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Effects of Neuromuscular Electrical Stimulation in Combination with Glutamine Administration on Skeletal Muscle Atrophy in Colon-26 Tumor-Bearing Mice

Daisuke Tatebayashi, Koichi Himori, Yuki Ashida, Takashi Yamada*

Graduate School of Health Sciences, Sapporo Medical University, Sapporo, Hokkaido 060-8556, Japan

***Corresponding author:** Takashi Yamada, takashi.yamda1976@sapmed.ac.jp

Abstract: The depressed protein synthetic response, a phenomenon termed anabolic resistance, has been shown to be involved in muscle wasting induced by cancer cachexia. Moreover, a positive relationship between the protein synthetic rate and intracellular glutamine (GLN) concentration has been found in skeletal muscles. This study investigated the effects of neuromuscular electrical stimulation (ES) and GLN administration on muscle wasting and GLN metabolism in colon-26 (C-26) tumor-bearing mice. CD2F1 mice were divided into 8 groups: control (CNT), CNT+ES, CNT+GLN, CNT+ES+GLN, C-26, C-26+ES, C-26+GLN, C-26+ES+GLN. Cancer cachexia was induced by subcutaneous injection of C-26 cells and developed for four weeks. ES was performed on the left plantar flexor muscles every other day, and GLN (1 g/kg) was administered daily intraperitoneally starting one day after the C-26 injection. Tumor-free body mass and fast-twitch gastrocnemius (Gas) muscle weight were lower in the C-26 group than in the CNT group (-19% and -17%, respectively). Neither ES training nor GLN administration, alone or in combination, ameliorated the loss of Gas muscle weight in the C-26 mice. However, ES training in combination with GLN administration inhibited the increased expression of GLN synthetase (GS) in the C-26 muscles. Thus, it is likely that GLN plays a critical role in muscle protein metabolism and, therefore, can be targeted as a tentative treatment of cancer cachexia.

Keywords: Cancer cachexia; Anabolic resistance; Muscle atrophy; Glutamine; Neuromuscular electrical stimulation

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1. Introduction

Cancer cachexia is a progressive metabolic disorder characterized by significant skeletal muscle atrophy [1]. Unlike the relatively preserved skeletal muscle mass and decreased fat tissue in a state of starvation [2], cancer-induced muscle atrophy is reported to be difficult to improve with conventional nutritional support [3]. Furthermore, muscle wasting due to cancer cachexia not only deteriorates physical function and quality of life but also leads to a loss of resilience to undergo comprehensive treatment [4]. Additionally, recent studies have

shown that the reduction in skeletal muscle mass in cancer patients and cancer model animals is associated with poor prognosis [5,6], making the inhibition of muscle atrophy associated with cancer cachexia an important challenge in cancer rehabilitation.

In cancer cachexia, resistance to muscle protein assimilation has been observed [7]. This resistance, which reduces the rate of muscle protein synthesis in addition to increased resistance training and amino acid intake, is a phenomenon where the synthesis of muscle protein decreases compared to normal conditions [8,9]. Muscle resistance to assimilation with age is believed to involve decreased translation from mRNA to protein, hindrance in amino acids uptake in muscle, and insulin resistance [9]. Furthermore, in cancer cachexia, it has been shown that the reactivity of the mammalian target of the rapamycin complex 1 (mTORC1) signaling pathway, which plays a crucial role in regulating muscle protein translation, is diminished in response to muscle contraction and glucose administration [10].

Glutamine (GLN), the most abundant non-essential amino acid in the body, is primarily produced in skeletal muscles through the action of glutamine synthetase (GS) and accounts for approximately 60% of the free amino acid pool within muscle cells [11]. In conditions like sepsis or trauma, the demand for GLN in the body exceeds the supply from skeletal muscles, causing a maximum decrease of up to 50% in muscle and up to 30% in blood GLN concentrations, necessitating external supplementation [12]. Therefore, despite being a non-essential amino acid, GLN is sometimes referred to as a conditionally essential amino acid. Interestingly, MacLennan *et al*. demonstrated that increasing GLN concentration in muscle cells by reinfusing a high-concentration GLN solution into muscles increased the muscle protein synthesis rate [13]. Furthermore, with tumor growth, the release of GLN from skeletal muscles increases twofold [14], leading to a decrease in muscle GLN concentration [15]. GLN supplementation has been shown to prevent the decrease in GLN concentration in skeletal muscles of cancer model rats and improve the reduced rate of muscle protein synthesis [16-18]. On the other hand, some reports suggest that there is no clear connection between muscle GLN concentration and protein synthesis rate. Damink *et al*. demonstrated that even when muscle GLN concentration was reduced by 40% using GS inhibitors, there was no change in muscle protein synthesis rate [19]. Therefore, the causal relationship between the increased GS expression in muscle cells and decreased GLN concentration in cancer cachexia and muscle protein assimilation resistance has not yet been fully elucidated.

