Original Article

Effects of neuromuscular electrical stimulation on pulmonary alveola and cytokines in chronic obstructive pulmonary disease (COPD) and skeletal muscle atrophy model mice

HIROSHI MARUOKA, RPT, PhD1)*, KEN-ICHI TANAKA, PhD1), MASA AKI TAKAYANAGI, PhD1), MASASHI ZENDA, RPT, MS1, 2)

1) School of Health and Social Services, Saitama Prefectural University: 820 Sannomiya, Koshigaya city, Saitama 343-8540, Japan
2) Division of Rehabilitation, International University of Health and Welfare Ichikawa Hospital, Japan

Abstract. [Purpose] It has been reported that exercise affects skeletal muscle in the chronic obstructive pulmonary disease (COPD) disease model. In this study, we examined the effects of neuromuscular electrical stimulation (NMES) in skeletal muscle on alveoli and cytokines. [Materials and Methods] We used twenty wild-type mice, randomly divided into three groups: Group A: Control (non-COPD, non-amyotrophia, non-NMES), Group B: COPD, amyotrophia with NMES and Group C: COPD, amyotrophia without NMES. Among those, a group of mice with ages from 12 to 14 weeks were used to create a chronic obstructive pulmonary disease (COPD) model, a group of mice with ages from 15 to 16 weeks was used to create a disuse syndrome by hind limb suspension, and a group of mice with ages from 17 to 28 weeks (12 weeks) were used to implement NMES. In this study, we used the real-time PCR method to assess the mRNA expression levels. We also conducted morphological analysis, assessed macrophage expression level by staining (general staining and immunostaining), and employed spirometry. [Results] Our study results showed significant decreases in Interleukin-6 (IL-6) levels in the lungs and muscle RING-finger protein-1 (MuRF1) in the muscles. Moreover, the pulmonary stromal macrophage marker (F4/80) and the protease marker (MMP12) showed significantly decreased expression, while no change was observed in the morphological of the alveolar spaces (mean linear intercept). [Conclusion] On the basis of these findings, our study reveals that NMES affects cytokines and macrophages in COPD skeletal muscle atrophy.

Key words: COPD, Skeletal muscle atrophy, Neuromuscular electrical stimulation

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a systemic inflammatory disease in which lung inflammation spread throughout the body. It has been indicated that COPD is associated with co-morbidities of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6) along with alveolar destruction, systemic inflammation, skeletal muscle dysfunction, and nutritional disorders along with increased oxidative stress1, 2. Therefore, managing its co-morbidities is extremely important to improve the prognosis of patients, as unveiling the pathology has remained insufficient thus far; hence, controlling inflammation and decrease in physical activities is considered critical1, 2.

In general, COPD causes skeletal muscle dysfunction, including a reduction in muscle strength that involves changes in muscle fiber composition, changes in enzyme activity, and a decrease in muscle capillaries number2. 3. Besides, skeletal
muscle dysfunction causes decreases in patients’ physical activity levels and exercise tolerance and is considered an essential factor related to prognosis. A report shows the causes of skeletal muscle dysfunction include an imbalance in protein synthesis and degradation and mitochondrial dysfunction. To address such skeletal muscle dysfunction, appropriately prescribed aerobic exercise, resistance training, and neuromuscular electrical stimulation (NMES) are recommended. It has been reported that exercise for prevention of skeletal muscle dysfunction has effects on skeletal muscle atrophy-related factors such as decreases in muscle RING-finger protein-1 (MuRF1) and TNF-α, mitochondrial division, oxidative stress, and cytokines that induce an inflammatory response. In contrast, many factors in the molecular mechanism remain unknown. Another report shows that NMES promotes mitochondrial fusion and enhances its function. This suggests that exercise and NMES can affect cytokine levels. On the other hand, it has been reported that in a COPD disease model created by smoking exposure, no change was observed in the inflammatory response in skeletal muscle, while reduction of Creatine Kinase activity and enhanced carbonylation levels were observed. Besides, although it has been reported that exercise affects skeletal muscle in the COPD disease animal model, the effects of NMES have not been included. Furthermore, it has been reported that exercise affects the lung tissues, including a reduction of the morphological of the alveolar spaces (mean linear intercept), with its mechanism involving cytokines, antiproteases, and oxidative stress. The lung is an organ in which numerous macrophages are found, and these macrophages play a central role in multiple processes, from inflammation to fibrosis, and is closely associated with cytokines. However, the effects of NMES on lung macrophages and cytokines have not been reported.

