 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Aldrich, MO, USA) at a ratio of 1:1. Then, 150 µl luminol-blood samples were layered on 50 µl S-TGP gel prepared in a tube at 37°C, and was promptly measured by LmCL (relative light unit: RLU) using a luminometer (Gene Light 55; Microtec Co., Ltd, Funabashi, Japan). The samples were incubated at 37°C, and the production of ROS from neutrophils was monitored in a kinetic mode for 60 min. After measurement of LmCL at 60 min, luminol-blood samples were removed and the tubes with 50 µl S-TGP gel in which neutrophils migrated were washed three times with PBS warmed at 37°C. Then, the tubes with gel were cooled on ice, and 50 µl Turk solution (Wako, Osaka, Japan) were added and mixed well. The liquid obtained in this way were set on the C-Chip (Disposable haemocytometer, Neubauer improved, DHC-No.1, Digital Bio, Seoul), and the migratory cell number was counted under the microscope. Migrated neutrophil number was calculated by 20 times multiplication of the counted cell number.

Statistical analysis
Data were analyzed using two-way analysis of variance. When significant time effects were evident, multiple comparisons were analyzed with Bonferroni adjustment. Associations between data were analyzed with Pearson's correlation coefficient (r). Statistical significance was set at $p<0.05$, and data were presented as means ± standard deviations (SD).

RESULTS

Delayed-onset muscle soreness and muscle damage markers

Muscle soreness developed on subsequent days after one-leg calf-raise exercise. It increased significantly 48 h and peaked around 72 h after exercise compared with the pre-exercise values ($p<0.01$) (Figure 1). Muscle soreness after exercise

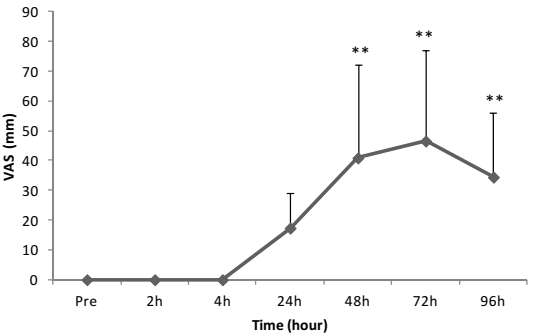

Figure 1. Time course of changes in delayed-onset muscle soreness (DOMS) following the calf-raise exercise as determined by a visual analogue scale (VAS).
Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment. **p<0.01: vs Pre.

decreased at 96 h from 72 h, but remained elevated compared with the pre-exercise values ($p<0.01$) (Figure 1). Concerning blood markers of muscle damage, Mb concentration significantly increased at 72 h after exercise ($p<0.05$) as compared with the pre-exercise values (Figure 2).

Differential leucocyte count

Peripheral leucocyte counts significantly increased at 4 h after exercise, due to the increase in neutrophils ($p<0.01$), and they returned to the pre-exercise values at

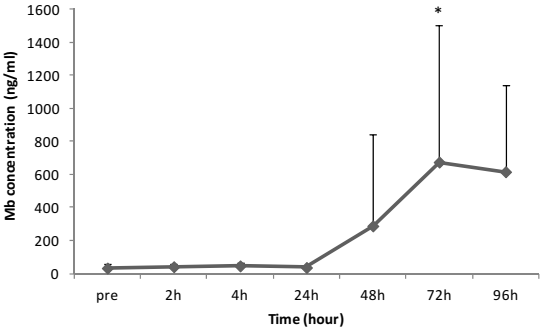

Figure 2. Changes in myoglobin (Mb) concentration following the calf-raise exercise. Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment. *p<0.05: vs Pre.

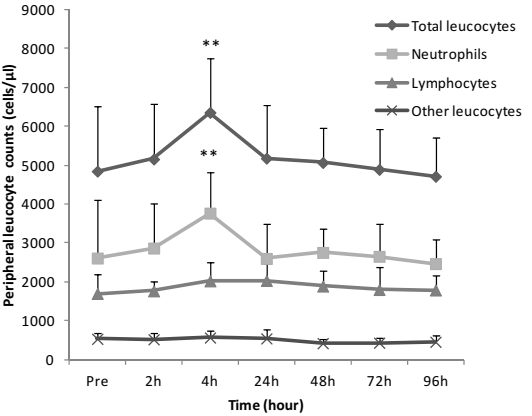

Figure 3. Changes in peripheral leucocyte counts following the calf-raise exercise. Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment. **p<0.01: vs Pre.

24 h after the exercise. Lymphocytes and other leucocytes showed no changes (Figure 3).

