Deterioration of Myofibrils Affected by Experimental Partial Immobilization in a Septic Rat Model

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Abstract
ICU-acquired weakness (ICU-AW) is an acute diffuse muscle weakness that is an important prognostic factor for patients in today’s intensive care units. In this study, we created a novel ICU-AW model of sepsis in which septic rats were immobilized in a lower limb cast for 96 hours. We investigated the structural changes in muscle tissue following lipopolysaccharide (LPS)-induced inflammation and cast immobilization from a pathological viewpoint. Ten-week-old male rats were divided into four groups: control group (C-group, n=6), cast-immobilized group (CC-group, n=6), LPS administration group (L-group, n=6), and LPS administered and cast immobilized group (LC-group, n=6). The LPS administration time and dose were determined based on the results of a study experiment performed first. After the LPS administration/cast-immobilization experiment, body weights and wet weights of both isolated soleus muscles were measured. Muscle tissue was analyzed from a pathological perspective using optical and transmission electron microscopy. Although the comparison of body weights between the four groups showed no significant differences after 96 h, a severe loss of soleus muscle wet weight and decrease in the relative soleus weight ratio were observed in the LC-group. Optical microscopy results showed that a significant increase in cross-sectional area (CSA) and decrease in the average number of muscle fibers were observed in the LC-group compared with all other groups (p<0.05), and the CSA showed a mixture of small and large muscle fibers. The average number of vacuoles in muscle fibers appeared to significantly increased in the CC and LC-groups (p<0.05, vs. the C, L-group). Longitudinal electron microscopic images showed distorted sarcomeres, Z-band disruption, and loss of actin-myosin filaments was present in the cross-sections. The present study suggested that from a pathological viewpoint, a combination of inflammation and immobilization causes more severe consequences in terms of structural changes in muscle tissue than either factor alone.

Key words
ICU-acquired weakness, immobilization, sepsis, muscle atrophy

Introduction
Recent advances in critical care medicine have improved the morbidity and mortality of patients treated in an intensive care unit (ICU). However, some critically ill patients exhibit motor dysfunction known as ICU-acquired weakness (ICU-AW)\(^1\), which manifests as symmetrical limb weakness and respiratory muscle weakness and may delay or impede social rehabilitation.

ICU-AW includes different etiologies—critical illness polyneuropathy (CIP), critical illness myopathy (CIM), and a mixture of both, critical illness neuromyopathy (CIPNM)—and several risk factors have been found, including ventilator management, hyperglycemia, sepsis, multiorgan system failure, and use of steroid and neuromuscular blocking agents. Especially, severely septic patients are prone to develop...
Skeletal muscle wasting occurs earlier and progresses rapidly over the first week in the ICU, and impairment in lower limb muscle strength, especially in the distal region, may remain 3 months after ICU discharge in severe cases of ICU-AW. It was shown that ICU-AW, which is detected in about 50% of patients with critical illness, is the most common neuromuscular disorder and that it affects the clinical course and outcomes in ICU patients.

The aims of this study were first, to establish a persistent severe septic model by comparing different doses of continuous intravenous infusion of lipopolysaccharide (LPS) in rats with an indwelling intravenous catheter, and second, to test our hypothesis of whether the combination of a persistent septic state and immobilized lower extremities that mimic patients in the earlier phase of severe sepsis in the critical care setting have not been fully analyzed in an animal model. Furthermore, it is unclear whether there is an additive or synergistic effect of the septic state and immobilization on muscle.

The study was approved by the St. Marianna Medical University Animal Ethics Committee (approval Nos.: 1902027, 1904009). Male Wistar rats (Japan SLC, Inc., Shizuoka, Japan) (age, 10 weeks; weight, 208–309 g) were housed in cages for 7 days and maintained at 22±0.2°C at a relative humidity of 55±5% under a 12-h light/dark cycle. The animals were anesthetized by intraperitoneal injection of a combination of anesthetics (medetomidine hydrochloride 0.15 mg/kg, midazolam 2 mg/kg, and butorphanol tartrate 2.5 mg/kg). An indwelling catheter (Micro-Renathane polyurethane catheter; SAJ Infusion Technologies, Lake Villa, IL, USA) was introduced into the right external jugular vein and connected to a Quick Connect Infusion Kit (SAJ Infusion Technologies), which was used during all experiments and permitted the animal to move freely and access food and water. The catheter was filled with heparin solution, and the end of the catheter was sealed with a plug. The catheter was flushed with heparin solution every few days to maintain patency. The weight and condition of the animals were observed every day. If any abnormal signs were noted, these animals were excluded from the experiments.

