

Perioperative oral β -hydroxy- β -methylbutyrate supplementation ameliorates sarcopenia in rats undergoing major hepatectomy

Running title: HMB ameliorates sarcopenia in rats undergoing hepatectomy

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Summary β -Hydroxy- β -methylbutyrate (HMB), a metabolite of leucine, is known to increase muscle mass and strength. However, the effect of perioperative HMB supplementation in liver surgery is unclear. Moreover, the impact of HMB on the skeletal muscle fiber type also remains unclear. We investigated the impact of HMB on the body composition and skeletal muscle fiber type in sarcopenic rats undergoing major hepatectomy. Nine-week-old male F344/NSlc rats were maintained in hindlimb suspension (HLS) and were forcedly supplemented with HMB calcium salt (HMB-Ca, 0.58 g/kg $\times$ 2 times) or distilled water in addition to free feeding. After two weeks of HLS, the rats underwent 70% hepatectomy and were sacrificed three days after surgery. Body composition factors and the proportion of slow-twitch fibers in hindlimb muscles were evaluated. HMB maintained the body composition and hindlimb force and acted against their deterioration in sarcopenic rats, exerting a particular effect on lean mass weight, which was significant. In the histological study, HMB significantly increased the proportion of slow-twitch fibers in the soleus ($p=0.044$) and plantaris ($p=0.001$) of sarcopenic rats. HMB ameliorated deterioration of the body composition and increased the proportion of slow-twitch fibers in sarcopenic rats undergoing major hepatectomy.

Key Words HMB, Hindlimb suspension, Lean mass weight, Hindlimb force, Slow-twitch fiber, Fast-twitch fiber, Liver surgery

¹ Sarcopenia, which is defined as an age-related decline in
² skeletal muscle mass and muscle function, has been
³ accepted as a new geriatric syndrome worldwide ¹. It has
⁴ been shown that sarcopenia is associated with increased
⁵ adverse outcomes such as falls, functional decline, frailty,

⁶ and mortality in elderly patients or the patients with
⁷ various diseases ²⁻⁴. Moreover, preoperative sarcopenia is
⁸ clarified to be a cause of morbidity and mortality after
⁹ surgery ⁵⁻⁹. For abdominal surgery, it has been reported
¹⁰ that preoperative sarcopenia causes not only postoperative

major complications but also poor long-term survival and disease-free survival postoperatively¹⁰. Recently, the role of perioperative nutritional therapy has been widely recognized to improve postoperative short- and long-term outcomes in various surgeries¹¹⁻¹³. However, the efficacy of perioperative nutritional therapy for liver surgery is still controversial.

β -Hydroxy- β -methylbutyrate (HMB) is a metabolite of leucine and is now widely used by athletes or bodybuilders, usually combined with physical training, to increase muscle mass and strength^{14,15}. Currently, the role of HMB in patients with sarcopenia has been explored to prevent worsening of the condition¹⁶. The following mechanisms of HMB in protein anabolism have been revealed: HMB progresses protein synthesis by stimulating the akt-mTOR pathway or inhibiting protein degradation by suppressing the ubiquitin proteasome pathway, in addition to inducing mitochondrial biogenesis or proliferation of satellite cells¹⁷. However, the impact of HMB on the skeletal muscle fiber type is not fully known. Recently, the efficacy of HMB for improving clinical outcomes in various diseases has been explored¹⁸. We reported that postoperative oral or enteral HMB supplementation after living donor liver transplantation significantly increased grip strength at 1 and 2 months and skeletal muscle mass index at 2 months and shortened the postoperative length of hospital stay¹⁹. Despite these findings, the impact of perioperative HMB supplementation in patients with sarcopenia in major liver surgery, especially on the skeletal muscle fiber type, remains unclear.

In the present study, we aimed to assess the impact of perioperative oral HMB supplementation on the body composition and skeletal muscle fiber type in sarcopenia rats undergoing major hepatectomy.

MATERIALS AND METHODS

Animals and materials.

Nine-week-old male F344/NSlc rats (specific pathogen free, Japan SLC, INC., Shizuoka, Japan) weighing approximately 160-200 g were kept in stainless cages in a temperature-, humidity- and light-controlled room ($23 \pm 3^\circ\text{C}$, $55 \pm 15\%$; 12-h light-dark cycle) for one week before the experiments. The rats were maintained on an AIN-93G diet²⁰ and were freely provided drinking water. HMB calcium salt (HMB-Ca) was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan) and was dissolved in distilled water (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). Distilled water was used as the vehicle. All procedures involving rats were complied with the National Research Council's Guide for the Humane Care and Use of Laboratory Animals. This study was approved by the Ethics Committee for Animal Experiments at Kyoto University (MEDKYO 20583).

Hindlimb suspension and major hepatectomy

We used a standard rodent hindlimb suspension (HLS) model reported by Morey-Holton & Globus to induce disuse muscle atrophy in rodents as a model of sarcopenia²¹. Briefly, rats were suspended from the ceiling of the cages by using an instrument designed to wrap a tail. This

allowed the forelimbs to move around the cage and freely to access food and water. In this model, rats were maintained at a suspension angle of approximately 30 degrees. Major hepatectomy was performed in the manner reported by Higgins and Anderson²². Briefly, a midline incision was made under volatile anesthesia, and then the left-lateral and left-medial lobes (approximately 70% of the total liver) were removed.

Experimental study design

The schematic diagram of the study design is demonstrated in Figure 1. Twenty rats were randomly assigned into the following two experimental groups: the control group (n=10) and the HMB group (n=10). All rats were maintained in HLS throughout the study. In addition to free feeding, the rats in the control group were forcibly supplemented with vehicle twice a day ($5.8 \text{ mL/kg} \times 2$ times: between 9 and 11 a.m. and between 3 and 5 p.m.), and those in the HMB group were forcibly supplemented with HMB-Ca twice a day ($0.58 \text{ g/kg} \times 2$ times between 9 and 11 a.m. and between 3 and 5 p.m.). The daily amount of food intake was measured. After two weeks of HLS, all rats underwent 70% hepatectomy under volatile anesthesia using 3.0% isoflurane on day 0. The resected livers were weighed. One rat in the HMB group died after surgery. On day 3, we performed a laparotomy on the rats and collected residual liver samples, hindlimb muscles and blood samples via the inferior vena cava; then, the rats were bled to death.