Neutrophil functions

Neutrophil migratory activity increased at 4 h after exercise ($p<0.05$) and ROS producing activity showed a trend to significantly increase ($p=0.07$). They both returned to the pre-exercise value at 24 h after exercise (Figure 4).

Inflammatory substances

Plasma and urinary concentrations of inflammatory substances such as pro-inflammatory cytokines, immunomodulatory cytokines, chemokines, anti-inflam-

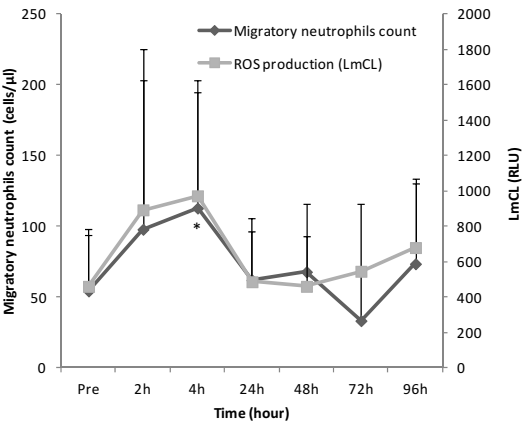

Figure 4. Changes in neutrophil migratory activity and producing activity of reactive oxygen species (ROS) as determined by Luminol-dependent chemiluminescence (LmCL) following the calf-raise exercise. Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment. LmCL p=0.07: vs Pre, migratory neutrophil counts *p<0.05: vs Pre.

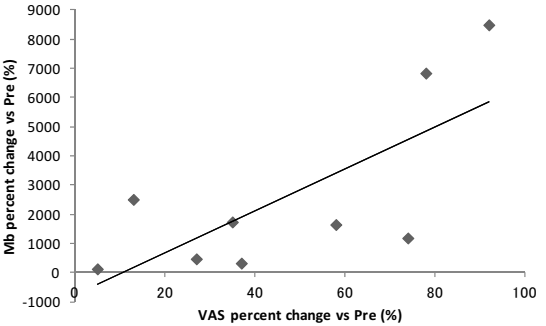

Figure 5. Associations between muscle soreness as determined by visual analogue scale (VAS) at 72 h and value of myoglobin (Mb) at 72 h. Values: percent changes of peak values vs Pre (n=9). Statistics: Pearson's correlation coefficient. VAS (Pre-72 h) vs Mb (Pre-72 h): r=0.73, p<0.05

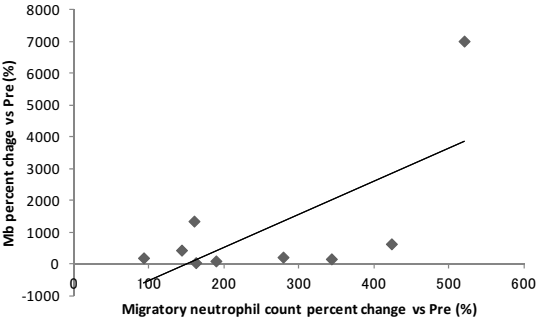

Figure 6. Associations between migratory neutrophil count at 4 h and myoglobin concentration at 48 h. Values: percent changes vs Pre (n=9). Statistics: Pearson's correlation coefficient. Migratory nuetrophil count (Pre-4 h) vs Mb (Pre-48 h) : r=0.67, p<0.05

matory cytokines, colony-stimulating factors, leucocyte activation markers, neutrophil chemotactic factor C5a and algesic substance PGE_2 showed no significant changes (Table 1).

Relationships between muscle soreness and muscle damage markers

We investigated the correlations between the peak VAS value of muscle soreness, and peak value of muscle damage marker, neutrophil dynamics and inflammatory mediators (all percent changes). There was a positive correlation between the percent changes of VAS at 72 h and Mb concentration at 72 h (r=0.73, p<0.05) (Figure 5). There was also a positive correlation between the percent changes of migrated neutrophil count at 4 h and Mb concentration at 48 h (r=0.67, p<0.05) (Figure 6).

