Perioperative Oral $\beta$-Hydroxy-$\beta$-Methylbutyrate Supplementation Ameliorates Sarcopenia in Rats Undergoing Major Hepatectomy

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Summary $\beta$-Hydroxy-$\beta$-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×2 times) or distilled water in addition to free feeding. After 2 wk of HLS, the rats underwent 70% hepatectomy and were sacrificed 3 d 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

Sarcopenia, which is defined as an age-related decline in skeletal muscle mass and muscle function, has been accepted as a new geriatric syndrome worldwide (1). It has been shown that sarcopenia is associated with increased adverse outcomes such as falls, functional decline, frailty, and mortality in elderly patients or the patients with various diseases (2–4). Moreover, preoperative sarcopenia is clarified to be a cause of morbidity and mortality after surgery (5–9). For abdominal surgery, it has been reported that preoperative sarcopenia causes not only postoperative major complications but also poor long-term survival and disease-free survival postoperatively (10). Recently, the role of perioperative nutritional therapy has been widely recognized to improve postoperative short- and long-term outcomes in various surgeries (11–13). However, the efficacy of perioperative nutritional therapy for liver surgery is still controversial.

$\beta$-Hydroxy-$\beta$-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 condition (16). 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 cells (17). 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 explored (18). We reported that postoperative oral or enteral HMB supplementation after living donor liver transplantation significantly increased grip strength at 1 and 2 mo and skeletal muscle mass index at 2 mo and shortened the postoperative length of hospital stay (19). 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 ± 3°C, 55 ± 15%; 12-h light-dark cycle) for 1 wk before the experiments. The rats were maintained on an AIN-93G diet (20) 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 Globus and Morey-Holton to induce disuse muscle atrophy in rodents as a model of sarcopenia (21). 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 Anderson (22). 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 Fig. 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 forcedly supplemented with vehicle twice a day (5.8 mL/kg × 2 times: between 9 and 11 a.m. and between 3 and 5 p.m.), and those in the HMB group were forcedly supplemented with HMB-Ca twice a day (0.58 g/kg × 2 times between 9 and 11 a.m. and between 3 and 5 p.m.). The daily amount of food intake was measured. After 2 wk 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 ×g using a refrigerated cen-

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**Table 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.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Hindlimb suspension (HLS)</th>
<th>HLS</th>
<th>HMB group (n = 10)</th>
<th>Hindlimb suspension (HLS)</th>
<th>HLS</th>
</tr>
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<tbody>
<tr>
<td>Control group (n = 10)</td>
<td>Free feeding (AIN-93G diet)</td>
<td>HMB-Ca twice a day (0.58 g/kg × 2 times)</td>
<td>Free feeding</td>
<td>Vehicle twice a day (0.58 g/kg × 2 times)</td>
<td></td>
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<tr>
<td>HMB group</td>
<td>Free feeding (AIN-93G diet)</td>
<td>HMB-Ca twice a day (0.58 g/kg × 2 times)</td>
<td>Free feeding</td>
<td>Vehicle twice a day</td>
<td></td>
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<table>
<thead>
<tr>
<th>Day</th>
<th>Body weight</th>
<th>Lean/Fat mass weight</th>
<th>Hindlimb force</th>
<th>Blood samples</th>
<th>Hindlimb muscles</th>
</tr>
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<tbody>
<tr>
<td>−14</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>−1</td>
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trifuge, 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), 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±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) (Fig. 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±3.04% vs 7.64±6.75%, p=0.052) (Fig. 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) (Fig. 2C); however, the preoperative rate of decrease in the
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mean lean mass weight from day −14 to day −1 was significantly lower in the HMB group than in the control group (−3.11 ± 2.51% vs −7.26 ± 5.52%, \( p = 0.044 \)) (Fig. 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 HMB 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 (Fig. 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 ± 6.56% vs 4.04 ± 16.5%, \( p = 0.113 \)) (Fig. 3B).

Blood biochemistry and liver regeneration

In the blood biochemistry test, the mean plasma CK level 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 (Fig. 4A and 4B) and the plantaris (Fig. 4C and 4D). The proportion of slow-twitch fibers in the HMB group was significantly larger than that in the control group (87.1% vs 80.2%, \( p = 0.044 \)) (Fig. 5A) and the plantaris (39.7% vs 26.4%, \( p = 0.001 \)) (Fig. 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.

### Table 1. Blood biochemical study on day 3.

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

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

The mean plasma CK level was significantly higher in the HMB group than in the control group (\( p = 0.019 \)).

\* \( p < 0.05 \).
Iwamura S et al. and is categorized into primary sarcopenia induced by aging and secondary sarcopenia induced by the other causes (1). 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, 2 wk of oral HMB supplementation decreased the loss of hindlimb force in HLS rats, although this difference was not significant. However, no significant changes in

Fig. 4. Myosin slow antibody staining of the soleus and plantaris. (A) Soleus in the control group; (B) Soleus in the HMB group; (C) Plantaris in the control group; (D) 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 (A and B) and the plantaris (C and D). Arrows indicate slow-twitch fibers. Arrow heads indicate fast-twitch fibers.

Fig. 5. The proportion of slow-twitch fibers in the soleus (A) and plantaris (B). 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).
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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 type (28), 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 ± 6.4% in the soleus and 39.1 ± 5.7% in the plantaris (Supplemental Online Material, Fig. S1A 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 planar mechanical stimulation prevents the slow-to-fast fiber type shift in rats with disuse muscle atrophy by disrupting calcineurin-NFATc1 inactivation (29) and that vibration stimulus to the plantar fascia prevents this shift via upregulation of MGF and YAP1 (30). We plan to conduct prospective studies to reveal the mechanisms of HMB on the skeletal muscle fiber type shift.

The role of HMB in liver disease

Studies of the impact of HMB supplementation on liver surgery in humans are scarce (19, 27). 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 observed (24). 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.

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 et al. reported that HMB suppressed the increase in fat mass caused by aging in middle-aged and old rats (33). On the other hand, Kim et al. reported that HMB significantly decreased fat mass weight from pre- to postresistance training in rats (34). 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 et al. (33). 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 (1.16 g/kg/d) was used for supplementation in this study and did not cause any adverse effects in the rats.

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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Supporting information
Supplemental online material is available on J-STAGE.

REFERENCES
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