Evaluation of serum leaking enzymes and investigation into new biomarkers for exercise-induced muscle damage

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ABSTRACT

This investigation determined whether existing muscle damage markers and organ damage markers respond to an acute eccentric exercise protocol and are associated with affected muscle symptoms. Nine healthy-young men completed 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 with a load corresponding to half of their body weight, with 3 min rest between sets. The tenderness of medial gastrocnemius, lateral gastrocnemius and soleus, and the ankle active range of motion (ROM) were assessed before, immediately after, 24 h and 48 h, 72 h, 96 h and 168 h after exercise. Blood and urine were collected pre-exercise and 2 h, 4 h, 24 h, 48 h, 72 h and 96 h post-exercise. Serum was analyzed for creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and aldolase (ALD) activities. We also determined heart-type fatty acid-binding protein (H-FABP), intestinal-type fatty acid-binding pro-

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tein (I-FABP) and liver-type fatty acid-binding protein (L-FABP), neutrophil gelatinase-associated lipocalin (NGAL), interleukin (IL)-17A, IL-23, nerve growth factor (NGF), soluble-Endothelial (sE)-selectin, s-Leukocyte (L)-selectin, s-Platelets (P)-selectin, and 8-isoprostane in plasma and urine. The tenderness of proximal and middle gastrocnemius increased significantly 72 h ($p<0.05$, $p<0.01$) after exercise. Ankle active ROM in dorsal flexion decreased significantly 48 h ($p<0.05$) and 72 h ($p<0.01$) after exercise. CK and ALD activities significantly increased at 72 h ($p<0.05$) and remained elevated at 96 h ($p<0.01$) post-exercise compared to pre-exercise values. Also, ALD which showed relatively lower interindividual variability was significantly correlated with tenderness of middle gastrocnemius at 72 h. LDH activity significantly increased 96 h post-exercise ($p<0.01$), whereas the increase in AST and ALT activities 96 h post-exercise was not significantly different from pre-exercise values. There were no significant changes in FABPs, NGAL, IL-17A, IL-23, NGF, selectins and 8-isoprostanes in plasma and urine. In conclusion, calf-raise exercise induced severe local muscle damage symptoms which were accompanied by increases in both serum CK and ALD activities, but we could not detect any changes in examined markers of organ damage, inflammation and oxidative stress. Further research is needed to determine other more sensitive biomarkers and the underlying mechanisms of exercise-induced muscle damage.

Key words: exercise-induced muscle damage, organ damage markers, acute eccentric exercise, creatine kinase, aldolase

INTRODUCTION

Exercise-induced muscle damage has been one of the most important targets in sport science research. Direct assessment of muscle damage involves histological examination of muscle tissue via biopsy. However, there is difficulty gathering tissue samples with biopsy technique and demonstrating disruption of muscle cells (1, 3, 48), in combination with the process being an invasive experience for research participants. Therefore, the less invasive measures for the accurate assessment of exercise-induced muscle damage are needed. As indirect indicator of muscle damage, delayed-onset muscle soreness (DOMS) is a poor reflector of eccentric exercise-induced muscle damage, and changes in other indicators of muscle damage are not necessarily accompanied by DOMS (27). On the other hand, the changes of range of motion (ROM) have been used as an indirect variable of DOMS (8, 9). Although a large number of studies have reported the effects of exercise on muscle damage, DOMS and inflammatory responses in humans (26, 30, 31, 34, 41, 44, 46), the mechanisms of exercise-induced muscle damage are not fully understood at present. DOMS is characterized by tenderness and movement-related pain, that is, mechanical hyperalgesia in the exercised muscle (22), and ROM might be affected by muscle soreness (33). Therefore, it is necessary to assess the muscle symptoms in a multifaceted manner, and investigate the associations with other muscle damage markers carefully.