Therefore, in this study, we examined the effects of NMES, including suppression of alveolar destruction, and improvement in inflammation and skeletal muscle atrophy, by creating a COPD model in mice combined with a disused treatment in skeletal muscle atrophy model mice by hindlimb suspension.

**MATERIALS AND METHODS**

This study was conducted with the approval of the research ethics committee of the institute to which we belong (approval number 30-16, Saitama Prefectural University). We used twenty wild-type mice (C57BL6, male, 12 weeks old) and randomly divided them into three groups: without any control, with NMES, and without NMES. Specific details of the respective groups are as follows (Table 1):

Group A: Control (non-COPD, non-amyotrophia, non-NMES) (n=8); Group B: COPD, amyotrophia with NMES, frequency of 3 times/week, a period of 12 weeks (n=6); and Group C: COPD, amyotrophia without NMES, a period of 12 weeks (n=6).

The study was commenced with the groups of mice at 12 weeks old, and a COPD disease model was created to perform prior studies on the mice aged from 12 to 14 weeks (21 days) in group B and group C; the mice aged from 15 to 16 weeks (14 days) were used to create a disuse model by suspending their hind limbs, and then NMES was applied to the mice aged 17–28 weeks (12 weeks) by using an Espurge, an electro stimulator (Itolator KK, Japan). NMES was applied percutaneously so as to exert the maximum isometric plantarflexion torque on the left and right gastrocnemius muscles under anesthesia with an inhalational anesthetic (isoflurane, flow rate 0.6 L/m, concentration 2%). Specifically, ten contractions/sets consisting of 3-s on and 7-s off intervals, and a total of six sets consisting of three consecutive sets with 5-min rest and another three consecutive sets were performed (150-μs pulse duration, 50-Hz frequency, 15.0-mA pulse intensity). NMES was applied every other day for a total of 36 times for 12 weeks, and tissues (lungs and gastrocnemius muscle) were collected 48 hours after the last NMES.

Many reports have been made on the setting and period of NMES. The setting was determined based on the method established as a resistance training model for the promotion of gene expression. Note that it has been reported that NMES application in 4-week-old rats resulted in muscle hypertrophy, while there have been fewer reports on muscle strength. All the mice were bred under an environment with a room temperature at 20 °C ± 1 °C, relative humidity at approximately 50%, and light-dark cycles for 12 hours (7–19 h), allowing them to freely eat solid food without setting any restrictions on their activities.

In this study, we combined the CDPD disease model and the COPD amyotrophia model mice. For the creation of the COPD disease model, we intratracheally administered a tobacco solution using a syringe under inhalation anesthetics (iso-

| Table 1. Specific details of the respective groups |
|-----------------------------------------------|
| COPD model (ages from 12 to 14 weeks) | Amyotrophia (hind limb suspension) (ages from 15 to 16 weeks) | NMES (ages from 17 to 28 weeks) |
| A | non | non | non |
| B | with | with | with |
| C | with | with | without |

COPD: chronic obstructive pulmonary disease; NMES: neuromuscular electrical stimulation. Group A (A): Control (non-COPD/non-amyotrophia/non-NMES), Group B (B): COPD/amyotrophia/with NMES, Group C (C): COPD/amyotrophia/without NMES (see the text in detail).
fluoride; flow rate 0.5 L/min; concentration 2%). The frequency of administration was five times/week, and each dosage volume was 50 µL. Administration of the mixture of tobacco solution (four-fold dilution) + Lipo polysaccharide (LPS; 10 µg) was performed when mice were 12 and 13 weeks old, followed by the administration of an only-tobacco solution (four-fold dilution) at 14 weeks old[12]. The tobacco solution was created by connecting the filter mouthpiece to the introduction tube of a suction pump and the connection tube was bubbled in saline (50 mL). The tobacco used to make tobacco solution was Peace 40 piece package (Nicotine 2.3 mg/piece), and the measurement value of absorbance was 0.902 (500-fold dilution). For the creation of the COPD amyotrophia model, we performed hind limb suspension, according to the previous studies[9].

