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Original Article

Changes in oxidative stress severity and antioxidant potential during muscle atrophy and reloading in mice

Wakana Katsuta, RPT[1, 2], Masahiro Aihara, RPT, MS[3, 4], Noboru Hirose, RPT, PhD[3, 5], Fumiaki Saito, MD, PhD[5], Hiroki Hagiwara, MD, PhD[1, 3, 5]*

[1] *Division of Biosciences, Graduate School of Science and Engineering, Teikyo University of Science: 2525 Yatsuzawa, Uenohara, Yamanashi 409-0193, Japan*
[2] *Department of Rehabilitation, National Center of Neurology and Psychiatry, Japan*
[3] *Department of Physical Therapy, Faculty of Medical Sciences, Teikyo University of Science, Japan*
[4] *Department of Health Science, International University of Health and Welfare Graduate School, Japan*
[5] *Department of Neurology, Teikyo University School of Medicine, Japan*

Abstract. [Purpose] Changes in oxidative stress severity and antioxidant potential are routinely used as oxidative stress markers. While several studies have reported the relationship between these markers and exercise, little is known about the dynamic nature of these markers during muscle atrophy and reloading. Therefore, we examined changes in oxidative stress severity and antioxidant potential during muscle atrophy and reloading. [Subjects and Methods] Muscle atrophy was induced in mice by casting the limb for 2 weeks. Mice were then subjected to reloading for 2 weeks. The severity of oxidative stress (hydroperoxide) and antioxidant potential (degree of reduction) were quantified. [Results] Muscle atrophy was induced by cast immobilization. The muscle mass of mice recovered to similar levels as the control group following 2 weeks of reloading. The degree of oxidative stress was within the normal range throughout the experimental period. The antioxidant potential decreased to the clinical borderline level 2 weeks after immobilization, further decreased after 1 day of reloading, and then recovered to within the normal range. [Conclusion] Performing d-ROMs and BAP tests may contribute to the understanding to atrophic process of skeletal muscle in clinical practice of physical therapy.

Key words: Skeletal muscle atrophy, Degree of oxidative stress, Antioxidant potential

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INTRODUCTION

Living organisms can efficiently extract energy using oxygen. However, during metabolic processes, active oxygen species are produced. This active oxygen plays an important role in maintaining life, but results in oxidative stress when the concentration of these species becomes excessive. Such oxidative stress, or the imbalance of oxidants and antioxidants in favor of the oxidants, can result in damage[1].

Oxidative stress severity and antioxidant potential are routinely assessed as oxidative stress markers. To date, various methods have been proposed to evaluate these markers; however, a majority of these methods require special techniques and equipment[2]. Unlike these methods, the Diacron-Reactive Oxygen Metabolites (d-ROMs) test and the Biological Antioxidant Potential (BAP) test are increasingly being used as simple and convenient methods of measuring these markers[3]. The d-ROMs test evaluates the oxidative stress state and is considered the "gold standard" for evaluating free radical levels *in vivo*.

*Corresponding author. Hiroki Hagiwara (E-mail: hagi@ntu.ac.jp)

It also has a high correlation with other oxidative stress markers. Meanwhile, the BAP test measures antioxidant potential and is considered a "supplementary" test that supports the results of the d-ROMs test.

In recent years, it has been shown that oxidative stress increases in response to physiological events and mental stress. Many studies have reported a relationship between oxidative stress and exercise[4–7]. Yet, the dynamics of oxidative stress markers associated with muscle atrophy and reloading are not fully understood[8]. This is particularly important for the elderly population, size of which will continue to increase in the future. Accordingly, there will be increasing relevance for countermeasures that prevent disuse muscle atrophy, which remain to be developed. Therefore, a detailed understanding of the mechanism of skeletal muscle change during muscle atrophy and reloading is crucial for the practice of physical therapy. In this study, we examined the changes in oxidative stress severity and antioxidant potential using the d-ROMs and BAP tests during mouse model of muscle atrophy induced by cast immobilization and reloading.

SUBJECTS AND METHODS

Twenty, 10-week-old male C57BL6 mice (Japan SLC, Hamamatsu, Japan) were housed in independent plastic cages under a 12-h light/dark cycle. This study was approved by the ethics committees on animal experimentation at Teikyo University of Science (14C037).