Material & Methods

Animal preparation

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Experiment 1: Lipopolysaccharide dose and administration periods

Twenty-four rats were randomly divided into four groups, each with 6 animals: control group (C-group), cast immobilized group (CC-group), LPS administered group (L-group), and LPS administered and cast immobilized group (LC-group). Animals were continuously infused with the LPS dose and over the administration period determined in experiment 1. The C-group and CC-group were continuously infused with the volume of saline equivalent to that administered for the LPS dose. The CC-group and LC-group were anesthetized with isoflurane (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) (4% induction, 2% maintenance), and then both hindlimbs were immobilized at the knee and ankle by stretching the limbs. Both hindlimbs were then immobilized with a cast stick (Castlight-α; ALCARE Co. Ltd., Tokyo, Japan) and a medical adhesive bandage, with reference to the methods of Madaro et al. The animals were observed for cast condition and presence of edema, and their weight was measured every day. If any abnormalities were observed in the cast, it was rewound with a medical bandage. After the end of the continuous infusion, the animals were weighed and

Experiment 2: Lipopolysaccharide administration and immobilization

Twenty-four rats were randomly divided into four groups, each with 6 animals: control group (C-group), cast immobilized group (CC-group), LPS administered group (L-group), and LPS administered and cast immobilized group (LC-group). Animals were continuously infused with the LPS dose and over the administration period determined in experiment 1. The C-group and CC-group were continuously infused with the volume of saline equivalent to that administered for the LPS dose. The CC-group and LC-group were anesthetized with isoflurane (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) (4% induction, 2% maintenance), and then both hindlimbs were immobilized at the knee and ankle by stretching the limbs. Both hindlimbs were then immobilized with a cast stick (Castlight-α; ALCARE Co. Ltd., Tokyo, Japan) and a medical adhesive bandage, with reference to the methods of Madaro et al. The animals were observed for cast condition and presence of edema, and their weight was measured every day. If any abnormalities were observed in the cast, it was rewound with a medical bandage. After the end of the continuous infusion, the animals were weighed and
then anesthetized with isoflurane, following which blood samples were collected for measurement. Following cast removal, after the additional inhalation of a high concentration of isoflurane to euthanize the animals, both soleus muscles were carefully isolated from the lower limbs. The wet weight of each soleus muscle was measured, and the relative wet weight ratios were calculated.

**Blood biochemical analysis**
Platelet counts of the blood samples were measured with an automatic blood cell calculator. After centrifugation, the serum was stored at -80°C until analysis. Serum levels of total protein and albumin were measured with a Dual Range BCA Protein Assay Kit (Visual Protein Biotechnology Corp, Taipei, Taiwan) and LBIS Rat Albumin ELISA Kit (FUJI-FILM Wako Shibayagi Corporation, Gunma, Japan) using a VARIOSKAN LUX (Thermo Fisher Scientific K.K., Tokyo, Japan).

**Morphological analysis**
At collection time, the soleus muscle samples were divided for two experiments: one for histochemical and the other for electron microscopic analysis. The histochemical analysis sample was frozen in isopentane cooled with liquid nitrogen and stored at -80°C until use. Cross-sections (10 μm) of the muscle were cut on a cryostat (CM1900; Leica, Wetzlar, Germany) at -20°C. Each section was stained with hematoxylin and eosin (H&E). The H&E-stained sections were used for analyses of cross-sectional area (CSA) in which more than 500 muscle fibers per field from at least six different fields were investigated with a light microscope (BZ-X710; KEYENCE Co., Osaka, Japan) and measured with NIH Image J software (version 1.52, National Institutes of Health, MD, USA). The electron microscopic analysis sample was fixed in 1/2 Karnovsky solution at 4°C overnight. The samples were then postfixed in 1% osmium tetroxide solution, dehydrated, and embedded in EPON resin. Quasi-ultrathin sections were created and stained with toluidine blue and then investigated with the light microscope (BZ-X710; KEYENCE). Ultra-thin sections cut with a diamond knife were electron stained and investigated with a transmission electron microscope (JEM-1200EX; Nippon Electronics Co., Ltd., Tokyo, Japan).