Assessment of body composition factors and treatment of samples

On day -14, day -1 and day 3, we measured the body weight using a gravimeter (MSE 1202S-100-D0, Sartorius, Japan) and the lean mass weight and fat mass weight using magnetic resonance imaging (MRI) (Echo-MRI-700, Echo Medical Systems LLC, USA). On day -14 and day 3, we measured the hindlimb force using a small-animal torque measurement device (T.K.K.5813, Takei Scientific Instruments Co., Ltd, Japan). On day 3, blood samples were collected and stored in heparinized tubes and immediately centrifuged for 10 min at $1,900 \times g$ using a refrigerated centrifuge, and blood plasma was transferred into a clean polypropylene tube. Liver samples were weighed. Left hindlimb muscles (gastrocnemius, soleus and extensor digitorum longus (EDL)) were weighed and frozen in liquid nitrogen. Right hindlimb muscles (soleus and plantaris) were fixed in 10% volume by volume neutral buffered formalin for histological evaluation.

Blood biochemical study and liver regeneration rate

On day 3, plasma levels of aspartate transaminase (AST), alanine transaminase (ALT), albumin (ALB), total bilirubin (T-Bil), creatine kinase (CK), albumin (ALB), total cholesterol (T-CHO), triglyceride (TG) and white blood cells (WBCs) were measured.

The liver regeneration rate was calculated as the remnant liver weight/the estimated whole liver weight. The estimated whole liver weight was calculated as the

resected liver weight/0.7. The liver regeneration rate was calculated on day 3.

Histological study

Some sections of right hindlimb muscles (soleus and plantaris) were stained using myosin skeletal slow antibody (Anti-Slow Skeletal Myosin Heavy-Chain-Antibody [EPR22697-17], Abcam, Japan) to stain slow-twitch fibers. Antibody binding was visualized with a light microscope (BX-53, Olympus Corp., Japan). The proportion of slow-twitch fibers was calculated using ImageJ software (version 1.52; NIH, Bethesda, MD, USA).

Statistics

Data are expressed as the means $\pm$ standard deviation and were compared statistically using Student's t-test. Changes in values over time among the same sample were compared using paired t-tests. P value < 0.05 were considered as a threshold. JMP Pro, version 14.0.0 (SAS Institute, Inc., Cary, NC) was used to perform statistical analyses.

RESULTS

Food intake, body weight, lean mass weight and fat mass weight

There was no significant difference in the total amount of food intake between the control group and the HMB group. The mean body weights in the HMB group and in the control group were significantly decreased from day -14 to day -1 ($p=0.029$ and $p<0.001$, respectively) and to day 3 (both values of $p<0.001$) (Figure 2A); however, the preoperative decrease in body weight in the HMB group from day -14 to day -1 showed a tendency to be lower than that in the control group ($-2.76 \pm 3.04\%$ vs $-7.64 \pm 6.75\%$, $p=0.052$) (Figure 2B).

The mean lean mass weight in the HMB group and in the control group was significantly decreased from day -14 to day -1 ($p=0.008$ and $p<0.001$, respectively) and to day 3 (both values of $p<0.001$ on day 3) (Figure 2C); however, the preoperative rate of decrease in the mean lean mass weight from day -14 to day -1 was significantly lower in the HMB group than in the control group ($-3.11 \pm 2.51\%$ vs $-7.26 \pm 5.52\%$, $p=0.044$) (Figure 2D).

The mean fat mass weight in the control group was significantly decreased from day -14 to day -1 (18.91 ± 2.13 g vs 14.77 ± 6.14 g, $p=0.043$); however, no significant decrease was seen in the HMB group (19.47 ± 3.51 g vs 18.88 ± 4.82 g, $p=0.605$).

Hindlimb muscle weight and hindlimb force

No significant differences were seen in the mean weight of the gastrocnemius (0.776 ± 0.081 g in the control group vs 0.800 ± 0.037 g in the HMB group, $p=0.431$), soleus (0.043 ± 0.006 g in the control group vs 0.042 ± 0.003 g in the control group, $p=0.644$) or EDL (0.083 ± 0.008 g in the control group and 0.084 ± 0.004 g in the HMB group, $p=0.763$) between groups. On the other hand, the mean hindlimb force in the HMB group was increased from day -14 to day 3, whereas it was decreased in the control group (Figure 3A). Although not significant, the rate of change

in the hindlimb force from day -14 to day 3 was higher in the HMB group than in the control group ($+5.89 \pm 6.56\%$ vs $-4.04 \pm 16.5\%$, $p=0.113$) (Figure 3B).

Blood biochemistry and liver regeneration

In the blood biochemistry test, the mean plasma CK level in the HMB group was significantly higher than that in the control group (Table 1). No significant difference was seen in the plasma levels of liver factors (AST, ALT, and T-Bil) and nutritional factors (ALB, T-CHO and TG) between the groups. Moreover, no significant difference was seen in the liver regeneration rate between groups (66.8% in the HMB group and 68.2% in the control group, $p=0.718$).

Histological study

Myosin slow antibody staining showed that there were smaller number of slow-twitch fibers to be seen in the control group than in the HMB group in both the soleus (Figure 4A and 4B) and the plantaris (Figure 4C and 4D). The proportion of slow-twitch fibers in the HMB group was significantly larger than that in the control group in both the soleus (87.1% vs 80.2%, $p=0.044$) (Figure 5A) and the plantaris (39.7% vs 26.4%, $p=0.001$) (Figure 5B).