Cancer-induced muscle atrophy is notably observed in fast-twitch muscles, which are generally less prone to inactivity-induced atrophy [20]. Therefore, to prevent muscle atrophy through exercise, it is believed that high-intensity training that recruits fast-twitch muscles is necessary. However, in many cases, it is difficult to actively prescribe high-intensity exercise therapy that can mobilize fast-twitch muscles to cancer patients who experience a decline in physical strength and fatigue. Neuromuscular electrical stimulation (ES) is a method that induces involuntary muscle contractions by electrically stimulating peripheral nerves and muscles and is widely used for rehabilitation and training purposes [21]. ES has the advantage of recruiting both slow and fast-twitch muscle fibers without following the size principle and can be performed even in a resting state [22]. However, in reports where ES training was applied to the quadriceps muscles of lung cancer patients, no suppression of muscle atrophy was observed [23]. Furthermore, in previous studies that investigated the effects of isometric and concentric contractions by ES training in cancer model mice, it was reported that muscle atrophy was not reduced [24,25]. It is believed that the involvement of muscle protein assimilation resistance in cancer cachexia is responsible for these results, but whether muscle cell GLN metabolism is involved in this mechanism has not been sufficiently studied.

Thus, this study aimed to clarify the role of GLN metabolism in cancer-induced muscle atrophy and investigate the effects of ES training and GLN administration, alone and in combination, on muscle mass and

GS expression in colon-26 (C-26) tumor-bearing mice, a widely used model for cancer cachexia.

2. Methods

2.1. Ethical Considerations

This study was conducted with the approval of the Sapporo Medical University Animal Experiment Committee (Approval No. 15-044_17-078) and in accordance with the regulations set by the committee.

2.2. Experimental animals and protocol

Five-week-old male CD2F1 mice were used in this study. They were randomly divided into control (CNT) mice and C-26 mice, further subdivided into GLN non-administration groups (CNT group and C-26 group) and GLN administration groups (CNT+GLN group and C-26+GLN group), with each group containing 5 mice. The right hind limbs of these groups were designated as the non-training side, and the left hind limbs were designated as the training side (CNT+ES group, C-26+ES group, CNT+GLN+ES group, and C-26+GLN+ES group), resulting in a total of 8 groups. The mice had *ad libitum* access to water and food and were kept in a room maintained at a constant temperature of 24 ± 2°C with a 12-h light-dark cycle. C-26 cells were cultured at 37°C under 5% CO_2 in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and tumor induction was performed by injecting 5×10^5 C-26 cells suspended in phosphate-buffered saline (PBS) into the subcutaneous region of the abdomen, following the method of Murphy *et al*. [26]. The GLN administration group received GLN (1 g/kg; HyClone) dissolved in PBS via intraperitoneal injection daily from the day following C-26 seeding until the day of dissection, following the method of Meador *et al*. [27]. The group without GLN administration received an equivalent amount of PBS in the same manner. ES was performed every other day from the day following C-26 seeding for a total of 14 sessions. After 24 hours from the final ES session (28 days after cancer cell seeding), the body weight of each mouse was measured, and the tumor mass and gastrocnemius muscle were collected. Additionally, the weight of the collected tumor mass and gastrocnemius muscle was measured, and the gastrocnemius muscle was rapidly frozen in liquid nitrogen and stored at -80°C until analysis.