Serum enzyme activities such as creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase

(LDH), aldolase (ALD) and myoglobin which leak into circulation from damaged muscle have been used as indirect markers of exercised-induced muscle damage (5, 28). Serum levels of these damage indicators depend on gender, muscle mass, exercise intensity and duration in addition to the individual training state, and there is a remarkable interindividual variability in the degree to which serum enzyme activities increase with exercise (30). Instead of the existing leaking enzymes, fatty acid-binding protein (FABP) and neutrophil gelatinase-associated lipocalin (NGAL) have been introduced as organ damage markers. FABPs are present in almost all tissues, and were named after the tissue in which they were discovered or are prominently expressed: L-FABP, liver-type fatty acid-binding protein; I-FABP, intestinal-type fatty acid-binding protein; H-FABP, heart-type fatty acid-binding protein (50). H-FABP is mainly expressed in the heart, but to a lesser extent also in the skeletal muscle (47). NGAL was identified as a 25 kDa protein secreted by neutrophils (45), and is expressed in human tissues, including kidneys, lungs, stomach, and colon (10). NGAL is focused in recent years as a biomarker in several benign and malignant diseases, especially as biomarker in acute kidney injuries (11), and may have the potential to protect against cellular injury mediated by reactive oxygen species (ROS) (36, 37). Although we have observed that neutrophils produce ROS following exercise (14, 42-44), NGAL might become a good alternative variable which is easier to measure simply in plasma and urine by conventional enzyme-linked immunosorbent assays (ELISA) than the existing neutrophil functional analyses that contain rather complex procedures and need to be determined *ex vivo* soon after blood sampling.

Exercise-induced muscle damage causes local inflammation which degenerates and regenerates muscle and surrounding connective tissue (30). Briefly, neutrophils are mobilized into the circulation after exercise, and soon infiltrate into the damaged tissue (2). Neutrophils are primed by the chemoattractants such as complement 5a (C5a) and interleukin (IL)-8 from the exercise-induced damaged muscle, causing rolling, arrest and transmigration which is mediated by intercellular adhesion molecules, selectins (16), initially. Neutrophils are present in muscle within a day after exercise (3, 17, 18, 19, 34, 40), and after neutrophils' infiltration, macrophages are replaced and present in muscle from 1 to 14 days after exercise (2, 3, 12, 13, 19, 32, 38). Neutrophils and macrophages produce ROS to degrade the damaged muscle tissue (23, 24), and may produce pro-inflammatory cytokines (6). The pro-inflammatory cytokines such as IL-1β, tumor necrosis factor (TNF)-α, and IL-6 are expressed in skeletal muscle following eccentric exercise (30), but we could not detect any changes of these cytokines in plasma and urine in our previous study (14). On the other hand, Sugama et al. reported that IL-17 induced by IL-6 and activated by IL-23 might promote neutrophil activation and muscle damage in a different way from the classical pro-inflammatory cytokines IL-1β and TNF-α following prolonged endurance exercise (41). Furthermore, nerve growth factor (NGF) which is involved in pathological pain conditions (15, 49) increased in the muscle after lengthening contraction in rats (22). 8-isoprostane is a prototypical biomarker of oxidative stress by exercise-induced free radical production (21). Fluctuations of these substances may be detected following exercise-induced muscle damage and inflammation, and can be one of the underlying mechanisms of it.

We have shown in a previous study that increased myoglobin concentration at 72 h after exercise was correlated with the appearance of DOMS at 72 h ($r=0.73$, $p<0.05$), and there was a positive correlation between exercise-induced increases in neutrophil migratory activity at 4 h and increases in myoglobin at 48 h ($r=0.67$, $p<0.05$) following one-leg calf-raise exercise (14), suggesting that neutrophils may be involved in muscle damage and that myoglobin is a very sensitive muscle damage marker. Based on these findings, the aims of the present study were to examine 1) changes in serum leaking enzyme activities and their correlation with DOMS, 2) concentrations of novel organ damage markers, novel pro-inflammatory cytokines and an oxidative stress marker and their relationship to muscle damage, and 3) urinary excretion rates of these markers as potential surrogate non-invasive indicators of muscle damage, using a local muscle damage model of one-leg calf-raise exercise.

METHODS

Subjects

Nine untrained healthy males (age 24.8±1.3 (mean±SD) yrs, body mass 62.3±6.3 kg and height 1.72±0.05 m) volunteered for this study. 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. All subjects completed a medical questionnaire and gave written informed consent. The experimental procedure was approved by the ethics committee of Waseda University.