DISCUSSION

HMB suppressed deterioration of the body composition

This study demonstrated that perioperative oral HMB supplementation suppressed the loss of body weight, lean mass weight and fat mass weight against their deterioration in sarcopenic rats, and its effect on lean mass weight was significant. In addition, although not significant, HMB increased the hindlimb force against its decrease in sarcopenic rats. Sarcopenia is defined as loss of skeletal muscle mass and physical performance and is categorized into primary sarcopenia induced by aging and secondary sarcopenia induced by the other causes¹. In this study, we used the rat HLS model to mimic secondary sarcopenia. The rats in the control group showed decreases in both lean mass weight and hindlimb force from day -14 to day 3, thus validating the rat HLS model as a mimic of secondary sarcopenia. Moreover, in the histological study, the proportion of slow-twitch fibers in the HMB group was significantly larger than that in the control group in both the soleus and plantaris. To the best of our knowledge, this is the first study assessing the impact of HMB not only on the body composition but also on the skeletal muscle fiber type in sarcopenic rats undergoing major hepatectomy. In this study, the hindlimb force of rats in the HMB group increased, but no significant differences in soleus and plantaris mass were observed. Evidence on the impact of HMB on hindlimb muscle weight and hindlimb force in rats with disused muscle atrophy is limited. The small number of available studies report data supporting the present study^{23,24}. In one study, two weeks of oral HMB supplementation decreased the loss of hindlimb force in HLS rats, although this difference was not significant. However, no significant changes in soleus and plantaris weights were observed²³. In another study, oral

HMB supplementation was shown to ameliorate the decrease in hindlimb force in rats with dexamethasone-induced muscle atrophy, whereas no significant differences in soleus weight were observed²⁴. Although these studies used different models, when taken together with the present study, the findings suggest that HMB contributes to hindlimb muscle force rather than hindlimb muscle weight in rats with muscle atrophy. Furthermore, in a blood biochemical study, no significant differences were observed in the plasma levels of nutritional factors, including T-CHO, TG and ALB, or in the amount of food intake between groups. These results suggest that the effects of HMB on hindlimb muscle weight and hindlimb force might be induced not by improving nutritional parameters but by affecting the skeletal muscles directly. Although it has been reported that perioperative oral HMB supplementation improves postoperative outcomes after surgery^{19,25-27}, it remains unclear how perioperative oral HMB supplementation affects patients with sarcopenia who are undergoing liver surgery. The present study revealed that HMB might exert its effect by preventing the worsening of sarcopenia in sarcopenic patients undergoing liver surgery.

HMB increased slow-twitch fibers in hindlimb muscles

No significant difference was seen in hindlimb muscle weight between groups, whereas the proportion of slow-twitch fibers in the soleus and plantaris was significantly larger in the HMB group than in the control group, which gave us a hypothesis that HMB might have effects on increasing the proportion of slow-twitch fibers in skeletal muscle. Although it has been reported that muscle disuse causes a slow-to-fast shift in fiber type²⁸, it remains unclear how HMB affects the skeletal muscle fiber type in muscle-disuse atrophy. To evaluate the change in muscle fiber type in the hindlimb muscles of sarcopenic rats, we evaluated the muscle fiber type using five normal rats growing under the same conditions as the rats in the present study. An additional study showed that the proportion of slow-twitch fibers was $86.2 \pm 6.4\%$ in the soleus and $39.1 \pm 5.7\%$ in the plantaris (Supplemental figure 1A and 1B). These results supported our hypothesis and implied that HMB might contribute to increased hindlimb force by ameliorating the proportion of slow-twitch fibers in the hindlimb muscles of sarcopenic rats. The mechanisms by which HMB prevents the shift in fiber type from slow to fast in disuse muscle atrophy remains unclear. However, there are reports that plantar mechanical stimulation prevents the slow-to-fast fiber type shift in rats with disuse muscle atrophy by disrupting calcineurin-NFATc1 inactivation²⁹ and that vibration stimulus to the plantar fascia prevents this shift via upregulation of MGF and YAP1³⁰. We plan to conduct prospective studies to reveal the mechanisms of HMB on the skeletal muscle fiber type shift.

Blood biochemical study showed that HMB activated muscle metabolism

In a blood biochemical study, the plasma CK level in rats in the HMB group on day 3 was significantly higher than that in the control group, which suggested that HMB might activate muscle metabolism in sarcopenic rats,

resulting in significant suppression of the loss of lean mass weight. No significant differences were seen in the plasma levels of nutritional factors, including T-CHO, TG and ALB, or in the amount of food intake between groups. These results indicated that the antisarcopenic effects of HMB were induced not by increasing food intake but by affecting the skeletal muscles directly.

The role of HMB in liver disease

Studies of the impact of HMB supplementation on liver surgery in humans are scarce^{19,27}, and the effects of HMB supplementation on animal or human liver disease remain unclear. It has been reported that muscle-wasting disorders increase the activity of BCKD, the rate-limiting enzyme in leucine oxidation. Liver diseases such as liver cirrhosis may impair HMB synthesis and induce HMB deficiency. Most HMB is synthesized from leucine in the liver, and the activation of leucine oxidation in skeletal muscle causes a decrease in plasma leucine levels¹⁷, emphasizing the need of studies of the impact of HMB supplementation on liver disease. Rats were sacrificed on the third postoperative day in this study for two reasons. First, rat liver regeneration after 70% hepatectomy is nearly complete within 7 days after surgery³¹, and we planned to evaluate liver regeneration in the early phase after hepatectomy. Second, two weeks of oral HMB supplementation before liver surgery is thought to be long enough to evaluate the effect of HMB on physical deterioration in HLS rats. Holeček M et al.³² reported that partial hepatectomized rats given oral HMB supplementation had significantly higher BCAA concentrations and DNA fragmentation in the liver than rats supplemented with saline, suggesting that HMB might have a stimulatory effect on DNA synthesis in the early period of liver regeneration. In this study, there was no significant difference in the liver regeneration rate on day 3 between groups. However, we can not conclude that there was no impact of HMB on liver regeneration, as we evaluated liver regeneration not from the viewpoint of liver function but from the viewpoint of liver volume. This study also showed that HMB had no adverse effects on food intake, plasma levels of liver transaminases and metabolic factors in HLS rats undergoing major hepatectomy. These results suggest that oral HMB supplementation may be safely introduced in rats undergoing major hepatectomy and may have beneficial anti-sarcopenic effects.

The impact of HMB on fat mass

How HMB affects fat mass remains unclear. Surprisingly, the results of this study showed that the fat mass weight of rats was maintained in the HMB group compared to the control group. There are few reports assessing the effects of HMB on fat mass in rats^{33,34}. Wilson JM et al. reported that HMB suppressed the increase in fat mass caused by aging in middle-aged and old rats³³. On the other hand, Kim JS et al. reported that HMB significantly decreased fat mass weight from pre- to postresistance training in rats³⁴. The present study showed that perioperative oral HMB supplementation maintained the fat mass weight against a significant decrease in fat mass in sarcopenic rats, which suggested that HMB might

have an anti-sarcopenic effect that maintains the fat mass weight against its loss caused by sarcopenia, as reported by Wilson JM et al³³. Further studies will be needed to evaluate the impact of HMB on fat mass from the viewpoint of visceral and subcutaneous fat mass.