2.3. ES load

The left hind limb of the mouse was fixed using tape in the dorsally recumbent position in an ankle dorsiflexion torque measurement system under isoflurane inhalation anesthesia (2%, 2 L/min). The dorsiflexion angle of the ankle joint during fixation was set at 20° dorsiflexion (**Figure 1**). To minimize the influence of body movement induced by exercise, the trunk was taped to immobilize it. To reduce skin resistance to electrical stimulation, the left hind limb of the mouse was shaved immediately before each ES session, and surface electrodes connected to an electrical stimulator (Nihon Kohden Co., Ltd.) were attached to the posterior and anterior aspects of the lower leg. The electrical signal of the maximal isometric torque (MIT) for ankle dorsiflexion was amplified by an amplifier and observed as a waveform using data analysis software (LabChart, Ver. 8, ADInstruments). ES training was modified based on the method of Gondin *et al*. [28]. MIT was measured under the stimulation conditions of a stimulation frequency of 50 Hz and a stimulation intensity of 45 V (maximal upper stimulation). Subsequently, MIT was gradually increased to 60% of the obtained MIT as the target torque under the conditions of a stimulation frequency of 50 Hz, a stimulation time of 2 s, and a rest time of 4 s, until the voltage reached 45 V. The stimulation was terminated when the torque of 60% MIT could no longer be exerted even at 45 V. This was considered one set, and three sets were performed in 1 day, with a 1-minute break between sets.

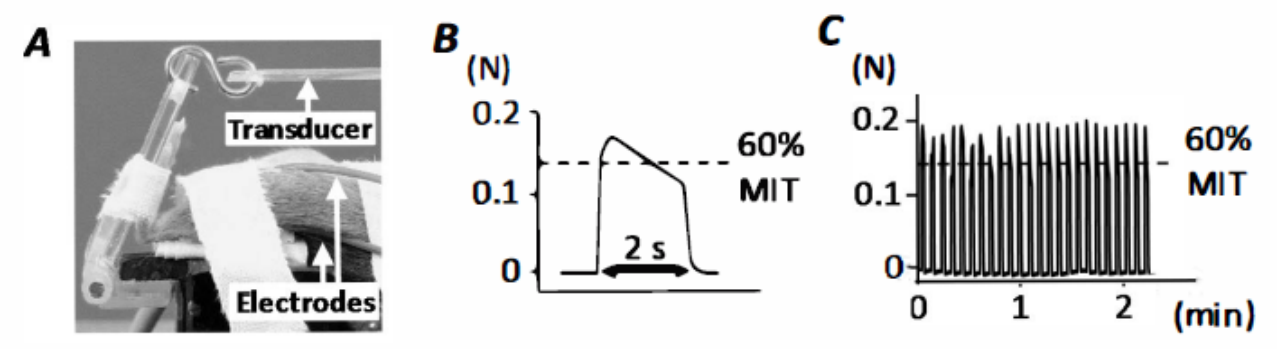

Figure 1. Procedure for neuromuscular electrical stimulation training on mouse plantar flexor muscles. **(A)** Under anesthesia, the animal was placed in a supine position and the experimental limb was attached to a footplate connected to a force transducer. The foot was placed at 20° angle of dorsal flexion. Surface electrodes were placed on the front and back surfaces of plantar flexor muscles. **(B and C)** Torque traces were displayed on a monitor and the stimulation intensity was progressively increased throughout the stimulation period to maintain a peak torque corresponding to 60% of the maximal isometric torque (MIT).

2.4. Open field test

To account for the influence of ES training, the measurement day was set as 2 days before the dissection day without ES training (26 days after cancer cell seeding). Since mice are nocturnal, the open field test was conducted in the dark at night. The method was modified from Shimada *et al.* [29]. A 60 cm^2 field was divided into nine regions of 3×3 (each 20 cm^2), and the number of times the mouse crossed the dividing lines within 10 minutes was recorded. During the measurement, the observer avoided moving and took great care not to affect the mouse's behavior. The equipment was cleaned after each measurement to eliminate any lingering odors.