**Statistics analysis**
After testing for normality and homoscedasticity, differences between multiple groups were assessed by a non-parametric test (Kruskal Wallis) with Steel-Dwass post hoc test. Non-parametric data are reported as the median, minimum, and maximum. The average numbers of muscle fibers and vacuoles were assessed by one-way ANOVA with Tukey post hoc test. Parametric data are reported as the mean ±SD. The comparison of cross-sectional areas was performed by summing the number of structures in areas in which muscle fiber counts should be 500 or more per one field from 6 rats in each group. Histograms are expressed as the percentage of total muscle fiber numbers of each size in each group. Differences in CSA distribution were assessed by the Kolmogorov-Smirnov two-sample test with post-hoc Bonferroni’s multiple comparison test. All statistical analyses were performed with R software (version 3.6.3), and p<0.05 was considered to indicate statistical significance.

**Results**

**Experiment 1: Lipopolysaccharide dose and administration periods**
We investigated the resistance of the animals to endotoxin administration for 120 h. The control group showed normal activities throughout the 120-h observation period. The L1, L2, and L3-group animals gradually became more lethargic, but then the L1-group animals gradually became more active. Rats in the L3-group either died or were humanely euthanized when breathing patterns regarded as indicative of respiratory failure and/or severe weight loss was observed within 72 h. Body weight in the L1-group dropped transiently but returned to the pre-infusion weight at 120 h, whereas that in the L2-group remained reduced at 120 h. In the L1, L2, and L3-groups, platelet counts dropped dramatically after endotoxin infusion, reaching their nadirs at 24 h in the L1 and L2-groups and at 48 h in the L3-group, and then gradually recovered. Platelet counts in the L2-group showed individual differences and signs of endotoxin resistance at 120 h. Resistance to endotoxin was significantly greater in the L1-group (Table 1). From the results of this experiment, we determined an LPS dose of 2 mg/kg/day and an administration period of 96 h for use in the subsequent experiment.

**Experiment 2: Lipopolysaccharide administration and immobilization**
The immobilized hindlimbs showed no edema. Comparison of body weights of the CC-group, L-
group, and LC-group with the C-group showed no significant differences after 96 h. We observed a loss in soleus muscle wet weight and the relative soleus weight ratio in both the CC-group and L-group and observed a severe loss of soleus muscle wet weight and the relative soleus weight ratio in the LC-group (Table 2).

**Blood biochemical analysis**

Platelet counts decreased significantly with LPS administration but did not change with cast immobilization. Serum albumin and serum total protein showed no significant differences between the CC-group and C-group, indicating that immobilization had no effect on nutritional status. However, a significant difference in serum albumin was observed with LPS administration (Table 2).

**Morphological analysis**

In the histochemical analysis under H&E staining, the CSA and the average number of muscle fibers per one field of view were analyzed. A significant increase in CSA and decrease in the average number of muscle fibers were observed in the LC-group compared with all other groups (Table 3, Figure 1). The average number of vacuoles per one visual field was analyzed under toluidine blue staining. Immobilization resulted in the creation of multiple vacuoles in the CC and LC-groups (Table 3, Figure 2). The distribution of the CSAs was analyzed with histograms. Compared with all other groups, the distribution of CSAs in the LC-group was quite sig-