For muscular strength, maximum values of limbs were continuously measured three times (10 second rest between measurements) with small animal grip measuring equipment (GPM-100B, Melquest K.K., Japan), and average values were calculated[7]. Measurements were performed before hindlimb suspension (Pre) and at the time of sacrifice (Post). Note that muscle strength was measured not only in the lower limbs that underwent NMES but in all four limbs.

For respiratory function, minute volume, tidal volume, and respiratory rate at rest were measured using a respiratory flow heads for mice (AD Instruments K.K., USA). The body weight was measured using an electrical animal balance KN type (Natsume Seisakusho K.K., Japan). The muscle strength and respiratory function were measured at 12 weeks of age (Pre) and at the time of euthanization (Post), and the body weight was measured every week.

After washing the collected tissues (the right middle lobe) with saline, they were fixed with formalin to create paraffin blocks, and then horizontal slice sections (6 µm thick) were created. For morphological analysis of the alveolar spaces (mean linear intercept), we performed Victoria Blue staining, which is commonly used. We took images of the stained slice sections using the fluorescence microscope BZ-X700 (KEYENCE Co., Japan). We used the Hybrid cell count BZ-H3C (KEYENCE Co., Japan) for analysis to measure the mean alveolar diameter in the randomized tissue images (350 × 280 µm). The paraffin sections were deparaffinized using xylene and ethanol and then the antigens were retrieved using Proteinase K (OMEGA bio-tek, USA). For the primary antibody, the F4/80 antibody, tissue sections were boiled in 1 mM EDTA, pH 8.0 for 10 min, followed by cooling at room temperature for 20 min.

Next, for suppression of endogenous peroxidase activation, we soaked them in a PBS solution including 3% hydrogen peroxide solution (FUJIFILM, Japan) for ten minutes, performed avidin blocking (VEC Labo., USA) for 15 minutes for non-specific staining blocking, and made them react in the PBS solution for 30 minutes by adding goat serum for non-specific reaction blocking. As the primary antibody, we used the CD206 antibody (ab64693, Abcam plc., Japan; diluted concentration 1/1000) and F4-80 antibody (ab100790, Abcam plc.; diluted concentration 1/150), Matrix metalloproteinases12 (MMP12) antibody (ab128030, Abcam plc., Japan; diluted concentration 1/150). For the secondary antibody, after dropping the IgG antibody derived from goat (VEC Labo.). For the amplification reaction, we performed the ABC process on the VECTA ABC Rabbit IgG Kit (VEC Labo.) to emit colors. We used goat serum added PBS without the primary antibody during the staining of the primary antibody to create negative controls. The images of the stained sections using fluoroscope BZ-X700 (KEYENCE Co., Japan). During the image analysis, we removed the nucleus, defined a threshold by using Photoshop (Adobe Inc., USA), and measured the positive cell count semiquantitatively. Two independent co-investigators measured the randomly picked images and calculated their mean values. The primary antibodies used at this point were CD68 and CD206, as the alveolar macrophage markers, and F4/80 as the interstitial lung marker.