2.5. Western blotting

Gastrocnemius muscles (approximately 20 mg) were homogenized in 40-fold Tris solution (10 mM Tris maleate, 35 mM NaF, 1 mM $NaVO_4$, 1% Triton X 100, 1 tablet of protease inhibitor/50 mL). Protein concentration was quantified using the Bradford method [30]. Total muscle proteins were loaded onto gels (4–15% Mini-PROTEAN TGX Stain-Free™ Gels, Bio-Rad) at 20 µg per lane and electrophoresed at 150 V for 45 minutes. All bands on the gel were visualized using a Stain-Free imager (Bio-Rad) and the band intensity of the myosin heavy chain (MyHC) was calculated as a ratio to the total band intensity of each lane using Image Lab Software (Bio-Rad). Subsequently, proteins were transferred from the gel to a membrane using a cell (Mini PROTEAN II Cell, Bio-Rad) at 100 V for 180 minutes. The membrane was blocked in a washing solution (40.3 mM Tris-HCl, 9.7 mM Tris-base, 150 mM NaCl, 0.02% Tween 20) containing 3% (w/v) skim milk for 1 hour and then incubated overnight at 4°C with primary antibodies (anti-GS antibody, 20,000-fold dilution, GeneTex; anti-GAPDH antibody, 2,000-fold dilution, Wako). After three washes with washing solution for 10 minutes each, the membrane was incubated with a secondary antibody, diluted 10,000-fold, at room temperature for 1 hour, followed by further washing. A chemiluminescence reagent (Immobilon, Millipore) was used for visualizing the target proteins, and band detection and quantification were performed using a chemiluminescence detection device. The expression level of GS was corrected by the expression level of GAPDH.

2.6. Statistical analysis

All data are presented as mean ± standard error (SEM). The comparison of tumor weights in the C-26 group and

C-26+GLN groups was performed using an unpaired *t*-test. On the other hand, a two-way analysis of variance (ANOVA) was used to compare the body weight and activity level in each group, while a three-way ANOVA was used to compare gastrocnemius muscle weight, GS, and MyHC expression levels. In case of significance, a Student-Newmann-Keuls test was performed as a post-hoc test. Statistical analysis was conducted using Statview 5 (SAS Institute Inc.), and the significance level was set at 5%.

3. Result

3.1. Changes in body mass and tumor mass

Table 1 shows the values of body mass and tumor mass in each group. In terms of body mass, a two-way analysis of variance with C-26 seeding and GLN administration as factors showed a significant main effect of C-26 seeding ($P < 0.05$), but no significant interaction was observed ($P > 0.05$). Multiple comparison tests revealed that the body mass in C-26 mice was 19% lower compared to CNT mice, and C-26+GLN mice had a 21% lower body mass compared to CNT mice ($P < 0.05$).

Table 1. Effects of tumor-bearing and glutamine administration on body and tumor mass in control and colon-26 mice

	CNT ($n = 5$)	CNT+GLN ($n = 5$)	C-26 ($n = 5$)	C-26+GLN ($n = 5$)
Tumor-free body mass (g)	23.8 ± 0.4	23.2 ± 0.5	19.2 ± 1.0*†	18.7 ± 0.5*†
Tumor mass (g)	-	-	6.5 ± 0.7	4.5 ± 0.7

Values are means ± SEM. Abbreviations: CNT, control; C-26, colon-26; GLN, glutamine. $P < 0.05$ versus *CNT and †CNT+GLN.

In the case of gastrocnemius muscle wet weight, a three-way ANOVA with C-26 seeding, GLN administration, and ES load as factors showed significant main effects of C-26 seeing and GLN administration ($P < 0.05$), while no significant second-order interactions or first-order interactions were observed. Multiple comparison tests indicated that the gastrocnemius muscle wet weight in the C-26 group was 17% lower than the CNT group ($P < 0.05$) (**Figure 2**). Contrary to expectations, both GLN administration and the combination of ES training and GLN administration did not prevent the decrease in gastrocnemius muscle wet weight in C-26 mice.