Clinical application of HMB in liver surgery

Preoperative sarcopenia has been shown to cause poor outcomes in liver surgery^{35,36}, which indicates that the amelioration of preoperative sarcopenia can improve postoperative outcomes. Although a few reports have shown the efficacy of oral HMB supplementation after liver transplantation^{19,27}, there has been no study about the efficacy of preoperative HMB supplementation before major hepatectomy. The present investigation showed that preoperative oral HMB supplementation significantly suppressed the loss of preoperative lean mass weight, resulting in the maintenance of skeletal muscle mass before surgery. Moreover, this study also showed that oral HMB supplementation before and after surgery increased postoperative muscle strength. These results suggest that HMB supplementation for patients with preoperative sarcopenia not only after but also before surgery might be important because it might contribute to improving postoperative outcomes by maintaining the skeletal muscle mass before surgery against sarcopenia and improving the postoperative physical performance. Therefore, the clinical application of HMB in major hepatectomy seems promising.

Limitations

There are a few limitations that should be acknowledged. First, this study was conducted in rats, and its translatability to clinical settings should be addressed. Second, the particular dose of oral HMB supplementation is controversial. HMB-Ca (1.16g/kg/day) was used for supplementation in this study and did not cause any adverse effects in the rats.

Conclusion

In conclusion, this study revealed that HMB suppressed deterioration of the body composition by ameliorating the proportion of slow-twitch fibers in skeletal muscle in sarcopenic rats undergoing major hepatectomy. Further studies are needed to elucidate the underlying mechanisms.

Authorship

Research conception and design: TK, SK, DH, SU, and EH; statistical analysis of the data: SI, AW, and SM; interpretation of the data: SI, TK, AW, MH, YM, SYao, SYagi, and NK; writing of the manuscript: SI, TK, and AW.

Disclosure of state of COI

AW, SK, and DH are employees of Otsuka Pharmaceutical Factory, Inc (Tokushima, Japan). All the other authors declared no competing interests.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

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Figure Legends

Fig. 1. Schematic diagram of the study design. A total of 20 rats were divided into two groups: the control group (n=10) and the HMB group (n=10). Body composition factors (body weight, lean mass and fat mass weight) were measured, and blood sampling and collection of hindlimb muscles were conducted according to the schedule shown.

Fig. 2. Changes over time in body weight (2A) and their change rates from baseline (2B). The preoperative decrease in body weight in the HMB group from day -14 to day -1 showed a tendency to be lower than that in the control group ($p=0.052$). Changes over time in lean body mass weight (2C) and their change rates from baseline (2D). The preoperative rate of decrease in the mean lean mass weight from day -14 to day -1 was significantly lower in the HMB group than in the control group ($p=0.044$). $\dagger<0.05$ and $\dagger\dagger<0.01$ vs. day -14 in the same group.

Fig. 3. Hindlimb force (3A) and force change rate from baseline (3B). The mean hindlimb force increased from day -14 to day 3 in the HMB group but decreased in the control group.

Fig. 4. Myosin slow antibody staining of the soleus and plantaris. (4A) Soleus in the control group; (4B) Soleus in the HMB group; (4C) Plantaris in the control group; (4D) Plantaris in the HMB group. A smaller number of slow-twitch fibers were observed in the control group than in the HMB group in both the soleus (Figure 4A and 4B) and the plantaris (Figure 4C and 4D). Arrows

indicate slow-twitch fibers. Arrow heads indicate fast-twitch fibers.

Fig. 5. The proportion of slow-twitch fibers in the soleus (5A) and plantaris (5B). The proportion of slow-twitch fibers was significantly larger in the HMB group than in the control group in both the soleus ($p=0.044$) and the plantaris ($p=0.001$).

Supplemental Fig. 1. Myosin slow antibody staining in the soleus (1A) and plantaris (1B) in normal rats. The proportion of slow-twitch fibers was $86.2 \pm 6.4\%$ in the soleus and $39.1 \pm 5.7\%$ in the plantaris. Arrows indicate slow-twitch fibers. Arrow heads indicate fast-twitch fibers.

Table Legends

Table 1. Blood biochemical study on day 3. The mean plasma CK level was significantly higher in the HMB group than in the control group ($p=0.019$).

Figure 1.