Table 1. Changes in plasma and urinary inflammatory substances following the calf-raise exercise

	Unit	Pre	2h	4h	24h	48h	72h	96h
pro-inflammatory cytokines								
IL-1β-P	pg/ml	0.68 ± 0.11	0.57 ± 0.50	0.57 ± 0.39	0.72 ± 0.41	0.61 ± 0.35	0.61 ± 0.38	0.57 ± 0.38
IL-1β-U	pg/min	0.10 ± 0.09	0.58 ± 0.79	0.15 ± 0.12	0.30 ± 0.35	0.13 ± 0.07	0.13 ± 0.12	0.12 ± 0.04
TNF-α-P	pg/ml	0.46 ± 0.23	0.53 ± 0.26	0.64 ± 0.42	0.75 ± 0.35	0.44 ± 0.36	0.53 ± 0.12	0.51 ± 0.20
TNF-α-U	pg/min	0.77 ± 0.84	0.84 ± 1.05	1.33 ± 1.86	0.61 ± 0.69	1.04 ± 1.76	0.47 ± 0.67	0.27 ± 0.28
immunomodulatory cytokines								
IL-2-P	pg/ml	0.93 ± 0.17	0.83 ± 0.08	0.82 ± 0.07	0.78 ± 0.05	0.90 ± 0.30	0.84 ± 0.20	0.88 ± 0.20
IL-2-U	pg/min	0.79 ± 0.84	0.74 ± 0.43	0.40 ± 0.19	0.51 ± 0.18	0.67 ± 0.48	0.54 ± 0.57	0.30 ± 0.08
IL-12p70-P	pg/ml	0.59 ± 0.32	0.28 ± 0.27	0.59 ± 0.35	0.72 ± 0.45	0.80 ± 0.41	0.50 ± 0.23	0.55 ± 0.39
IL-12p70-U	pg/min	0.33 ± 0.24	0.34 ± 0.21	0.22 ± 0.09	0.31 ± 0.14	0.33 ± 0.23	0.56 ± 0.76	0.15 ± 0.07
IFN-γ-P	pg/ml	0.59 ± 0.16	0.60 ± 0.30	0.65 ± 0.32	0.63 ± 0.16	0.52 ± 0.16	0.54 ± 0.12	0.58 ± 0.17
IFN-γ-U	pg/min	0.79 ± 1.01	0.81 ± 0.65	0.30 ± 0.09	0.45 ± 0.16	0.42 ± 0.21	0.14 ± 0.08	0.23 ± 0.07
multifunctional cytokines								
IL-6-P	pg/ml	0.27 ± 0.16	0.38 ± 0.19	0.29 ± 0.18	0.22 ± 0.12	0.26 ± 0.12	0.30 ± 0.11	0.39 ± 0.23
IL-6-U	pg/min	0.14 ± 0.13	0.32 ± 0.15	0.15 ± 0.11	0.18 ± 0.10	0.16 ± 0.08	0.16 ± 0.14	0.29 ± 0.35
anti-inflammatory cytokines								
IL-1ra-P	pg/ml	35.6 ± 5.2	33.1 ± 5.5	34.4 ± 4.8	33.1 ± 8.4	29.3 ± 5.2	29.6 ± 2.6	32.3 ± 5.2
IL-1ra-U	pg/min	231.8 ± 173.2	401.2 ± 233.7	327.5 ± 230.7	837.6 ± 97.1	354.9 ± 192.6	204.0 ± 20.2	278.2 ± 155.0
IL-4-P	pg/ml	0.66 ± 0.35	0.70 ± 0.49	0.88 ± 0.56	1.00 ± 0.98	0.78 ± 0.66	0.76 ± 0.50	0.59 ± 0.31
IL-4-U	pg/min	0.60 ± 0.46	1.12 ± 0.72	0.95 ± 0.60	0.84 ± 0.31	0.65 ± 0.32	0.70 ± 0.69	0.77 ± 0.40
IL-10-P	pg/ml	0.71 ± 0.45	0.75 ± 0.52	0.50 ± 0.29	0.45 ± 0.21	0.57 ± 0.23	0.47 ± 0.31	0.66 ± 0.68
IL-10-U	pg/min	0.40 ± 0.44	0.56 ± 0.55	0.48 ± 0.50	0.56 ± 0.40	0.84 ± 1.11	0.46 ± 0.58	0.44 ± 0.42