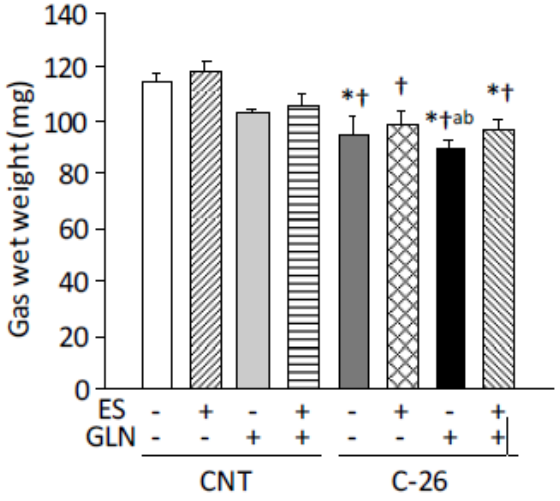

Figure 2. The wet weight of gastrocnemius (Gas) muscles from control (CNT) and colon-26 (C-26) mice with or without neuromuscular electrical stimulation (ES) training and/or glutamine (GLN) administration. Bars show the mean and SEM results from 5 mice per group. Statistical significance was set at $P < 0.05$ versus *CNT, †CNT+ES, [a]CNT+GLN, and [b]CNT+GLN+ES.

3.2. Changes in activity level

In the open field test, the number of times mice crossed the dividing lines within 10 minutes was 129 ± 12 times for CNT mice, 134 ± 15 times for CNT+GLN mice, 79 ± 21 times for C-26 mice, and 105 ± 11 times for C-26+GLN mice. A two-way ANOVA with C-26 seeding and GLN administration as factors showed a significant main effect of C-26 seeding ($P < 0.05$), but no significant interaction was observed ($P > 0.05$).

3.3. Changes in GS expression level

Figure 3A shows typical electrophoresis results of GS in each group. In a three-way ANOVA with C-26 seeding, ES load, and GLN administration as factors, significant main effects of C-26 seeding and ES load were observed ($P < 0.05$), and a first-order interaction between C-26 seeding and GLN administration was observed ($P < 0.05$). Multiple comparison tests revealed that GS expression in the CNT+ES group was approximately halved compared to the CNT group ($P < 0.05$). In contrast, the GS expression in the C-26 group, C-26+ES group, and C-26+GLN group was higher than that in the CNT group ($P < 0.05$). No significant difference was observed between the GS expression in the C-26+ES group as well as the C-26+GLN group and the C-26 group. However, GS expression in the C-26+GLN+ES group was lower than in the C-26 group ($P < 0.05$) (**Figure 3B**).

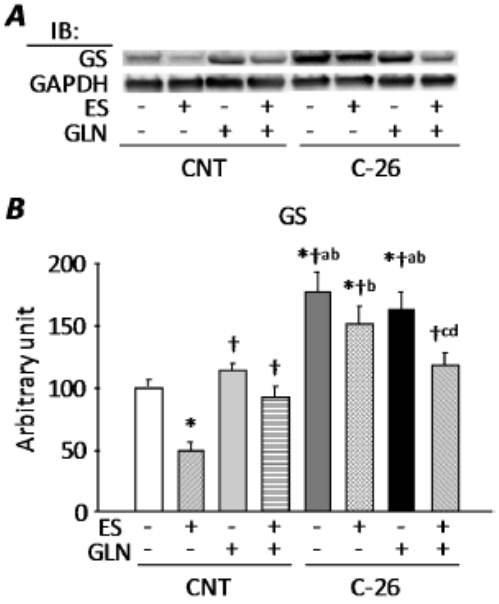

Figure 3. (A) Representative immunoblots (IB) for glutamine synthetase (GS) expression in gastrocnemius muscles from control (CNT) and colon-26 (C-26) mice with or without neuromuscular electrical stimulation (ES) training and/or glutamine (GLN) administration. **(B)** The expression levels of GS were quantified as arbitrary units normalized to GAPDH content. Data show the mean and SE< results from 5 muscles per group. Statistical significance was set at $P < 0.05$ versus *CNT, †CNT+ES, ^{a}CNT+GLN, ^{b}CNT+GLN+ES, ^{c}C-26, and ^{d}C-26+GLN.

3.4. Changes in MyHC expression level

Figure 4A displays typical electrophoresis results of MyHC in each group. The proportion of MyHc in total muscle protein quantified from Stain-Free images showed a significant main effect of GLN administration in a three-way ANOVA with GLN administration as a factor ($P < 0.05$), while no significant interactions were observed. The distribution of MyHC in whole muscle cell proteins is not altered in gastrocnemius muscles from colon-26 mice, as shown in **Figure 4B**.