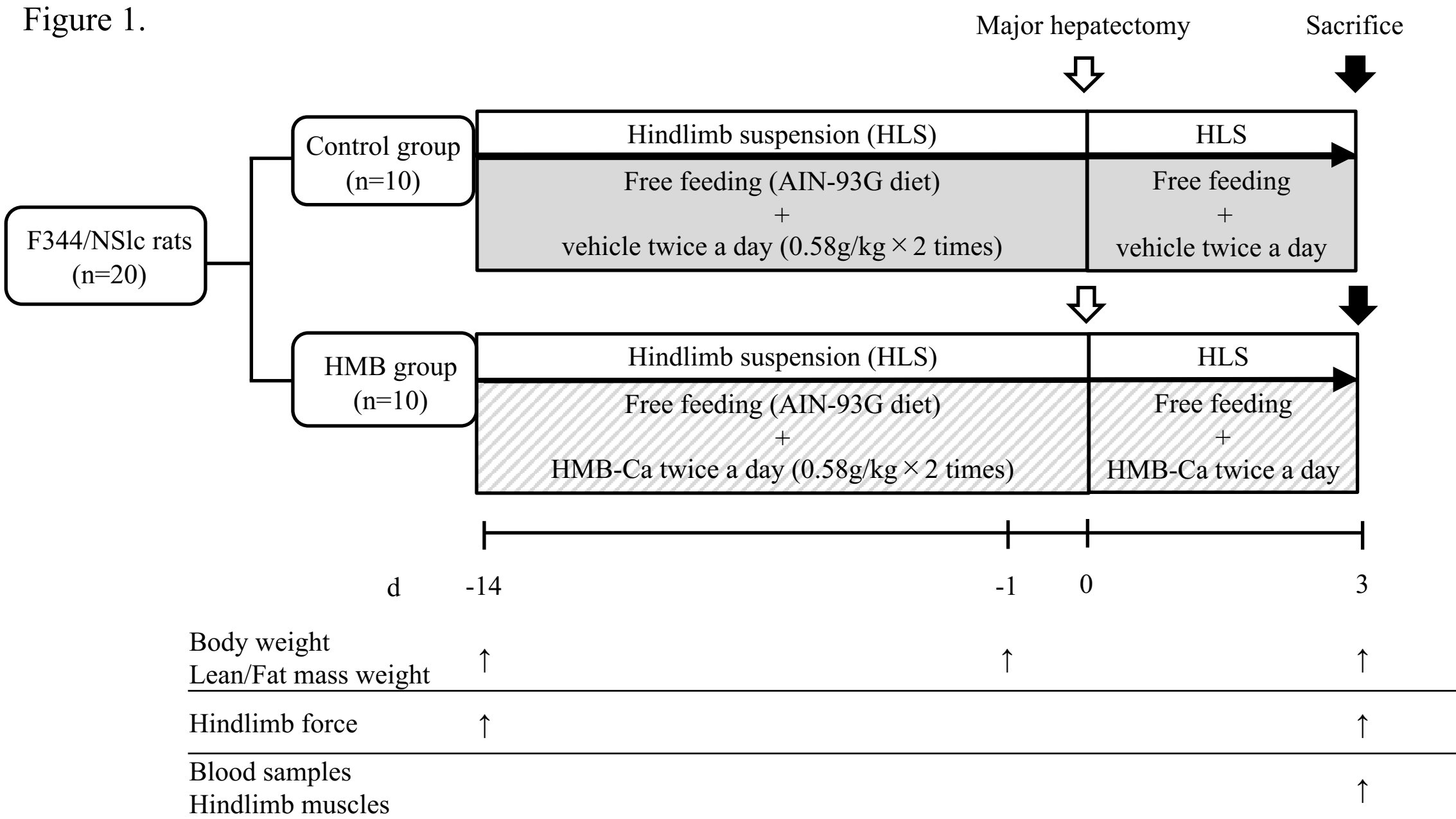


Figure 2.

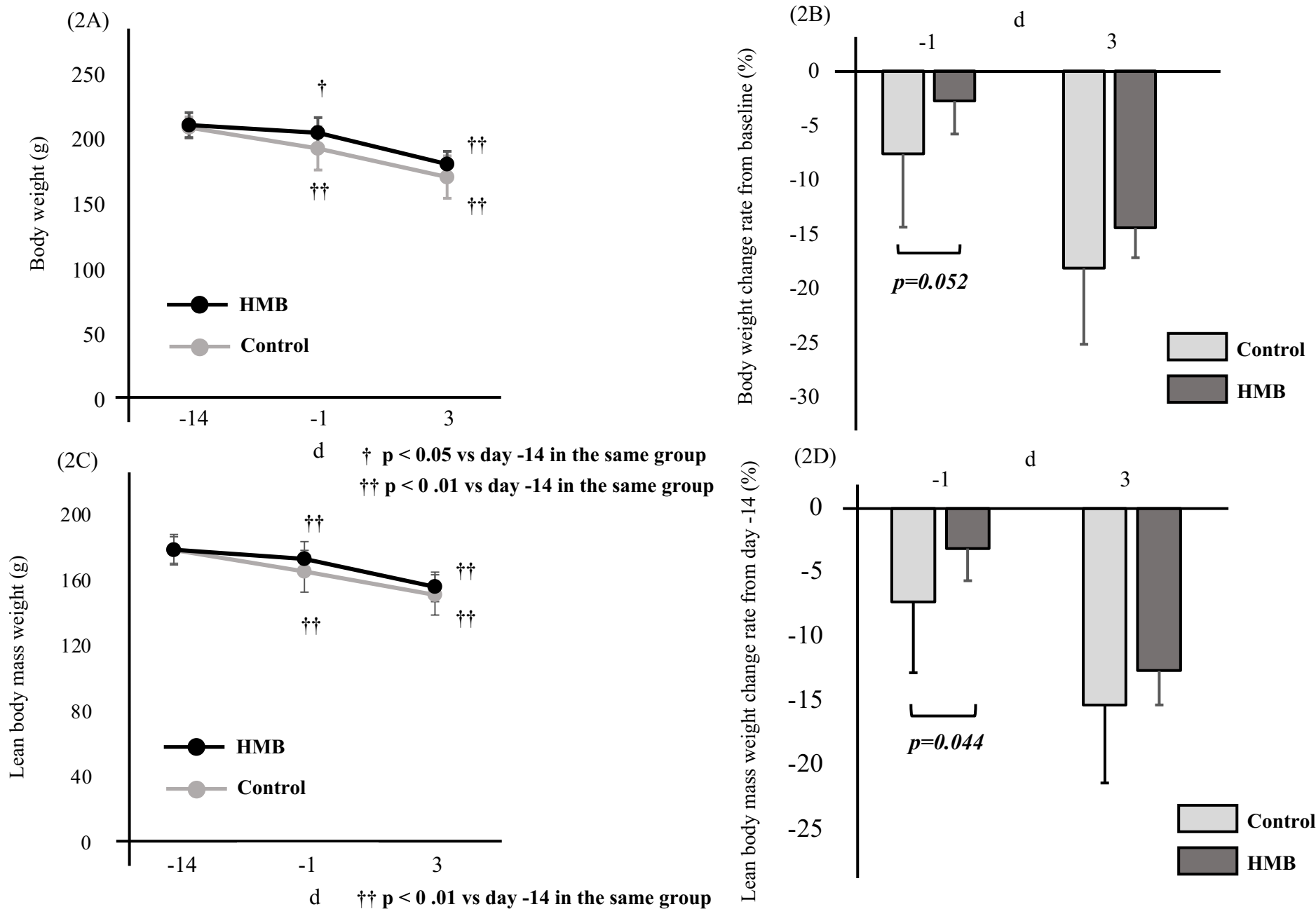


Figure 3.