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° (plantar 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 with a load corresponding to the half of their body weight, with 3 min rest between sets. The tenderness of the exercised muscle correlative to DOMS was assessed using the FP meter (SN-402, Navis, Japan) at 1 kg and rated subjectively using a visual analogue scale (VAS) that has a 100-mm line with “no pain” on one end and “extremely sore” on the other. The points of measurements were the proximal, the middle and the distal points of medial gastrocnemius (MG) and lateral gastrocnemius (LG), the middle points between MG and LG, and the middle and the distal points of soleus (SOL). The ankle active ROM was assessed using the goniometer. The tenderness of the exercised muscle and the ankle active ROM were assessed before, immediately after 24 h and 48 h, 72 h, 96 h and 168 h after and exercise. The blood and urine samples were collected before and 2 h, 4 h, 24 h and 48 h, 72 h and 96 h after the exercise. Participants were supposed to urinate 2 h before each sampling, and the urine samples were collected in measuring cylinders.

Blood and urine sampling and analyses

Approximately 12 ml of blood was drawn by 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 analyses. Serum CK, AST, ALT, LDH and ALD activities 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 concentration of creatinine was 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 H-FABP, I-FABP, L-FABP and NGAL (Human H-FABP, I-FABP, L-FABP and NGAL ELISA kit, Hycult biotechnology, Uden, The Netherlands), IL-17A (Human IL-17A ELISA kit, Gen-Probe Diaclone SAS, Besancon, France), IL-23 (Human IL-23, R&D Systems, Inc. MN, USA), nerve growth factor (NGF) (Human Nerve Growth Factor ELISA kit, Cusabio Biotech Co. Ltd., Wuhan, China), E-selectin (Soluble), sL-selectin and sP-selectin (Life technologies Co. CA, USA) and 8-isoprostane (Detroit R&D, Inc., MI, USA). The measurements were performed according to the instructions for each ELISA kit using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). The urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Statistical analysis

Data are presented as mean ± SD. Statistical validation was made using Friedman's test. When significant time effects were evident, multiple comparisons were analyzed with Scheffe test. Associations between data were analyzed with Pearson's correlation coefficient (r). Statistical significance was accepted at $p<0.05$.

RESULTS

Changes in the tenderness of the exercised muscle, and the ankle joint active range of motion (ROM)

As shown in Table 1, tenderness of the exercised muscle developed on subsequent days after calf-raise exercise and was principally sensed on the MG. It increased significantly 72 h (proximal MG; $p<0.05$, middle MG; $p<0.01$) after exercise compared with the pre-exercise values. The ankle joint active ROM in the dorsal flexion decreased significantly at 48 h ($p<0.05$) and 72 h ($p<0.01$). The lowest ROM was shown at 72 h after exercise in dorsal flexion, whereas there was no significant difference in plantar flexion compared with the pre-exercise values.

Changes in the muscle and the organ damage markers
As shown in Table 2, CK and ALD activities significantly increased at 72 h after exercise ($p<0.05$), thereafter, they remained elevated for 96 h after exercise ($p<0.01$). LDH activity significantly increased at 96 h after exercise ($p<0.01$). However, AST and ALT activities showed no significant difference when compared with pre-exercise values. As listed in Table 3, plasma concentrations of H-FABP, I-FABP and L-FABP were below the detectable levels of the assays and urinary concentrations exhibited no significant change. Plasma and urinary NGAL were not significantly different compared with pre-exercise values.

Changes in the pro-inflammatory cytokines and other indicators
As shown in Table 4, plasma concentration of IL-17A was not significantly changed and urinary excretion rate of IL-17A was below the detectable level. Plasma concentration of IL-23 was below the detectable level, whereas urinary excretion rate of IL-23 showed no significant change. Plasma sE-selectin concentration was below the detectable level, and sL-selectin and sP-selectin concentrations were not significantly different. Plasma concentration of 8-isoprostane was below the detectable level, and urinary excretion rate of 8-isoprostane and plasma concentration of NGF did not change significantly.

Relationships between enzyme activities and the tenderness of exercised muscle and the ankle active range of motions (ROM)
As shown in Table 5, there were no significant correlations between the tenderness of proximal MG 72 h and CK, LDH and ALD for the percent changes of the peak values. The tenderness of middle MG 72 h was significantly correlated with ALD 72 h ($r=0.78$, $p<0.05$). The dorsal flexion of ankle active ROM was not significantly correlated to the tenderness of MG or to CK, LDH and ALD.