The mice were divided into 4 groups, each containing 5 mice: control (Group Co), cast immobilization (Group CI), reload one day (Group RL1), and reload 14 days (Group RL14). Muscle atrophy was induced by cast immobilization[9, 10] in groups CI, RL1, and RL14. Mice had free access to food and water. Those mice whose limbs were immobilized were able to access food and water using their forelimb and contralateral hindlimb during the immobilization period. The mice were monitored on alternate days. The cast was replaced when it became loose or if any signs of adverse events (e.g., skin injury, edema, and necrosis) were observed in the fixed limb. Reloading was performed by removing the cast and re-grounding the lower limb of the treatment side and keeping it under normal breeding conditions. The reloading period was either one day (Group RL1) or 14 days (Group RL14).

The mice were euthanized after the experimental period. The tibialis anterior (TA), gastrocnemius (Gc), and soleus (Sol) muscles were isolated and weighed immediately after dissection. Samples were frozen in liquid nitrogen-cooled isopentane, cut into 10 μm cryosections using a cryostat (HM560; Microm, Walldorf, Germany)[11, 12], and stained using hematoxylin-eosin. In order to analyze muscle fiber area, the cross-sectional area of more than 300 muscle fibers in Gc of each group (n=3) was analyzed. The sections were immunostained with an anti-laminin α2 polyclonal antibody (L9393; Sigma, MO, USA), observed under an FSX100 fluorescence microscope (Olympus, Tokyo, Japan), and single muscle fiber area (μm^2) was measured by using ImageJ software[12].

We collected 150 μl of blood samples at days 1, 3, 7, and 14 during the cast immobilization period (CI1, 3, 7, and 14) and at days 1, 3, 7, and 14 during the reloading period (RL1, 3, 7, and 14) using 5 mm size of the Goldenrod animal lancet (MEDIpoint Inc., NY, USA), according to the manufacturer's instructions[13]. The animal lancet has several major advantages over conventional methods, such as the tail vein collecting method. It does not require special skills or equipment, and it can be performed frequently, such as immediately before and after the intervention. Then, we centrifuged them immediately (for 20 minutes at 3,500 rpm) to obtain blood serum for analysis.

For oxidative stress markers, the d-ROM test value (oxidative stress severity) and BAP test value (antioxidant potential) in the serum were measured using the FREE Carpe Diem system (Diacron International, Grosseto, Italy) according to the manufacturer's instructions. In the d-ROM test, the levels of hydroperoxide were measured in units of U.CARR, where 1 U.CARR is equal to 0.08 mg/dl of a solution of hydrogen peroxide. In the BAP test, the level of anti-oxidant substrate reduction was measured in μM. The latent antioxidant potential (B/R ratio) was then calculated on the basis of the values obtained in the BAP and d-ROM tests[3, 14].

Statistical analyses were performed using a one-way ANOVA and Newman-Keuls multiple comparison test, wherever appropriate. Significance was assumed when $p<0.05$.

RESULTS

First, we compared the muscle-wet weight in Groups Co, CI, RL1, and RL14. The weight of the TA, Gc, and Sol muscles in Groups CI and RL1 showed a significant reduction compared to Group Co ($p<0.05$). In group RL14, the muscle wet weight recovered to the same level as that of Group Co. The muscle wet-weight divided by body weight of the TA, Gc, and Sol muscles in Groups CI and RL1 showed a significant reduction compared to that of Group Co ($p<0.05$) (Table 1). Muscle atrophy was induced by cast immobilization and recovered to near the levels of the control group 2 weeks after reloading.

Upon histological analysis, we observed a reduction in muscle fiber area in Groups CI and RL1 in comparison with Group Co. In contrast, the muscle fiber area was regained in Group RL14 (Fig. 1).