The RNAs of the collected tissues (right upper lobe and right lateral head of gastrocnemius muscle) were stabilized by the RNA later Stabilization Solution (Thermo Fisher Scientific K.K., Japan) and stored at −20 °C. We extracted the total RNA for the RNA analysis of the tissues, according to the protocol of the RNeasy Fibrous Tissue Mini Kit (QIAGEN K.K., Japan). After extraction of the total RNA, we measured the total RNA concentration using NanoDrop Lite (Thermo Scientific, USA), and measured the total RNA concentration using NanoDrop Lite (Thermo Scientific, USA). Each sample was diluted until the total RNA concentration became even and cDNA was synthesized according to the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific K.K.) protocol. For real-time PCR, we performed the Taqman probe method using the PCR analysis system (Chromo4, BIO-RAD, USA) for 40 cycles. To do a comprehensive analysis based on the previous studies, we determined the target genes as 18 factors associated with inflammation (TNF-α, Interleukin-4, among others), nine factors of mitochondria (peroxisome proliferator-activated receptor gamma coactivator 1α: PGC-1α, among others), two factors associated with amyotrophy (MuRF1 and MAFBx), six factors of protease associated with emphysema (Cathepsins-L, etc.), and GAPDH as the endogenous standard gene (Table 2)[9]. The relative value was calculated from Ct values obtained by the comparative Ct method (ΔΔCt method), and they were compared setting the B group as the reference value (1.0). Furthermore, for the difference of exercise durations, we calculated the mean value from each Ct value from the B and the D groups to obtain the ratio of the A and the C groups (A/B and C/D). From the ratios obtained, we calculated the relative values and made comparisons by setting A/B as the reference value (1.0).

SPSS (Ver. 26 for Windows) was used as statistical software, and the Shapiro-Wilk test verified normality. A one-way analysis of variance, multiple comparisons (Tukey’s test), and t-test were employed to assess the presence of significant differences in muscle strength, respiratory function, body weight, and mean linear intercept; and the Kruskal-wallis test was employed to assess mRNA level. Note that all significance levels were defined as less than 5%.

**RESULTS**

Comparing Pre and Post-intervention, the body weight of all groups significantly increased in the post-intervention (p<0.01, Table 3). No significant difference in muscle strength was observed in the post-intervention group A, while a
significant reduction in muscle strength was observed in group B and group C (p<0.01, Table 3). That is, the muscle strength was reduced regardless of NMES application. Although there was no significant difference in respiratory function between group A and group B post-intervention, there was a significant rise in minute ventilation and respiratory rate in group C (p<0.05, Table 3).

The morphological of the alveolar spaces significantly raised in group B and group C as compared to that in group A (p<0.01–0.05, Table 4), while no significant difference was observed between group B and group C (Fig. 1). That is, the mean linear intercept increased regardless of NMES application. At the same time, a significant rise was observed with F4/80 and MMP12 levels in group B and group C compared to those in Group A (p<0.01, Table 4); a significant decrease in group B was observed in comparison with group B and group C (p<0.01–0.05). In other words, it suggested that F4/80 and MMP12 were affected by NMES application.

In the lungs, there was no significant difference in IL-6 levels between group B and group C compared to those in Group A, while a significant decrease was observed in group B when compared to that in group C (p<0.05, Fig. 2). Also, there was no significant significant decrease in Cathepsin-L levels in group B compared to those in group A (p<0.05), while no significant difference was observed between group B and group C. There was a significant increase in TIMP3 (Tissue inhibitor of metalloproteinases 3) levels in group C compared to those in group A (p<0.05), in contrast, no significant difference was observed by comparing group B and group C. That is, NMES contributed to a significant decrease in IL-6 levels in the lungs.

There was a difference in MuRF1 expression in skeletal muscle between group B and group C compared to that in group A, while a significant decrease was observed in group B compared to group C (p<0.05, Fig. 3). Furthermore, the MAFbx levels significantly decreased in group B compared to those in group A (p<0.05). At the same time, no difference was observed by comparing groups B and C. Opal1 levels (optic atrophy 1) significantly decreased in group B and C compared to those in group A (p<0.05, respectively), while no significant difference was observed by comparing group B and group C.