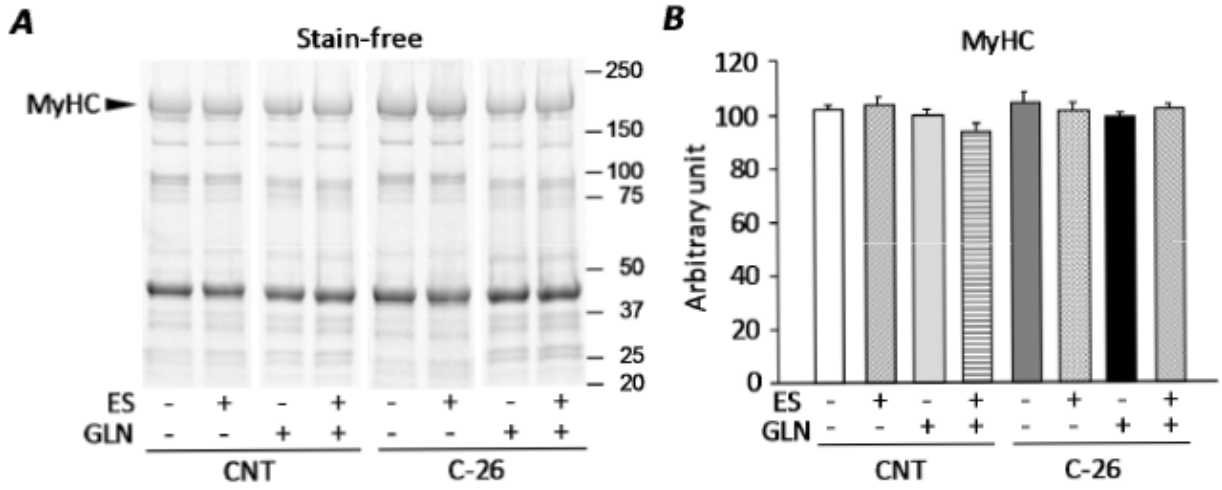

Figure 4. (A) Representative Stain-Free Image for myosin heavy chain (MyHC) in gastrocnemius muscles from control (CNT) and colon-26 (C-26) mice with or without neuromuscular electrical stimulation (ES) training and/or glutamine (GLN) administration. **(B)** The expression levels of MyHC were quantified as arbitrary units normalized to whole muscle proteins in Stain-Free images. Data show the mean and SEM results from 5 muscles per group.

4. Discussion

In this study, mice on the 28th day after seeding with C-26 cancer cells experienced a significant decrease in their body weight, approximately 20%, concomitant with tumor growth. Unintentional weight loss exceeding 5% within 6 months is one of the diagnostic criteria for cancer cachexia in humans [31]. Therefore, it is reasonable to assume that cancer cachexia occurred in this study due to the seeding of C-26 cells. It should be noted that mice subcutaneously seeded with C-26 cancer cells are reported to succumb to the disease in about 32 days [32], suggesting that the 28th day represents an advanced stage of the disease.

Under stressful conditions such as cancer cachexia, immune cells and the gastrointestinal tract have increased GLN consumption, leading to an increased release of GLN from the major GLN-producing organ, skeletal muscle [15,18]. Previous studies have demonstrated that increased GS activity in the skeletal muscles of methylcholanthrene-induced cancer model mice is associated with increased GLN release and decreased GLN concentration in the muscle [14]. This study is also consistent with previous studies as there was an increase in GS expression in the skeletal muscles of C-26 mice, a widely used model for cancer cachexia. Therefore, although this study did not measure GLN concentrations in the muscles, it is suggested that, in C-26 mice, the increased demand for GLN associated with cancer cachexia leads to an increase in GS synthesis in skeletal muscles and a decrease in intramuscular GLN concentration.

The mechanism behind this phenomenon is unclear, but it has been shown that endurance running exercise suppresses the increase in GS expression in mice skeletal muscle [33]. In line with these findings, this study showed that ES training suppressed GS expression in the skeletal muscles of CNT mice. However, in the skeletal muscles of C-26 mice, ES training showed a tendency to suppress the increase in GS expression, but no statistical difference was observed. Moreover, external GLN administration has been shown to prevent the decrease in intramuscular GLN concentration and the increase in GS activity in skeletal muscles of methylcholanthrene-induced cancer model mice [14]. However, in this study, GLN administration did not suppress the increase in GS expression in the skeletal muscles of C-26 mice. The reasons for these differences

are unclear but may be related to variations in GLN dosage and administration methods, as previous studies have utilized 2% GLN in free drinking water.