We further analyzed the changes in oxidative stress markers, oxidative stress severity (hydroperoxide) and antioxidant potential (degree of reduction). The hydroperoxide levels were within the normal range throughout the experimental period. In contrast, the antioxidant potential decreased to the clinical borderline level (2,000–2,200 μM/l) two weeks after the induction of atrophy, further decreased after 1 day of reloading, and then recovered to within the normal range. Since there is no

Table 1. Changes in muscle wet-weight and wet-weight divided by body weight of tibialis anterior (TA), gastrocnemius (Gc), and soleus (Sol) muscles

		Group Co (n=5)	Group CI (n=5)	Group RL1 (n=5)	Group RL14 (n=5)
Body weight (BW; g)		26.7 ± 1.1	25.0 ± 0.6	24.1 ± 0.6	25.6 ± 0.8
TA	Wet weight (WW; mg)	65.2 ± 17.6	53.2 ± 4.1*	41.6 ± 9.8*	47.4 ± 2.9*
	WW/BW	2.44 ± 0.66	2.12 ± 0.16*	1.79 ± 0.38*	1.85 ± 0.07*
Gc	Wet weight (WW; mg)	153.6 ± 13.3	113.4 ± 12.9*	104.2 ± 4.3*	135 ± 6.6*
	WW/BW	5.75 ± 0.47	4.54 ± 0.55*	4.51 ± 0.18*	5.28 ± 0.36*
Sol	Wet weight (WW; mg)	8.2 ± 1.92	5.6 ± 0.9*	4.0 ± 1.0*	7.4 ± 0.9**
	WW/BW	0.31 ± 0.06	0.22 ± 0.03*	0.17 ± 0.04*	0.28 ± 0.03

All values are given as mean ± SD.
*vs. group Co ($p<0.05$).
**vs. group RL1 ($p<0.05$).

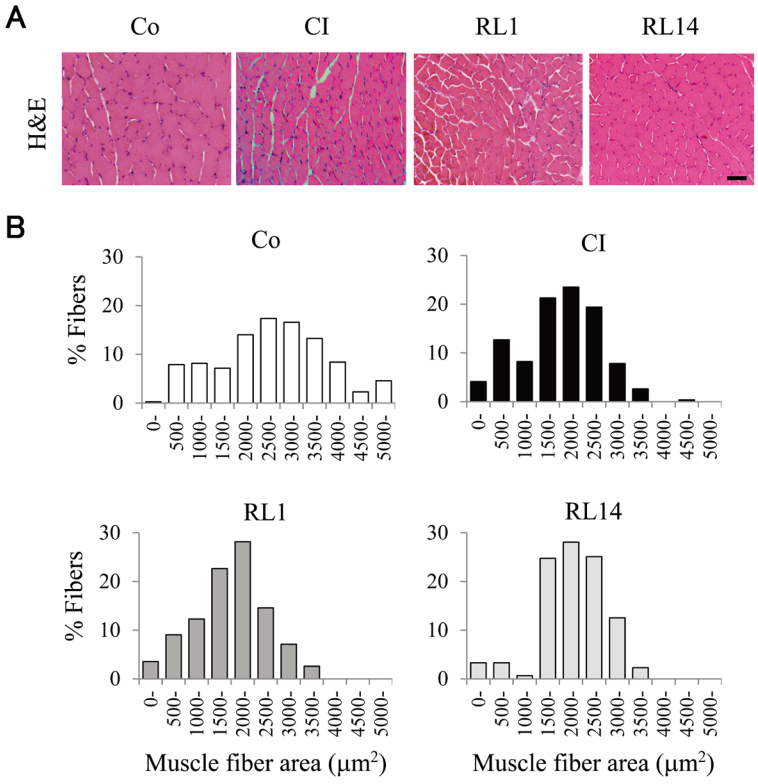

Fig. 1. Histological analysis of gastrocnemius (Gc) muscle.
(A) Representative cross-sections of Gc muscle. Cross-sectional images taken from the Co (control), CI (cast immobilization), RL1 (reload one day), and RL14 (reload 14 days) Groups stained with hematoxylin and eosin (H&E). Scale bar=100 μm. (B) Distribution of muscle fiber area of Gc muscle in each group. A reduction in muscle fiber area was observed in Groups CI and RL1 in comparison with Group Co. In contrast, the muscle fiber area was regained in Group RL14.

normal value reported for mice to date, we referred to normal values and borderline of human. The BAP/d-ROM ratio in Groups CI14 and RL1 showed a significant reduction compared to that of Group Co (both $p<0.05$) (Table 2).