There was a significant difference in IL-6 levels in group B compared to those in group A (p<0.01), while no significant difference was observed between group B and group C. That is, NMES application contributed to a significant decrease in MuRF1 expression in the skeletal muscle.

| Table 2. Primer sequences used for quantitative real-time PCR |
|---------------------------------------------------------------|
| **Mouse Sequence (ABI TaqMan Gene expression assays)** | **Tissues** |
| Gene name | TaqMan probe set ID |
| TNF-α | Mm00443258_m1 |
| IFN-γ | Mm01168134_m1 |
| IL-4 | Mm00445259_m1 |
| IL-6 | Mm00446190_m1 |
| IL-13 | Mm00434204_m1 |
| Nrf2 | Mm00477784_m1 |
| Cathepsin-L | Mm00515597_m1 |
| Cathepsin-S | Mm01255859_m1 |
| TIMP3 | Mm00441826_m1 |
| Cystatin-C | Mm00438347_m1 |
| MnRF1 | Mm01185221_m1 |
| MAFbx | Mm00499523_m1 |
| MEF2 | Mm00485897_m1 |
| Opal1 | Mm01349707_m1 |
| Mfn2 | Mm00500120_m1 |
| Fis1 | Mm00481580_m1 |
| Drpl | Mm01342903_m1 |
| IL-6 | Mm00446190_m1 |
| Nrf1 | Mm01135606_m1 |
| PGC-1α | Mm0047183_m1 |
| GAPDH | Mm99999915_g1 |

TNF-α: Tumor necrosis factor-α, IFN-γ: Interferon-γ, IL: Interleukin, Nrf2: Nuclear respiratory factor 2, TIMP3: Tissue inhibitor of metalloproteinases 3, MnRF1: Muscle RING finger protein-1, MAFbx: skeletal muscle atrophy F-box/Atrogin-1, MEF2: myocyt enhancer factor 2 (S100B), Opal1: optic atrophy 1, Mfn2: mitofusin 2, Fis1: fission protein 1, Drpl: dynamin related protein 1, Nrf1: nuclear respiratory factor 1, PGC-1α: PPAR gamma coactivator 1-α, GAPDH: housekeeping gene.
Table 3. Change in the Weight values and Muscular strength, Respiratory function

|          | Weight (g) | Muscular strength (/Weight*100, g) | Minute ventilation: VE (mL/min) | Average ventilation volume: VT (mL) | Number of breaths: f (min) |
|----------|------------|-----------------------------------|---------------------------------|-----------------------------------|--------------------------|
|          | Pre        | Post**                            | Pre    | Post**                           | Pre    | Post | Pre    | Post | Pre    | Post | Pre    | Post |
| A        | 26.2 ± 1.0 | 27.9 ± 1.9††                      | 9.6 ± 1.4 | 9.8 ± 1.8                         | 56.4 ± 7.9 | 58.2 ± 10.8 | 0.30 ± 0.03 | 0.28 ± 0.05 | 185.5 ± 18.0 | 206.7 ± 22.6 |
| B        | 25.3 ± 1.0 | 32.1 ± 1.9††                      | 10.4 ± 1.1 | 6.8 ± 0.8††                      | 65.2 ± 5.5 | 65.0 ± 11.5 | 0.28 ± 0.13 | 0.31 ± 0.03 | 194.5 ± 19.2 | 207.8 ± 26.3 |
| C        | 25.7 ± 1.2 | 38.7 ± 3.6††                      | 10.5 ± 0.6 | 6.4 ± 0.9††                      | 57.0 ± 11.5 | 73.5 ± 7.0†† | 0.20 ± 0.14 | 0.28 ± 0.12 | 194.3 ± 22.9 | 218.5 ± 9.9† |

Values are mean ± SD. *p Value by ANOVA test for the comparison between 3 groups and Tuky test for the Weight values and Muscular strength, Minute ventilation(VE), Average Ventilation volume (VT), Number of breaths(f), T test for the comparison between the Weight values and Muscular strength, VE, VT, f. Group A (A): Control (non-COPD/non-amyotrophia/non-NMES), Group B (B): COPD/amyotrophia/with NMES, Group C (C): COPD/amyotrophia/without NMES (see the text in detail). **p<0.01, *p<0.05, †p<0.05 in comparison with A, aa: p<0.01 in comparison with A, a: p<0.05 in comparison with B, bb: p<0.01 in comparison with B, Pre vs. Post, ††p<0.01, Pre vs. Post.