| Table 1. Alteration of Body Weight and Platelet Counts over 120 h during LPS Administration |
|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | 0 h              | 24 h             | 48 h             | 72 h             | 96 h             | 120 h            |
| **Body weight (g)** |                 |                  |                  |                  |                  |                  |
| Control (n=6)     | 274 (268-277)    | 277 (268-280)    | 277 (268-280)    | 280 (273-281)    | 281 (275-283)    | 281 (275-284)    |
| L1 (n=6)          | 276 (272-283)    | 273 (268-280)    | 270 (266-279)    | 269 (267-277)*   | 271 (267-279)*   | 272 (270-281)    |
| L2 (n=6)          | 276 (271-286)    | 271 (268-280)    | 268 (265-278)    | 266 (260-275)*   | 267 (259-279)*   | 263 (253-273)*   |
| L3 (n=6)          | 274 (266-279)    | 269 (259-275)    | 266 (256-275)    |                  |                  |                  |
| **Platelet counts (10^3/μl)** |                 |                  |                  |                  |                  |                  |
| Control (n=6)     | 707 (616-814)    | 769 (679-831)    | 749 (689-783)    | 763 (704-811)    | 850 (777-882)    | 878 (775-893)    |
| L1 (n=6)          | 690 (582-836)    | 69 (58-90)*      | 127 (157-219)*   | 351 (342-441)*   | 508 (437-560)*   | 546 (502-592)*   |
| L2 (n=6)          | 642 (620-861)    | 67 (60-89)*      | 107 (88-118)*†   | 154 (106-206)*†  | 209 (164-238)*†  | 295 (202-362)*†  |
| L3 (n=6)          | 705 (665-848)    | 79 (62-96)*      | 46 (32-55)*†‡    |                  |                  |                  |

Data are expressed as median (minimum-maximum value), with p<0.05 indicating statistical significance. LPS: lipopolysaccharide, C: Control group administered normal saline, L1: 1 mg/kg/day in LPS group, L2: 2 mg/kg/day in LPS group, L3: 3 mg/kg/day in LPS group. *: p<0.05 vs. C, †: p<0.05 vs. L1, ‡: p<0.05, vs. L2.

| Table 2. Alteration of Indicators at 96 h after LPS Administration and Immobilization |
|------------------|------------------|------------------|------------------|------------------|
|                  | C (n=6)          | CC (n=6)         | L (n=6)          | LC (n=6)         |
| **Body weight (g)** | 295 (280-308)    | 288 (277-292)    | 278 (260-286)    | 269 (261-290)    |
| **Soleus wet weight (mg)** | 174 (168-208)    | 127 (121-146)*   | 128 (110-147)    | 97 (78-106)††    |
| **Relative soleus wet weight ratio** | 0.60 (0.57-0.67) | 0.45 (0.42-0.50)* | 0.46 (0.42-0.54) | 0.35 (0.29-0.39) †† |
| **Platelet counts (10^3/μl)** | 908 (786-941)    | 860 (839-996)    | 332 (107-361)†   | 318 (129-397)††  |
| **Serum albumin (g/mL)** | 16.6 (13.3-20.4) | 15.0 (13.4-18.3) | 9.4 (8.8-13.3)†* | 8.10 (5.7-9.8) †† |
| **Serum total protein (g/mL)** | 68.2 (60.1-73.2) | 65.7 (64.1-71.1) | 58.2 (56.3-68.7) | 59.0 (56.0-66.2) |

The relative soleus wet weight ratio was calculated as soleus wet weight (mg) divided by body weight (g). Data are expressed as median (minimum-maximum value), with p<0.05 indicating statistical significance. LPS: lipopolysaccharide, C: Control group, CC: Control group in a cast, L: LPS group, LC: LPS group in a cast. *: p<0.05 vs. C, †: p<0.05 vs. CC, ‡: p<0.05 vs. L.
Table 3. Optical Microscopic Changes at 96 h after LPS Administration and Immobilization

|                          | C (n=6)       | CC (n=6)      | L (n=6)       | LC (n=6)      |
|--------------------------|---------------|---------------|---------------|---------------|
| Average number of muscle fibers | 100.2±13.8    | 95.7±16.5     | 95.0±15.6     | 67.5±7.1*†‡  |
| Cross-sectional area (µm²) | 2538.6 (563.3-6428.7) | 2521.4 (166.1-5452.5) | 2530.59 (219.1-6258.8) | 3274.4 (141.6-9443.7)*†‡ |
| Average number of vacuoles in muscle fiber | 0±0           | 6.5±4.4*      | 0.2±0.2†      | 17.4±5.0*†   |

Data are expressed as mean ±SD for average numbers of muscle fibers and vacuoles in muscle fibers and as median (minimum-maximum value) for cross-sectional area, with p<0.05 indicating statistical significance. LPS: lipopolysaccharide, C: Control group, CC: Control group in a cast, L: LPS group, LC: LPS group in a cast. *: p<0.05 vs. C, †: p<0.05 vs. CC, ‡: p<0.05 vs. L.