IL-12p40-P	pg/ml	10.7 ± 17.8	13.2 ± 24.4	13.2 ± 24.7	10.7 ± 18.1	8.8 ± 13.7	7.7 ± 11.6	8.5 ± 13.2
IL-12p40-U	pg/min	0.81 ± 0.91	0.82 ± 0.64	0.56 ± 0.51	0.76 ± 0.83	1.06 ± 1.19	0.45 ± 0.48	0.33 ± 0.21
chemokines								
IL-8-P	pg/ml	4.5 ± 2.7	4.2 ± 2.4	4.4 ± 1.5	3.7 ± 0.9	3.7 ± 0.9	5.3 ± 4.3	4.3 ± 1.6
IL-8-U	pg/min	0.59 ± 0.42	1.20 ± 0.54	0.76 ± 0.43	1.3 ± 1.5	0.87 ± 0.69	0.65 ± 0.45	0.95 ± 0.80
MCP-1-P	pg/ml	95.4 ± 19.0	90.5 ± 13.8	94.3 ± 19.4	101.5 ± 19.1	89.8 ± 13.1	102.3 ± 13.2	96.8 ± 10.6
MCP-1-U	pg/min	87.5 ± 82.6	258.5 ± 197.9	199.4 ± 122.4	235.3 ± 146.3	189.8 ± 146.2	76.1 ± 29.2	128.3 ± 47.3
colony-stimulating factors								
G-CSF-P	pg/ml	1.8 ± 0.9	2.2 ± 1.4	2.0 ± 1.4	1.9 ± 1.0	2.3 ± 1.7	1.9 ± 1.3	2.0 ± 1.4
G-CSF-U	pg/min	0.19 ± 0.12	0.24 ± 0.13	0.27 ± 0.17	0.27 ± 0.17	0.48 ± 0.54	0.24 ± 0.24	0.16 ± 0.05
M-CSF-P	pg/ml	19.3 ± 2.5	23.1 ± 7.8	22.6 ± 5.4	21.8 ± 7.6	22.5 ± 6.3	17.0 ± 6.2	20.1 ± 3.0
M-CSF-U	pg/min	25.7 ± 23.1	130.8 ± 108.9	121.8 ± 84.9	281.4 ± 310.7	230.6 ± 298.1	40.4 ± 29.1	218.6 ± 280.9
GM-CSF-P	pg/ml	0.27 ± 0.10	0.21 ± 0.37	0.20 ± 0.06	0.28 ± 0.13	0.24 ± 0.09	0.17 ± 0.02	0.22 ± 0.09
GM-CSF-U	pg/min	0.41 ± 0.40	0.66 ± 0.61	0.54 ± 0.38	0.42 ± 0.29	0.69 ± 0.48	0.48 ± 0.50	0.41 ± 0.29
leucocyte activation markers								
calprotectin-P	ng/ml	15.7 ± 12.9	9.9 ± 6.8	10.5 ± 9.7	9.2 ± 7.0	11.7 ± 8.0	12.1 ± 6.3	12.3 ± 8.9
calprotectin-U	ng/min	0.15 ± 0.13	0.33 ± 0.19	0.27 ± 0.23	0.57 ± 0.68	0.45 ± 0.42	0.24 ± 0.23	0.20 ± 0.14
MPO-P	ng/ml	12.7 ± 7.8	12.6 ± 6.9	15.2 ± 9.8	13.2 ± 10.0	13.7 ± 8.1	12.2 ± 7.7	12.2 ± 5.9
MPO-U	ng/min	0.06 ± 0.05	0.10 ± 0.08	0.08 ± 0.06	0.10 ± 0.09	0.12 ± 0.13	0.08 ± 0.08	0.08 ± 0.07
neutrophil chemotactic factors								
C5a-P	ng/ml	3.8 ± 4.3	3.6 ± 3.6	3.2 ± 3.2	3.3 ± 3.5	3.4 ± 3.8	3.5 ± 4.0	3.5 ± 3.8
C5a-U	ng/min	0.03 ± 0.01	0.06 ± 0.02	0.09 ± 0.11	0.09 ± 0.11	0.08 ± 0.10	0.04 ± 0.02	0.06 ± 0.03
algesic substances								
PGE_2-P	pg/ml	64.1 ± 11.7	69.7 ± 12.6	79.0 ± 15.8	60.4 ± 18.0	58.9 ± 24.4	59.9 ± 7.7	58.9 ± 16.9
PGE_2-U	pg/min	1075 ± 886.4	1271 ± 732.9	1372 ± 1044	1354 ± 906.9	991.0 ± 643.2	758.3 ± 630.8	777.1 ± 535.1