Table 4. Change in the mean linear intercept values and Immunol staining

|          | Mean linear intercept** (μm) | Immunol staining CD206 (count)** | Immunol staining F4/80 (count)** | Immunol staining MMP12 (count)** |
|----------|------------------------------|---------------------------------|----------------------------------|---------------------------------|
| A        | 31.5 ± 37.8                  | 7.5 ± 2.4                       | 9.3 ± 1.7                        | 5.8 ± 13.0                      |
| B        | 50.8 ± 42.0††                | 17.2 ± 7.4                      | 19.8 ± 1.8††                     | 16.7 ± 2.3††                    |
| C        | 54.3 ± 42.7††                | 17.8 ± 5.6††                    | 25.7 ± 3.9††                     | 20.7 ± 3.1††                    |

Values are mean ± SD. p value by ANOVA test for the comparison between 3 groups and Tuky test for the Mean linear intercept and Immunol staining. Group A (A): Control (non-COPD/non-amyotrophia/non-NMES), Group B (B): COPD/amyotrophia/with NMES, Group C (C): COPD/amyotrophia/without NMES (see the text in detail). **p<0.01, *p<0.05 in comparison with A, aa: p<0.01 in comparison with A, a: p<0.05 in comparison with B, bb: p<0.01 in comparison with B.

Fig. 1. a–c) Representative photomicrographs of Victoria Blue Staining pulmonary parenchyma of a) Group A (A): Control (non-COPD/non-amyotrophia/non-NMES), b) Group B (B): COPD/amyotrophia/with NMES, c) Group C (C): COPD/amyotrophia/without NMES (see the text in detail). Scale bars: 50 μm.
DISCUSSION

Based on skeletal muscle loss and qualitative changes, COPD has been found to cause skeletal muscle dysfunction\(^2\). In particular, approximately one-third of COPD patients have reduced muscle strength in their lower limbs, and this reduction in muscle strength is considered an important determinant factor of exercise tolerance\(^2,19\). It has been reported that NMES, which is applied to improve such reduced muscle strength, has a muscle-strengthening effect even in the poor systemic condition\(^19\). Besides, NMES has the same effect as exercise, and it has been reported that eight weeks of NMES implementation increases muscle strength by approximately 10\(^\circ\)\(^20\). In the present study, our test results of NMES application indicated different results from previous studies\(^13–17,19,20\) as a significant decrease in muscle strength was observed. However, muscle strength measurement by treadmill running for 12 weeks on COPD mice was performed in the same manner in the present study, and the results showed that muscle strength values were maintained\(^21\). In other words, it was possible that the difference in the intervention methods affected muscle strength because only the gastrocnemius muscle was stimulated by NMES.
while running on the treadmill was a full-body exercise.

Generally, expression of skeletal muscle atrophy-related factors such as MuRF1 was enhanced, and muscle proteins were degraded in skeletal muscle dysfunction, thereby causing skeletal muscle atrophy. Additionally, it has been reported that NMES application for denervation-induced skeletal muscle atrophy reduces the factors related to skeletal muscle atrophy. In the present study, MuRF1, a skeletal muscle atrophy-related factor, showed significantly decreased expression in group B compared to that in group C. Hence, this indicates that NMES suppresses the muscle protein degrading system, the results were similar to previous studies on muscle exercise. However, this time, the results were different from previous studies because no changes have been observed in factors such as IL-6. Previous studies have reported that NMES in wild-type mice increases mitofusin 2 (Mfn2) expression, which is involved in the fusion of IL-6 and mitochondria. However, the use of different types of mice might influence the results, as the COPD mice were used this time, whereas wild-type mice were used in our previous studies. It has been reported that COPD skeletal muscle dysfunction does not increase carboxylation of carbonic anhydrase, decreases muscle creatine kinase activity, and increases inflammatory cytokines such as IL-6. Thus, it was assumed that skeletal muscle dysfunction in COPD had an oxidative proteins modification effect on muscle proteins without causing intramuscular inflammation. Therefore, it was possible that COPD mice had a direct skeletal muscle disorder due to tobacco use, and the mitochondrial function or inflammatory cytokines in skeletal muscle may not be affected. In the future, it is necessary to study the oxidative modification effect on muscle proteins.