Figure 1. Hematoxylin and eosin staining of cross-sections of the soleus muscle in the four groups
Optical microscopic images show the change in the cross-sections of muscle fibers at 96 h in each group (n=6, respectively). C: Control group, CC: Control group in a cast, L: LPS group, LC: LPS group in a cast.

Significantly different (p<0.05, vs. all other groups). In the LC-group, the peak shifted to the right, the percentage of the CSA values of the muscle fibers decreased significantly by about 10% from the value shown in the C-group, and the tail was spread more widely to the left and the right, which indicated variation in the fiber sizes (Table 3, Figure 3).

Electron microscopical analysis of longitudinal sections in the LC-group showed myofibrillar disorganization with distorted sarcomeres and unclear sarcomere lengths. The myofibrillar structures showed disrupted Z-bands and the loss of A-band density, and these differences were significant when compared to the other groups (Table 4). Widening of the internyofibrillar space and narrowing of the width of the myofibrils were also observed. These changes were due to the loss of filaments, indicating the collapse of sarcomeres. However, significant mitochondrial degeneration was not apparent (Table 4). Myofibrillar spaces with distorted sarcomeres and vacuole-like structures were also observed in the CC-group, again indicating the effects of immobilization. Z-streaming was also present in the L-group, which was affected by LPS administration (Figure 4). Z-streaming was not seen in the CC-group. Cross-sectional structures in the LC-group showing variation in fiber size matched with the H&E-stained sections (Figure 5). Disruption of the hexagonal lattice structure
Figure 2. Toluidine blue staining of cross-sections of the soleus muscle in the four groups
Optical microscopic images show the change in the cross-sections of muscle fibers at 96 h in each group (n=6, respectively). Evaluation of vacuoles in images was analyzed in 3 to 16 fields per one rat in each group. The images reveal the appearance of vacuoles in muscle fibers in the CC and LC groups (arrowheads). C: Control group, CC: Control group in a cast, L: LPS group, LC: LPS group in a cast.

was seen due to the loss of filaments (Figure 5), along with vacuole-like structures in the LC-group. These differences from the electron micrography images in the LC-group were significant when compared with those in the other groups (Table 4, p<0.05, vs. all other groups).

Discussion
The aim of this study was to investigate the effects of immobilization of the lower extremities in a septicemic rat model to create septicemic ICU-AW models. This study simultaneously investigated the relationship between septicemia and muscle mass, muscle fiber size, number, and size distribution due to immobilization, as well as pathological changes in myofibrils. The results showed that septicemic rat models that were also immobilized exhibited the most severe effects on muscle fibers.

The results of the present study indicated that histological changes in the muscles caused by immobilization are dependent on the method and duration of immobilization, the observed time points. Overall, significant muscle atrophy was observed in the flexion models of the lower limbs. By contrast, atrophic changes differed and depended on the muscle type in limbs in the extended position. Atrophic changes occurred in type II muscles and progressed in severe cases, whereas type I muscles temporarily exhibited muscle hypertrophy. Some reports have indicated that these hypertrophic changes are due to the effects of insulin growth factor 1 expression, which is induced by the stretch reflex. When muscles are subjected to prolonged immobilization and consistent stretching, reports indicate that hypertrophic type I muscles progress to atrophic changes. These types of changes are most commonly observed in the soleus muscles, which function as antigravity muscles.

Among the septicemic animal models, most research has involved cecal ligation and puncture models and LPS models. Intraperitoneal injection of LPS resembles septicemia in that it induces an increase in cytokines that peak between 1.5 and 4 h and began to decline at 8 h after LPS administration, whereas septicemia is a condition that persists over the long term, which makes a direct comparison difficult. However, continuous administration of LPS re-
results in increased mortality rates, so it is difficult to create models that exhibit the histopathological changes observed during ICU-AW while maintaining a septicemic state over several hours.