Multiple previous studies have reported a high correlation between the synthesis rate of muscle protein and intramuscular GLN concentration [13,34]. Additionally, GLN administration has been shown to improve ribosome size and distribution in skeletal muscle after surgical trauma [35], prevent the reduction of GLN content in the skeletal muscles of diabetic model mice, and suppress the inactivation of the mTORC1 signaling pathway, a major muscle protein synthesis pathway [36]. On the other hand, the fact that muscle protein synthesis rate does not change when intramuscular GLN concentration is significantly decreased over a certain period by GS inhibitors suggests that muscle protein synthesis rate may not be determined by intramuscular GLN concentration [19]. Supporting this idea, in this study, the combination of ES training and GLN administration suppressed the increase in GS expression in the skeletal muscles of C-26 mice but did not prevent the decrease in muscle weight. Additionally, the combination of GLN administration and low-intensity swimming exercise did not improve the atrophy of the gastrocnemius muscle in Walker 256 cancer model mice [37]. Therefore, based on these findings, it is highly likely that there is no causal relationship between intracellular GLN concentration and muscle protein synthesis rate.

Consistent with the results of this study, previous studies have reported that isometric or concentric contraction ES training does not prevent the atrophy of the gastrocnemius muscle in C-26 mice [24,25]. On the other hand, recent research has shown that ES training involving eccentric contractions, which can increase mechanical stress on muscles more than isometric contractions, enhances the mTORC1 signaling in the skeletal muscles of C-26 cancer model mice without causing muscle damage and counteracts muscle protein anabolic resistance [24,38,39]. Furthermore, a study comparing the effects of ES training with different contraction modes on muscle hypertrophy in normal mice demonstrated that eccentric contractions, as opposed to isometric contractions, result in higher activation of the mTORC1 signaling pathway and muscle hypertrophy, and this is determined not by the type of contraction but by the torque exerted during contraction [40]. Thus, if ES training is carried out to reduce muscle atrophy due to anabolic resistance in cancer, it is important to increase the intensity of the load, and training using eccentric contractions is suggested to be effective. Nevertheless, it should be noted that with the progression of cancer cachexia, the susceptibility of skeletal muscles to damage from eccentric contractions may increase, and hence, this aspect should be carefully considered in future studies.

In cancer-related muscle atrophy, reports indicated both selective reductions in MyHC and uniform decreases in muscle proteins other than MyHC [41-43]. In this study, no significant changes in the proportion of MyHC in total muscle proteins were observed. Hence, it is suggested that the muscle atrophy observed in C-26 mice does not involve selective reductions in MyHC and instead results in a uniform decrease in cytoplasmic and myofibrillar proteins. Supporting this notion, glucocorticoids, which are considered one of the factors contributing to muscle protein anabolic resistance in cancer cachexia, have been shown not to affect MyHC mRNA and protein expression levels [44].

5. Research limitations

The balance between muscle protein synthesis and degradation is regulated by food intake. Previous studies have reported that C-26 mice are a cancer model that does not exhibit reduced food intake [39,45]. Thus, while it is considered unlikely that differences in food intake played a role in the observed changes in muscle mass in this study, the food intake was not measured, so its involvement cannot be completely ruled out.

6. Conclusion

In this study, the effects of isometric ES training and GLN supplementation on skeletal muscle were investigated in C-26 cancer model mice. ES training and GLN supplementation individually did not suppress the increased expression of GS in C-26 mice's skeletal muscles. However, when used in combination, they prevented this increase. On the other hand, neither ES training nor GLN supplementation, alone or in combination, prevented muscle atrophy in C-26 mice. While there have been reports suggesting a relationship between increased GS expression and decreased GLN concentration in skeletal muscle and resistance to muscle protein anabolism in muscle atrophy [13,46], there are also reports suggesting no relationship [19,47]. The results of this study support the latter. Therefore, nutritional and exercise therapies aimed at normalizing GLN metabolism in skeletal muscle do not appear to be effective in improving muscle atrophy associated with cancer cachexia.

Disclosure statement

The authors declare no conflict of interest.

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