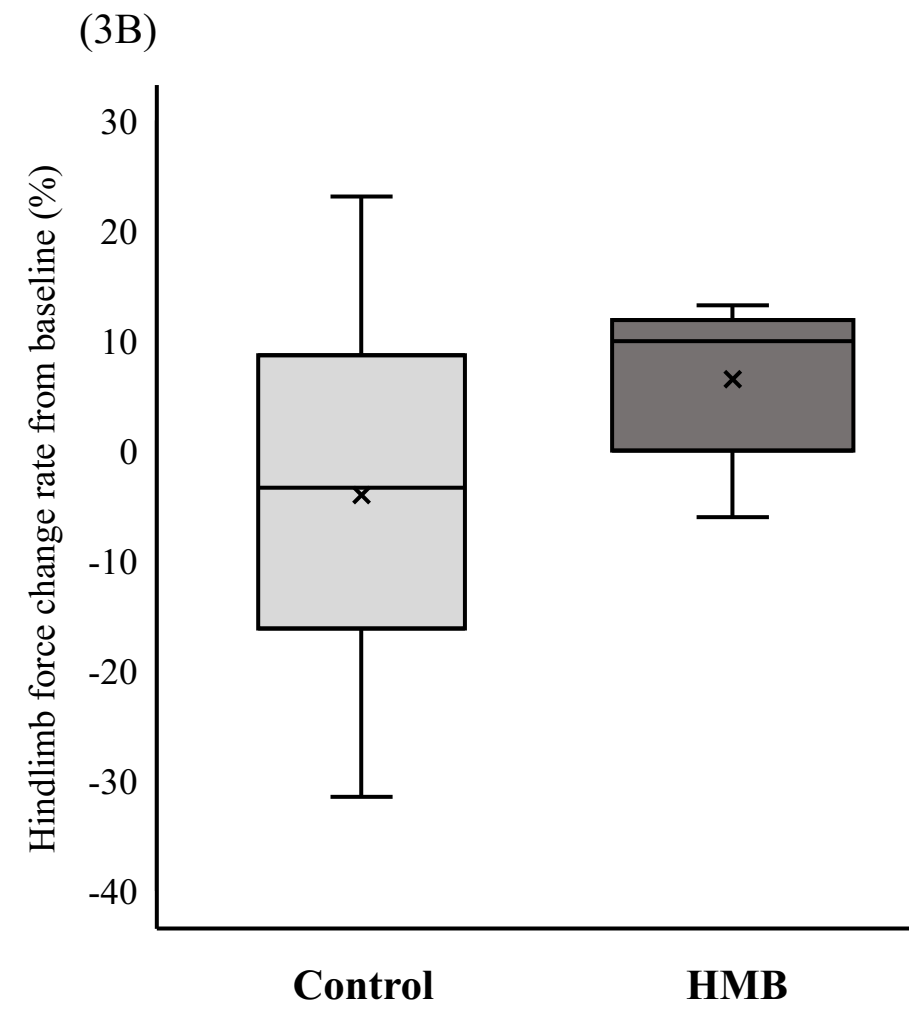
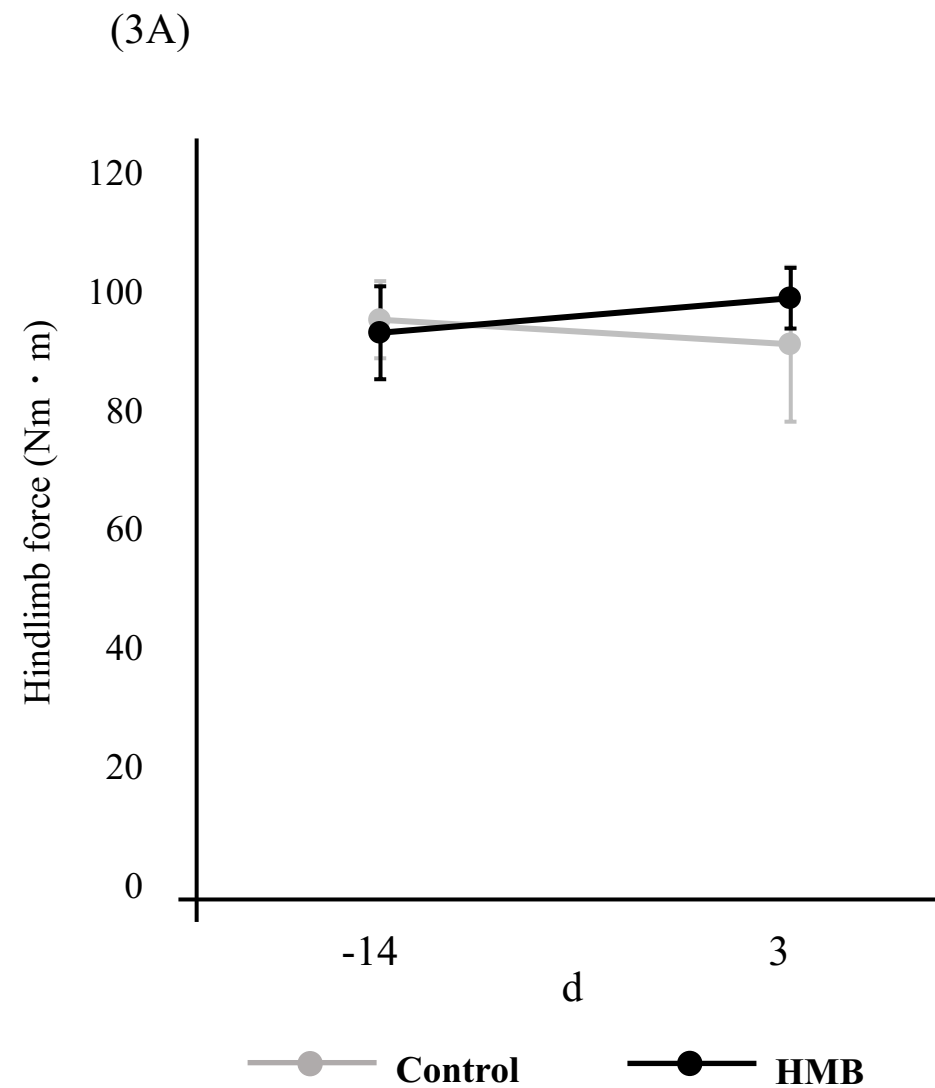
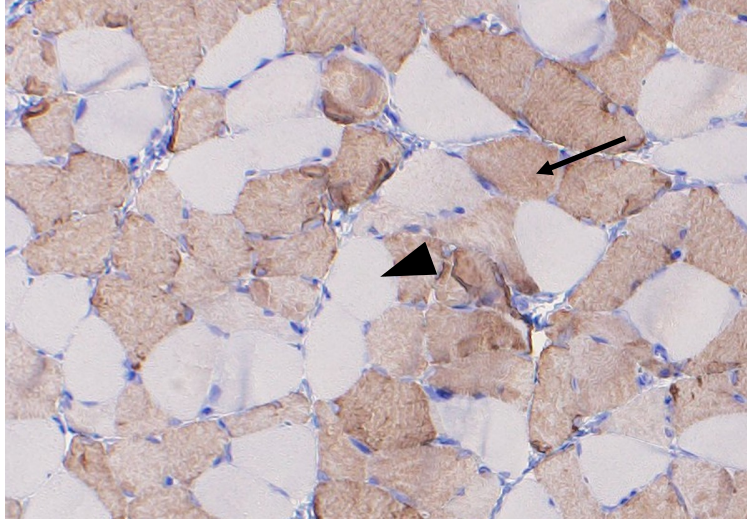
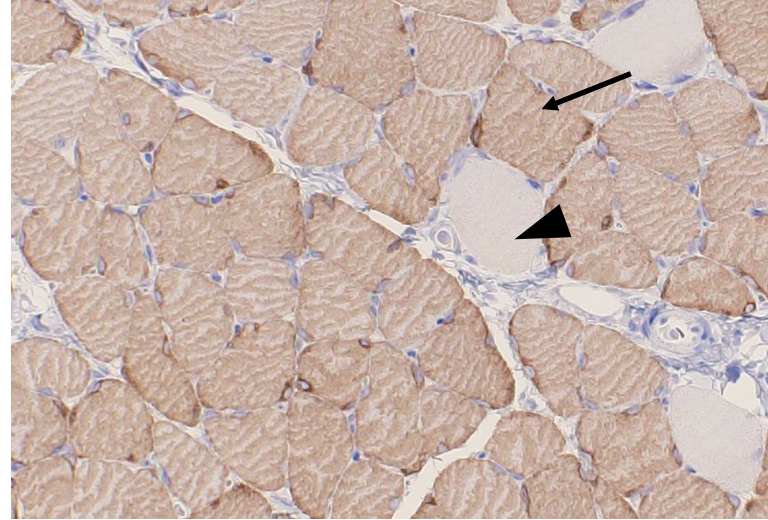