In the present study, we observed no differences in body weight between the four groups after 96 h, but the soleus muscle mass decreased to the greatest extent in the LPS administered (L) group and cast immobilized (CC) group. In the LC group, which was subjected to both of these factors simultaneously, there were sometimes more significant decreases in muscle mass based on individual factors. Hypoalbuminemia is an important indicator of a hyponutritional state in septicemic cases\(^\text{11}\), and we also observed significantly lower serum albumin values in the LPS administered groups (L and LC groups) in the present study. Meanwhile, irrespective of the absence of a significant difference in albumin values between the L and LC groups, the soleus muscle mass decreased significantly in the LC group. These results suggest that poor nutrition alone is not the main factor and that immobilization during septicemia directly impacts the soleus muscle. Severe disorders such as septicemia usually result in a hypermetabolic state. The electron microscopy examination in this study showed breakdown of the sarcomere structure and disappearance of actin and myosin. Skeletal muscle proteolysis first involves the isolation of actin and myosin from muscle fibers via the calpain proteolysis pathway, then ubiquitination of the isolated actin and myosin, followed by degradation into 26S proteasomes\(^\text{12}\). In addition, ubiquitinated proteolysis pathways have also been shown to be involved in immobilization\(^\text{13}\), and we believe that the decrease in soleus muscle mass during the early stages of ICU-AW occurs as a result of these two conditions that occur simultaneously.

We found that LPS and immobilization alone did not affect the number of muscle fibers. However, the simultaneous presence of both conditions resulted in significantly fewer muscle fibers, enlarged muscle CSA and increased variation in muscle size. We previously published\(^\text{14}\) the results of a nerve conduction velocity study conducted during 48 h of continuous LPS administration and found that this may cause neurogenic changes in the soleus muscle. However,
Table 4. Electron Micrograph Findings of Longitudinal and Cross-Sections of the Soleus Muscle at 96 h after LPS Administration

|                           | C      | CC     | L      | LC     |
|---------------------------|--------|--------|--------|--------|
| **Longitudinal section**  |        |        |        |        |
| Sarcomere length          | Orderly| Irregular, unclear | Orderly| Unclear disruption |
| Sarcomere distorted       | –      | – ~ +  | – ~±   | + ~ + + + *†‡     |
| Z-band streaming/disruption| –      | –      | – ~ +  | + ~ + + + *†‡     |
| Z-band split              | –      | – ~ +  | – ~±   | – ~ +  |
| Vacuole-like structures   | –      | –      | – ~±   | – ~ +  |
| Mitochondrial degeneration| –      | –      | – ~±   | –      |
| Glycogen granules         | +      | – ~ +  | + ~ +  | ± ~ +  |
| **Cross-section**         |        |        |        |        |
| Fiber size                | Uniform| Uniform + variation | Uniform| Variation |
| Myofibrillar disorganization| –      | – ~ +  | – ~ +  | + ~ + + + *     |
| Actin loss                | –      | – ~ +  | – ~ +  | – ~ +  |
| Myosin loss               | –      | –      | – ~ +  | – ~ + *†       |
| Vacuole-like structures   | –      | – ~ +  | – ~ + + | – ~ + + |

Grading was determined according to the following findings: -: no detectable change, ±: minimal detectable change (almost absent change per one field), +: mild detectable change (partially detectable change per one field), ++: moderate detectable change (detectable change in more than half of one field), +++: Severe detectable change (detectable change in almost all of one field). LPS: lipopolysaccharide, C: Control group, CC: Control group in a cast, L: LPS group, LC: LPS group in a cast. *: p<0.05 vs. C, †: p<0.05 vs. CC, ‡: p<0.05 vs. L.

on the basis of the results of the present study, we did not observe small angular fibers or group atrophy as neurogenic changes when we examined the pathological changes among the early ICU-AW models, and as the changes affected the muscles overall, we consider them to be myogenic changes. This information may show why CIM comprises a greater percentage of patients with ICU-AW and is more severe than critical illness polyneuropathy. Fink et al. performed a study similar to ours in which they immobilized septicemic models and despite the absence of a decrease in muscle CSA, they also found no changes in the percentage of muscle fiber types when compared to the control group. We did not verify differences in muscle type in the present study, but we did show that there were changes in atrophic muscle types due to septicemia and immobilization. We therefore presume that atrophy of both muscle fiber types occurred during the long-term (12 days) administration performed in the Fink et al. study, which explains the differences with our study. In addition, unlike Fink et al., we observed increased CSA in our experimental results. Wollersheim et al. performed skeletal muscle biopsies on patients with ICU-AW and observed an increase in CSA after five days and a decrease after 15 days. The cause of this increase in CSA is unknown, although studies that performed immobilization of septic shock models showed that this resulted in muscle fiber edema in skeletal muscles. The increase in CSA that we observed may therefore reflect early changes that occur during ICU-AW. In addition, the inconsistency in muscle size during ICU-AW that was confirmed on the histograms is characteristic of sarcopenia, even when it is caused by septicemia or immobilization. The results of the present study therefore suggested...
that the muscle abnormalities start during the early stages of ICU-AW, based on the distribution of CSAs in the muscle fibers.