DISCUSSION

In this study, we examined changes in oxidative stress severity and antioxidant potential during muscle atrophy and reloading. Our results show that muscle atrophy was induced by two weeks of cast immobilization and that muscle mass recovered to a level similar to that of the control group after two weeks of reloading. We found that the degree of oxidative stress was within the normal range throughout the experiment period. Meanwhile, the antioxidant potential decreased to the

Table 2. Changes in oxidative stress markers (the d-ROM test value, BAP test value) and the BAP/d-ROM ratio (B/R ratio)

	d-ROMs test (U.CARR)	BAP test (μM/l)	BAP/d-ROM ratio
Co	102.7 ± 19.4	2,596.5 ± 208.7	25.3 ± 2.6
CI1	105.5 ± 8.9	2,888.6 ± 214.8	27.5 ± 4.1
CI3	113.2± 31.9	2,723.4 ± 107.2	27.4 ± 11.8
CI7	119.8 ± 5.3	2,675.7 ± 225.3	22.4 ± 2.5
CI14	94.4 ± 29.9	2,006.6 ± 358.1*	18.7 ± 5.4*
RL1	105.3 ± 12.7	1,760.4 ± 412.7*	17.6 ± 5.4*
RL3	106.2 ± 7.3	2,626.6 ± 137.3	24.9 ± 2.3
RL7	92.7 ± 9.0	2,401.9 ± 178.3	26.8 ± 2.7
RL14	96.6 ± 15.9	2,340.3 ± 414.6	25.1 ± 6.4

Co: control; CI1, 3, 7, and 14: at days 1, 3, 7, and 14 during the cast immobilization period; RL1, 3, 7, and 14: at days 1, 3, 7, and 14 during the reloading period.
All values are given as mean ± SD.
*vs. group Co ($p<0.05$).

clinical borderline level two weeks after induction of atrophy, further decreased after one day of reloading, and then recovered to within the normal range. In this study, we revealed that oxidative stress severity and antioxidant potential changed differently during muscle atrophy and reloading.

At present, there are a variety of markers to measure oxidative stress. Among them, the d-ROMs and BAP tests used in this study are simple and requires less time in comparison with the conventional measuring methods. In addition, these tests can evaluate oxidative stress with a high reproducibility. The hydroperoxide, the target product of the d-ROM test, is the primary product of lipids, which is the most vulnerable substrate to oxidation *in vivo*[15]. Therefore, it is reasonable to use the d-ROM and BAP tests to measure oxidative stress markers when it is unclear what type of active oxygen species is resulting in oxidative stress. In the present study, we confirmed that the d-ROM test and the BAP test are useful for measuring reactive oxygen species during muscle atrophy and reloading.

To our knowledge, this is the first report to investigate the effects of oxidative stress on skeletal muscles during muscle atrophy and reloading using the d-ROMs and BAP tests. Oxidative stress is known to be increased by exercise and is understood to reflect muscle injury due to exercise. It has been reported that fine muscle injury occurs during reloading after muscle atrophy[16]. Further histological study using immunohistochemistry is necessary to elucidate the relationship between fine muscle injury and oxidative stress. In this study, a muscle atrophy model by cast immobilization was used. Further studies with alternative muscle atrophy models such as hindlimb suspension and muscle disease models are required to better interpret our results.

During physical therapy in the clinic, early intervention for disuse muscle atrophy is crucial. We speculate that it is possible to assess the oxidative stress levels in the skeletal muscle of patients by performing the d-ROMs and BAP tests. This can inform subsequent interventions, such as adjusting the optimal load and prescribing antioxidants. By performing these protocols in the early stages, it is possible to delay or prevent the progression of disuse muscle atrophy and promote more effective intervention.

Based on the results of this study, oxidative stress may be involved in changes to skeletal muscle mass due to disuse muscle atrophy during the early stages of reloading. Performing d-ROMs and BAP tests may contribute to increased understanding of the pathology of muscle atrophy and the evaluation of the effects of interventions used during physical therapy.

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