DISCUSSION

Muscle-derived leaking enzymes such as CK, AST, ALT, LDH and ALD have been used in many studies as indirect markers of exercise-induced muscle damage. However, there are generally poor correlations between DOMS and muscle damage indicators (27). In this study, CK and ALD were significantly increased earlier than LDH. Also, ALD showed relatively lower interindividual variations and was significantly correlated with tenderness of middle MG at 72 h, whereas there were only moderate, non-significant correlations between tenderness and the other leaking enzyme activities. These results suggest that ALD rather than CK and LDH might be a more accurate and objective muscle damage indicator.

There are a few studies that have investigated the organ damage markers, FABPs and NGAL following exercise-induced muscle damage. FABPs are present in almost all tissues (50), but H-FABP is present in skeletal muscle (47). Sorichter et al. reported that plasma H-FABP and myoglobin increased earlier (30 min) than CK (2 h) following 20 min of downhill running (39). These results suggest that plasma H-FABP concentrations reflect exercise-induced muscle damage earlier, but plasma H-FABP seems to have no advantage in view of its similarity of changes in the appearance of myoglobin. Also, the change might be attributed

Table 1. Changes in the tenderness of exercised muscle and active range of motion (ROM).

	unit	Pre	0 h	24 h	48 h	72 h	96 h	168 h
medial gastrocnemius								
proximal	mm	16 ± 9	21 ± 10	35 ± 20	40 ± 31	54 ± 24*	36 ± 18	20 ± 15
middle	mm	19 ± 10	23 ± 12	34 ± 18	48 ± 24	64 ± 22**	49 ± 18	23 ± 14
distal	mm	20 ± 11	19 ± 14	30 ± 14	45 ± 23	49 ± 31	40 ± 19	19 ± 12
medial/lateral gastrocnemius								
proximal	mm	15 ± 15	14 ± 12	26 ± 16	24 ± 18	25 ± 23	19 ± 15	11 ± 14
middle	mm	16 ± 13	15 ± 12	18 ± 10	25 ± 24	26 ± 28	16 ± 15	11 ± 11
distal	mm	15 ± 9	16 ± 11	23 ± 15	21 ± 15	24 ± 18	18 ± 15	15 ± 14
lateral gastrocnemius								
proximal	mm	11 ± 12	8 ± 10	15 ± 13	14 ± 11	26 ± 24	18 ± 15	8 ± 12
middle	mm	14 ± 12	15 ± 12	18 ± 13	26 ± 18	39 ± 29	26 ± 19	10 ± 13
distal	mm	13 ± 10	10 ± 11	15 ± 12	19 ± 16	21 ± 17	16 ± 14	6 ± 11
soleus								
middle	mm	21 ± 14	18 ± 13	24 ± 15	18 ± 10	21 ± 14	14 ± 12	14 ± 15
distal	mm	7 ± 8	10 ± 10	11 ± 11	7 ± 11	9 ± 12	10 ± 14	6 ± 8
ROM of ankle joint								
plantar flexion	degree of an angle	33 ± 4	34 ± 4	32 ± 3	32 ± 10	32 ± 5	32 ± 6	33 ± 4
dorsal flexion	degree of an angle	-25 ± 5	-20 ± 5	-20 ± 5	-15 ± 4*	-12 ± 7**	-16 ± 5	-23 ± 7

Data are presented as means ± SD (n=9). Statistics: * $p<0.05$, ** $p<0.01$, significantly different from each Pre values. before exercise (Pre), immediately post-exercise (0 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), 168 hour post-exercise (168 h), range of motion (ROM).