In addition, the histograms from the present study showed a lower percentage of muscle fibers with CSA values between 2000 and 3000 μm² in the LC group when compared to the control group. In addition to the decreased sarcolemma membrane excitability²⁰ and decreased myofibril function²¹ indicated by previous studies of CIM, the differences in the decreases in muscle mass and distribution of muscle fibers that produce the maximum tensile force, and the myofibril damage observed in the present study, are presumed to significantly affect muscle strength.

Septicemia not only activates the calpain system and ubiquitin proteasome proteolytic pathways but also induces autophagy triggered by increased oxidative stress in muscle²². Hussain et al.²³ showed that autophagy is promoted by atrophy of the diaphragmatic muscles due to ventilation. Meanwhile, another study showed that a significantly greater extent of autophagy occurs in the appendicular muscles than in the diaphragmatic muscles in LPS injection rat models²⁴. The fact that vacuoles were created to a significantly greater extent in the CC and LC groups in the present study may indicate that autophagy is already being promoted in the early stages of ICU-AW, i.e., within the first 96 h. The early initiation of rehabilitation in septicemic patients suppresses the onset of ubiquitin protease activity and autophagy and skeletal muscle atrophy²⁵. However, the suppression of autophagy paradoxically impairs neuromuscular function²⁶, and the drastic increase and subsequent decrease in autophagy observed during the acute phase of septicemia appears to have a protective effect on the body²⁷, so we believe that the changes in the number of vacuoles must be monitored during the late phase. The fact that we did not observe mitochondrial degeneration or glycogen granules during the electron microscopy examination shows that severe muscle injury has already occurred within the first 96 h of ICU-AW but that these changes have not yet resulted in muscle denaturation. This further supports the importance of initiating multifaceted treatment during the early stages of ICU-AW to prevent the muscle denaturation that may subsequently occur.

Figure 4. Electron micrographs of longitudinal sections of the soleus muscle in the four groups

Electron micrographs of longitudinal sections of the soleus muscle in each group (n=6, respectively) reveal expansion of the intermyofibrillar spaces in the LC group (thin arrows), the disintegration of sarcomeres showing streaming of the Z-line (arrowheads) in the L group, and vacuole-like structures (thick arrows) in the CC and LC groups. C: Control group, CC: Control group in a cast, L: LPS group, LC: LPS group in a cast.
There are several limitations in this study. First, the models that we created were early ICU-AW models, which meant that we were unable to assess changes caused by septicemia and muscle immobilization over a medium- to long-term period. Second, we did not perform morphological assessment of the muscle fiber types, and third, we did not perform electrophysiological tests or measurements of muscle strength, and therefore were unable to perform simultaneous assessment of tensile strength. These aspects will need to be verified in future studies.

In the present study, we observed more severe decreases in muscle mass and muscle fiber numbers, and more extreme destruction of myofibrils, due to individual differences in the LC group. Meanwhile, we also observed enlargement of the muscle fibers, which may indicate muscle fiber edema prior to muscular atrophy. Despite the destruction of muscle tissue observed in the present study, there were no effects on cytoplasmic organelles, and no findings suggested the presence of muscle denaturation. However, if these changes persist, this may result in the establishment of CIM, and as a result, we believe that early intervention targeting immobilization and septicemia should form part of the treatment strategy. The present study provides numerous outstanding topics for future research in this area, and we believe that the present findings will be helpful in elucidating the pathology of ICU-AW.

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**Conflicts of Interest**

The authors have nothing to disclose.

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