Values: means±SD. Statistics: Two-way ANOVA was not significant.
P: Plasma
U: Urine. Data are the gross amount in the volume of urinary excretion per one minute.
Abbreviations: IL: interleukin, TNF: tumour necrosis factor, IFN: interferon, IL-1ra: IL- 1 receptor antagonist, MPO: myeloperoxidase, MCP-1: monocyte chemotactic protein-1, G-CSF: granulocyte colony-stimulating factor, GM-CSF: granulocyte macrophage colony-stimulating factor, M-CSF: macrophage colony-stimulating factor, PGE_2: prostaglandin E_2.

DISCUSSION

The aims of this study were at first to investigate the associations among DOMS caused by calf-raise exercise, muscle damage marker in peripheral blood, and inflammatory mediators. Muscle soreness appeared at 24 h after exercise and subsequently developed from 48 h to 72 h ($p<0.01$), and decreased at 96 h though still high above compared with pre-exercise ($p<0.01$). Generally, DOMS appears from several hours or one day after exercise and peaks at two or three days after exercise (1, 14, 16). This study confirmed that calf-raise exercise with eccentric contractions caused DOMS. As a marker of muscle damage, serum Mb concentration significantly increased at 72 h after exercise compared with pre-exercise. Numerous studies have reported on muscle damage and muscle soreness, however, they did not always occur simultaneously according to the degree of muscle damage and muscle soreness (4, 5, 13, 14). In this study, we observed the similar time course and Mb was closely correlated with muscle soreness at 72 h after exercise ($r=0.73$, $p<0.05$). These results confirm that the present calf-raise exercise caused sufficient muscle damage for the purpose of the present study.

Several studies report that circulating leucocyte and neutrophil count increase within several hours after eccentric exercise, but that is dependent on the intensity, duration, and type of exercise and muscle mass (16, 20). Saxton *et al.* reported that circulating neutrophil count increased 1.48 fold (Pre:2.9±0.4, 4h:4.3±0.5) at 4 h after low systemic stress (repeated eccentric muscle action) and caused 1.76 fold (Pre:2.9±0.3, 4h:5.1±0.4) increase in neutrophils at 4 h after high systemic stress (bench-stepping) (20). Gleeson *et al.* reported the circulating leucocyte number decreased two and three days after a bench-stepping exercise, although it increased immediately after and still more increased at 1 to 4 h (5). We demonstrated that total leucocyte count increased by 31 % ($p<0.01$) and circulating neutrophil count by 44 % ($p<0.05$) above pre-exercise level, respectively. Neutrophilia returned to pre-exercise values at 24 h after exercise. Neutrophil migratory activity was also significantly increased ($p<0.05$) at 4 h after exercise. There have been several studies that investigated the neutrophil migration into the muscle after the muscle-damaging exercise (0-6 h) (2, 3, 8, 9, 11, 33), but only Fielding *et al.* demonstrated neutrophil accumulation in the muscle (3). This could be partly attributable to the fact that biopsy samples can depict quite limited parts of the muscle tissue. Paulsen *et al.* investigated leucocyte accumulation into the exercised-muscle using radiolabeled leucocytes by scintigraphy (15). Although they measured high radioactivity in the exercised-muscle, large individual differences were observed. We demonstrated a positive correlation between the increase in neutrophil migratory activity at 4 h and the increase in Mb concentration at 48 h ($r=0.67$, $p<0.05$). This result shows the possibility that neutrophil is involved in the muscle damage and the inflammatory processes.

ROS production from neutrophils migrated into the hydrogel likened to muscle tissue was assessed with LmCL. There was a trend for this to be increased ($p=0.07$) at 4 h after exercise. Pizza *et al.* reported that neutrophil O_2^- production was increased ($p<0.05$) at 4 h following one-arm eccentric exercise (18). Suzuki

et al. also observed increased neutrophil count with a left shift, enhanced spontaneous mobility and ROS production after 90-min bicycling, suggesting that mobilized neutrophils from the bone marrow reserve have higher activity (23). They also reported that the increases in Mb values at post 3 h were correlated closely with the raise in neutrophil count at post and LmCL response at post and 1 h post 90-min bicycling, suggesting that neutrophil mobilization and activation might affect muscle damage (24). The result in this study was similar, but we could demonstrate the enhanced neutrophil migration by use of newly-developed *ex vivo* methodology in imitation of tissue damage.

Although DOMS, muscle damage marker, circulating neutrophil counts and their functions changed significantly following the eccentric exercise, plasma and urinary inflammatory mediators which we investigated were not changed significantly. Uchida *et al.* reported increasing serum PGE_2 following bench press exercise despite no changes in plasma IL-1β, IL-6 or TNF-α (30). Although we also investigated plasma and urinary PGE_2 concentrations, they did not change. Serum PGE_2 may be produced by leucocytes during the time left to removal of the fibrin clot and blood cells where neutrophilia occurs after exercise that might affect the higher values. These results demonstrated that the one-leg calf-raise exercise caused local inflammation and that neutrophil mobilization and migration were the most affected variables among the wide range of tested inflammatory mediators. However, further research is needed to clarify the mediators that mobilize neutrophils into the circulation and substances which are produced by migrated neutrophils and promote inflammation.

In conclusion, one-leg calf-raise exercise caused DOMS, muscle damage, increases in circulating neutrophil number and migratory activity without changes in the other inflammatory mediators. Neutrophils may be involved in the early stage of muscle damage and the inflammatory processes after eccentric exercise.

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