Figure 4.

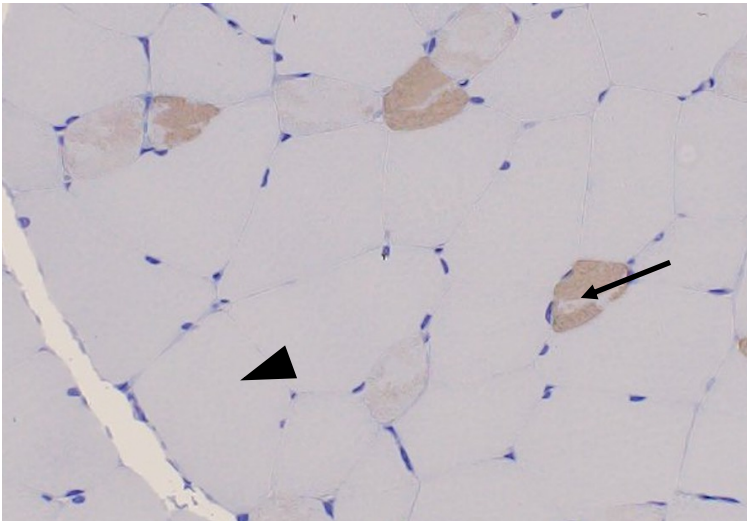
(4A)



(4B)



(4C)



(4D)

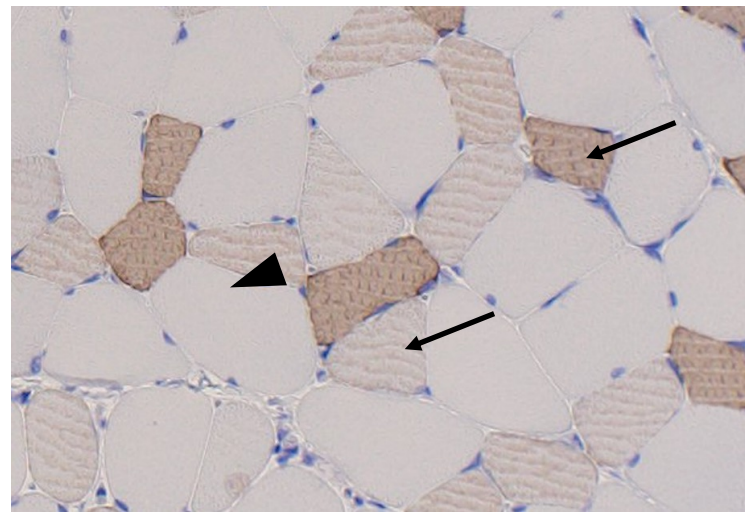
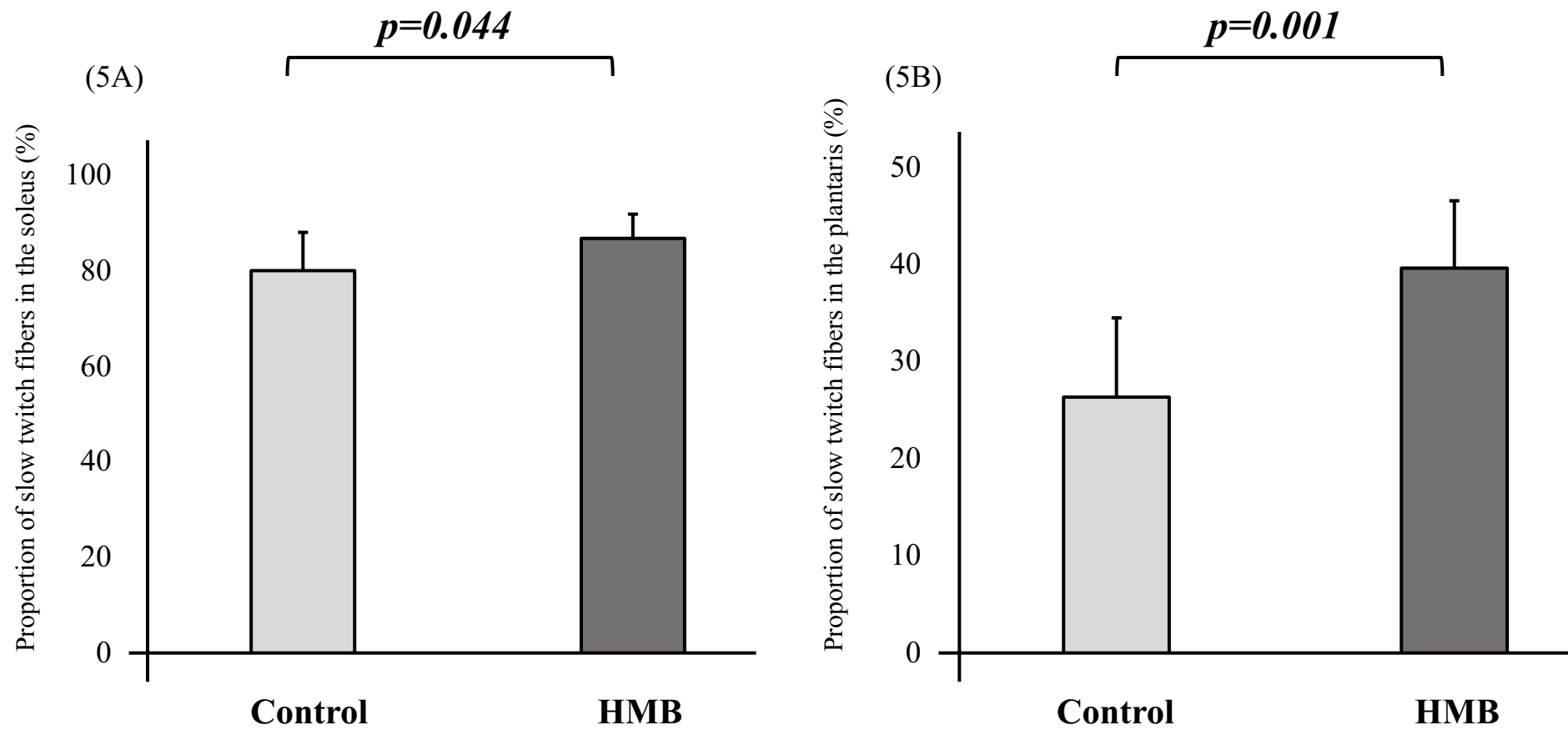