Table 2. Changes in the muscle damage markers following the calf-raise exercise.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
CK	U/l	131.7 ± 94.0	133.0 ± 84.2	139.3 ± 81.4	172.6 ± 150.7	766.1 ± 1474.0	3245 ± 4648*	6069 ± 6498**
AST	U/l	19.4 ± 5.9	18.4 ± 5.1	18.6 ± 4.8	19.2 ± 7.0	29.3 ± 26.2	75.9 ± 88.1	126.2 ± 130.2
ALT	U/l	16.8 ± 6.0	16.3 ± 5.9	15.8 ± 5.0	16.0 ± 6.3	17.7 ± 7.1	27.8 ± 19.6	40.0 ± 29.4
LDH	U/l	139.8 ± 26.9	137.8 ± 24.7	138.1 ± 21.9	137.9 ± 26.9	146.3 ± 24.7	187.8 ± 79.9	241.7 ± 103.8**
ALD	U/l	3.7 ± 0.7	3.7 ± 0.1	3.8 ± 0.6	3.8 ± 0.6	6.2 ± 6.1	21.0 ± 26.7*	47.2 ± 44.7**

Data are presented as means ± SD (n=9). Statistics: * $p < 0.05$, ** $p < 0.01$, significantly different from each Pre values. before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aldolase (ALD).

Table 3. Changes in plasma and urinary novel markers of organ damage.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
H-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
H-FABP-U	pg/min	1242 ± 970	1156 ± 1114	1106 ± 1011	3261 ± 4243	2559 ± 3815	3857 ± 4835	3072 ± 2523
I-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
I-FABP-U	pg/min	45.8 ± 47.8	53.7 ± 35.7	87.9 ± 95.2	54.8 ± 48.2	81.9 ± 83.0	37.8 ± 31.8	40.5 ± 19.2
L-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
L-FABP-U	pg/min	3988 ± 3280	5323 ± 5031	4378 ± 2609	6846 ± 6631	5847 ± 6369	5539 ± 5480	6727 ± 3595
NGAL-P	ng/ml	13.2 ± 5.0	14.3 ± 7.7	13.8 ± 8.2	14.2 ± 9.6	13.6 ± 7.0	17.1 ± 12.9	15.5 ± 11.2
NGAL-U	ng/min	0.36 ± 0.51	0.76 ± 1.13	0.35 ± 0.20	0.39 ± 0.38	0.52 ± 0.55	0.35 ± 0.33	0.35 ± 0.29

Data are presented as means ± SD (n=9). before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), not detected below the detection limits (ND), plasma (P), urine (U), heart (H), Intestine (I), liver (L), fatty acid-binding protein (FABP), neutrophil gelatinase-associated lipocalin (NGAL).

Urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Table 4. Changes in plasma and urinary inflammatory biomarkers.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
IL-17A-P	pg/ml	31.1 ± 36.7	29.9 ± 35.1	29.1 ± 33.4	27.8 ± 32.7	30.9 ± 31.8	33.3 ± 33.5	30.1 ± 29.9
IL-17A-U	pg/min	ND	ND	ND	ND	ND	ND	ND
IL-23-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
IL-23-U	pg/min	0.9 ± 1.2	1.2 ± 1.5	1.4 ± 1.8	1.4 ± 2.0	1.3 ± 2.0	0.8 ± 1.3	0.4 ± 0.4
8-isoprostane-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
8-isoprostane-U	pg/min	243.9 ± 151.9	501.3 ± 397.2	443.7 ± 293.5	792.5 ± 867.7	688.0 ± 969.8	267.1 ± 116.3	490.5 ± 372.3
sE-selectin-P	ng/ml	ND	ND	ND	ND	ND	ND	ND
sL-selectin-P	ng/ml	1341.0 ± 158.0	1348.0 ± 254.1	1276.0 ± 250.9	1263.0 ± 353.1	1370.7 ± 275.9	1440.0 ± 208.7	1332.0 ± 133.9
sP-selectin-P	ng/ml	32.7 ± 2.3	31.9 ± 9.1	33.6 ± 10.9	31.6 ± 5.3	29.1 ± 7.3	27.9 ± 5.5	30.8 ± 8.5
Nerve growth factor-P	pg/ml	2.4 ± 3.1	4.2 ± 5.9	2.6 ± 2.7	9.7 ± 17.6	9.3 ± 17.7	6.1 ± 8.2	9.7 ± 21.1

Data are presented as means ± SD (n=9). before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), not detected below the detection limits (ND), plasma (P), urine (U), soluble Endothelial (sE), soluble Leukocyte-Endothelial (sL), soluble Platelet (sP). Urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Table 5. Pearson's correlation coefficient matrix of muscle damage markers, tenderness of medial gastrocnemius and ankle active range of motion in dorsal flexion.