COPD is caused by chronic inflammation of the lungs in which neutrophils, macrophages, and lymphocytes are involved. There are alveolar macrophages and pulmonary interstitial macrophages present in the lungs, and the hyperfunction of macrophages is involved in the development of emphysema in the lungs. Shibata et al. reported the process of emphysema formation in COPD model mice that is produced using elastase. According to the report, IL-4 secreted by basophils transforms lung-infiltrating monocytes into pulmonary interstitial macrophages and is involved in the protease (MMP12) produced by the pulmonary interstitial macrophages. Besides, it has been reported that exercise in COPD mice causes changes in alveolar morphology, and leads to a decrease in antiprotease (tissue inhibitor of metalloproteinases (TIMP) expression and a decrease in the morphological of the alveolar spaces and macrophages). In COPD, the balance between protease and antiprotease levels are lost, and proteases are dominant. Such a deteriorated balance between protease and antiprotease can be observed in lung emphysema. In this study, the morphological of the alveolar spaces of group B was no different from that of group C, while the reduction of F4/80 and MMP12 and the tendency of reduced TIMP3 were observed. In other words, NMES led to reduced levels of pulmonary interstitial macrophages (F4/80) as well as decreases in protease (MMP12) and antiprotease (TIMP3) produced by macrophages. It has been generally understood that the mechanism of alveolar destruction involves an imbalance of protease and antiproteases, oxidative stress, and alveolar cell apoptosis. It has been reported that oxidative stress is caused by the disrupted balance between oxidants and antioxidants, as well as activation of inflammatory genes and inactivation of antiproteases. Moreover, it has been assumed that it may be associated with the destruction of the extracellular matrix due to the mechanisms that cause cell death and abnormal clearance. Therefore, the fact that NMES does not affect the morphological of the alveolar spaces needs to be examined from the aspects of oxidative stress and apoptosis along with protease expression levels.

COPD produces inflammatory cytokines from inflammatory cells such as macrophages and epithelial cells to enhance the inflammatory response, as well as release proteases and oxidants that damage the lungs. It has been reported that such inflammatory cytokines include TNF-α and IL-1, IL-4, IL-6, and IL-13. In this study, the data indicates that NMES significantly decreases IL-6 levels in the lungs along with a decreasing trend in IL-4 and IL-13 levels. IL-6 is typically one of the systemic inflammation markers and previous study has reported that administration of elastase to IL-6 gene-deficient mice reduces lung inflammation. Furthermore, it has been indicated that IL-6 is highly expressed in patients with COPD, and it is a prognostic biomarker. For this reason, it was assumed that NMES reduced lung inflammation and acted as an anti-inflammatory agent. On the other hand, IL-6 protects cells against exposure to oxidative stress in IL-6 gene-deficient mice. It has also been reported that IL-6 activates regeneration, growth, and proliferation of muscle tissues in the skeletal muscle, thereby enabling tissues to grow and maintain. Thus, many aspects of how the changes in IL-6 caused by NMES affect other organs, including the lungs, have to be unveiled.

IL-4 and 13 generally share receptors and signal transduction pathways and indicate similar physiological activities. It has been reported that transgenic mice in which IL-13 is overexpressed explicitly in the lungs exhibit morphological changes in the alveoli due to an increase in proteases such as Cathepsin-L. Also, our previous study reported that treadmill running in COPD mice reduced IL-4 and IL-13, TNF-α levels, which are inflammatory cytokines in the lungs. In the present study, NMES showed a tendency to reduce Cathepsin-L levels. However, despite the fact that NMES is a stimulus for skeletal muscle growth, the underlying mechanism by which NMES affects inflammatory cytokine levels in the lungs has not been elucidated. Therefore, it was considered necessary to study the factors such as antiprotease SERPINE, which is a COPD susceptibility gene, the protease MMP9, and the pulmonary surfactant protein-D (SP-D), which is immunoregulatory in nature.

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**Conflict of interest**

There are no conflicts of interest in this study.
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