Figure 5.



Supplemental figure 1.

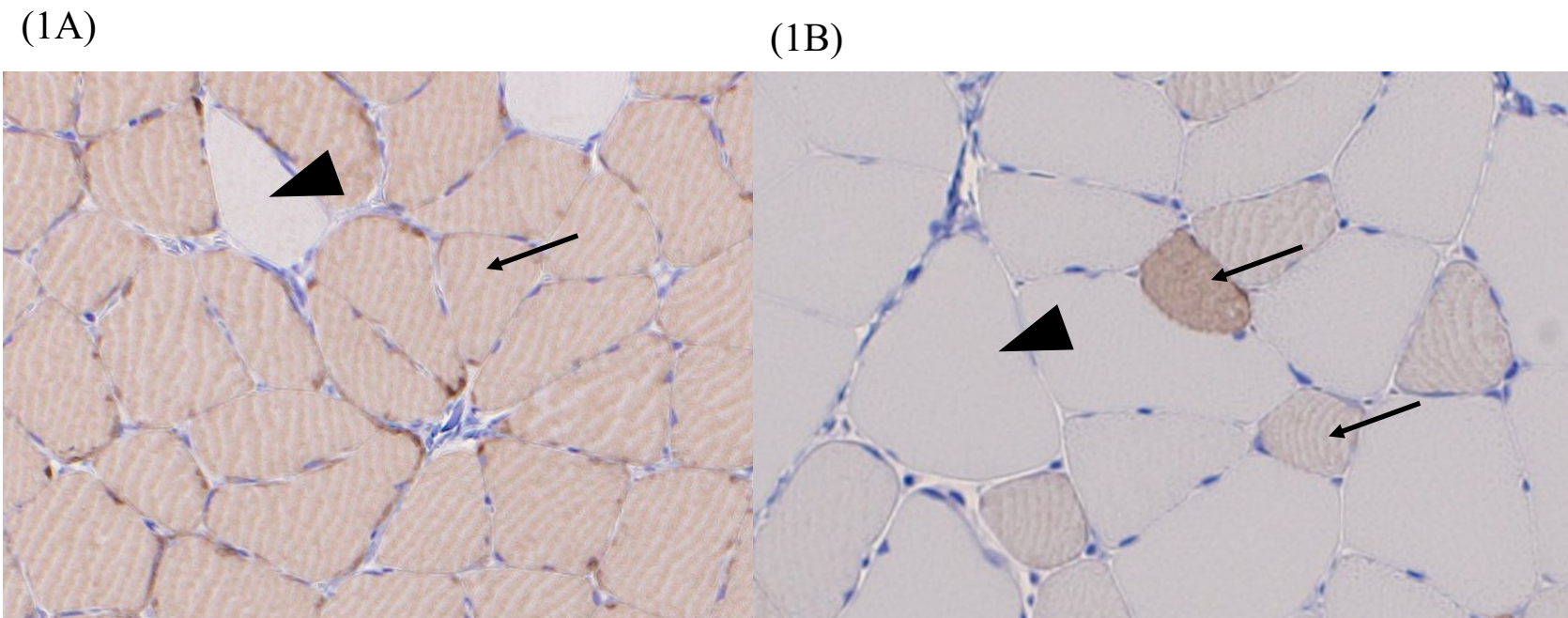


Table 1. Blood biochemical study on day 3.

	Control group	HMB group	p value
AST, IU/L	130.30 ± 34.41	118.67 ± 29.59	0.443
ALT, IU/L	71.80 ± 17.45	70.33 ± 28.13	0.892
T-Bil, mg/dl	0.13 ± 0.03	0.14 ± 0.03	0.429
CK, IU/L	381.40 ± 49.53	485.56 ± 115.98	0.019*
ALB, mg/dl	2.32 ± 0.18	2.33 ± 0.11	0.854
T-CHO, mg/dl	50.60 ± 6.62	49.56 ± 5.20	0.709
TG, mg/dl	25.30 ± 6.09	30.44 ± 11.39	0.230
WBC, 10 ² /μL	56.60 ± 8.94	59.23 ± 8.34	0.517

ALB, albumin; ALT, alanine transferase; AST, aspartate transferase; CK, creatinine kinase; HMB, β-hydroxy-β-methylbutyrate; TG, triglyceride; T-Bil, total bilirubin; T-CHO, total cholesterol; WBC, white blood cell.

* **p < 0.05**