	CK-72 h	CK-96 h	LDH-96 h	ALD-72 h	ALD-96 h	PMG-72 h	MMG-72 h	dROM-48 h	dROM-72 h
CK-72 h		0.24	0.32	0.94	0.48	0.22	0.59	-0.34	-0.43
CK-96 h	0.24		0.94	0.38	0.93	-0.04	0.13	0.19	-0.05
LDH-96 h	0.32	0.94		0.46	0.95	-0.04	0.20	0.31	0.13
ALD-72 h	0.94	0.38	0.46		0.64	0.37	0.78*	-0.15	-0.25
ALD-96 h	0.48	0.93	0.95	0.64		0.06	0.42	0.22	0.04
PMG-72 h	0.22	-0.04	-0.04	0.37	0.06		0.37	0.25	0.03
MMG-72 h	0.59	0.13	0.20	0.78*	0.42	0.37		0.02	0.12
dROM-48 h	-0.34	0.19	0.31	-0.15	0.22	0.25	0.02		0.89
dROM-72 h	-0.43	-0.05	0.13	-0.25	0.04	0.03	0.12	0.89	

All data are calculated as percent changes for the pre-exercise values.

Statistics: * Significant correlation between serum leaking enzyme activity and muscle tenderness score ($p<0.05$), creatine kinase (CK), lactate dehydrogenase (LDH), aldolase (ALD), proximal medial gastrocnemius (PMG), middle medial gastrocnemius (MMG), ankle range of motion in dorsal flexion (dROM), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h).

to not only eccentric exercise-induced local muscle damage, but also systemic factors such as dynamic exercise-induced haemoconcentration. In this study, one-leg calf-raise exercise caused severe muscle damage as shown by the precise local assessment of the muscle symptoms, but we could not detect any changes in FABPs and NGAL nor any correlations with the symptoms. In any case, these organ damage markers are not considered to be indicative of muscle damage resulting from eccentric exercise loading on the calf, thus they cannot be used as local muscle damage markers at least.

Endurance exercise induces peripheral blood neutrophilia (41-44), and enhances the capacity of neutrophils to produce ROS (29, 42-44). In a study using myeloperoxidase (MPO) knockout mice, exercise-induced muscle damage was facilitated by MPO-containing neutrophils and their activating factors such as proinflammatory cytokines (25). On the other hand, Maruhashi et al. reported that the antioxidant capacity was affected by the type and intensity of exercise, specifically, low-load eccentric exercise did not reduce antioxidant capacity, but conversely low-load concentric exercise temporarily reduced antioxidant capacity (20). We investigated neutrophil activation-related markers such as selectins, 8-isoprostane, IL-17A and IL-23, but they were not significantly changed, suggesting that inflammatory responses and oxidative stress were not changed, at least, in the examined markers following one-leg calf-raise exercise. The enhanced eccentric exercise-induced neutrophil migratory activity independent of ROS production and MPO degranulation observed in our previous study (14) might be due to the mobilization of functionally different heterogeneous neutrophils possibly from the bone marrow reserve (42, 44). Since we could not detect any significant changes of a wide range of proinflammatory and oxidative stress markers in this exercise mode other than neutrophil mobilization and migration (14), it is necessary to focus more on the involvement of this earlier step of inflammation as the underlying mechanisms and the point of target for potential preventive countermeasures against exercise-induced muscle damage in the future studies.

In conclusion, it is confirmed in the present study that not only are serum CK but also ALD activities more reliable indicators for exercise-induced muscle damage than the other examined variables, but there are lower correlations with muscle symptoms, and novel organ damage markers of FABPs and NGAL could not be alternative indicators for muscle disruption, neutrophil mobilization and migration. Also, we could not detect any perturbations of novel proinflammatory cytokines and soluble adhesion molecules, and the inflammatory mechanisms are still not clear. Therefore, further research is needed to determine whether there are more sensitive indicators including urinary biomarkers as non-invasive assessment of exercise-induced muscle damage and the underlying mechanisms as well.

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