

β -hydroxybutyrate enhances structural repair but not early functional recovery in septic muscle: implications for phased interventions in sepsis-associated ICUAW

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Article

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Abstract

Background

Intensive care unit-acquired weakness (ICUAW) is a prevalent neuromuscular complication in critically ill patients. β -hydroxybutyrate (BHB), a ketone body with pleiotropic functions, has shown protective effects in several muscle-wasting models. However, its impact on skeletal muscle regeneration in sepsis-associated ICUAW and the underlying mechanisms remain elusive.

Methods

Sepsis was induced in male C57BL/6 mice by cecal ligation and puncture (CLP), with a sham group as control. To determine the optimal β -hydroxybutyrate (BHB) dose, CLP mice received high-, medium-, or low-dose BHB or normal saline subcutaneously for five days. Using the identified optimal dose (2 g/kg BHB), body weight, lean mass, grip strength, tibialis anterior muscle cross-sectional area (CSA), and satellite cell markers (PAX7, MYOD1, Myogenin) were dynamically assessed on days 1, 3, 5, and 10 post-CLP. The role of Wnt signaling was examined using the inhibitor IWP-2.

Results

Medium-dose BHB (2 g/kg) reduced serum IL-6 and increased lean mass and tibialis anterior muscle CSA on day 5, without increasing mortality. Over 10 days, BHB better preserved body weight and lean mass; grip strength did not differ, but tibialis anterior muscle CSA recovery was accelerated by day 5. BHB increased PAX7⁺ and MYOD1⁺ satellite cells on days 3 and 5, and Myogenin⁺ cells on day 5. These protective effects on lean tissue and satellite cell markers were completely abolished by the Wnt inhibitor IWP-2.

Conclusions

Exogenous BHB accelerates the early structural regeneration of skeletal muscle in septic mice through Wnt pathway activation. However, the dissociation between structural recovery and functional gain highlights the multi-phasic nature of ICUAW recovery, suggesting that functional rehabilitation lags behind myogenesis and may require additional interventions targeting neuromuscular reinnervation.

1 Introduction

Intensive care unit-acquired weakness (ICUAW) is a prevalent neuromuscular complication in critically ill patients, characterized by symmetric limb weakness and respiratory muscle dysfunction that cannot be explained by other etiologies ^[1]. Epidemiological data indicate that the incidence of ICUAW reaches 43% among patients receiving mechanical ventilation for more than 7 days, with an even higher proportion in

patients with sepsis ^[2]. ICUAW not only prolongs the duration of mechanical ventilation, increases length of hospital stay and healthcare costs, but is also closely associated with elevated short-term mortality ^[3, 4]. More importantly, ICU survivors exhibit persistent impairment of exercise capacity and quality of life up to 5 years after discharge ^[5], underscoring that the impact of ICUAW extends far beyond the hospitalization period.

Sepsis is the most important risk factor for ICUAW^[6]. Under septic conditions, pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) are massively released ^[7, 8], activating the ubiquitin-proteasome system (UPS) via the NF- κ B and p38 MAPK signaling axes, leading to upregulation of the muscle-specific E3 ubiquitin ligases *Atrogin-1* and *MURF1*, which catalyze the degradation of myofibrillar proteins^[9–11]. Concurrently, inflammation-induced IGF-1 resistance inhibits PI3K/Akt signaling pathway^[12–14], creating a pathological landscape characterized by both "anabolic resistance" and "catabolic hyperactivity." This mechanistic model, centered on disrupted protein metabolism, can explain the rapid loss of muscle mass during the ICU stay, but it fails to address a key question: why do a substantial proportion of ICU survivors continue to suffer from persistent muscle weakness long after sepsis is controlled and inflammatory cytokine levels have returned to baseline?

Emerging evidence suggests that impaired muscle regenerative capacity may contribute to the chronic-phase pathology of ICUAW. Dos Santos et al. performed quadriceps muscle biopsies in patients 6 months after ICU discharge and found that inflammatory cytokines, autophagy markers, and proteasome activity had all returned to baseline levels in patients with persistent weakness, whereas muscle satellite cell content was significantly decreased compared with healthy controls^[15]. This finding implicates satellite cell pool depletion or functional inactivation in the chronic-phase pathology of ICUAW.

Notably, animal studies have demonstrated that obese mice exhibit significantly less skeletal muscle atrophy than lean mice following sepsis induction, accompanied by markedly elevated blood ketone body levels, particularly β -hydroxybutyrate (BHB)^[16, 17]. Exogenous BHB administration promoted the expression of muscle regeneration markers ^[17]. BHB, a ketone body produced by the liver during starvation or intense exercise, not only serves as an alternative fuel but also possesses pleiotropic functions including epigenetic regulation, anti-inflammatory, and antioxidant activities^[18–20]. In models of cancer cachexia, Duchenne muscular dystrophy, and sarcopenia, BHB has been shown to improve muscle structure and function^[21–23], suggesting that it may act on a common pathway of muscle regeneration.

However, no study to date has systematically evaluated the interventional effects of BHB in a sepsis-associated ICUAW model, nor has there been mechanistic investigation with satellite cell function as an endpoint. Therefore, this study aims to explore the effects of exogenous BHB supplementation on muscle regeneration in septic mice, to monitor the dynamic changes of the satellite cell pool, and to investigate the potential mediating pathways. We hypothesize that BHB may accelerate the myogenic

progression via Wnt signaling; however, given the inherent complexity of neuromuscular recovery, we also postulate that structural regeneration and functional restoration may follow distinct temporal trajectories. Elucidating this dissociation is critical for understanding the multi-phasic pathophysiology of ICUAW and for designing stage-specific therapeutic strategies.

2 Results

2.1 BHB Intervention Reduces Pro-inflammatory Cytokine Levels; Moderate Doses Attenuate Lean Tissue Loss, Whereas High Doses May Increase Mortality in Septic Mice

We first examined the dynamic changes in blood BHB levels following administration. The results showed that blood BHB levels increased in a significant dose- and time-dependent manner after BHB administration, peaking at 60 minutes post-injection in all BHB-treated groups. The peak concentrations in the high-, medium-, and low-dose BHB groups were 7.720 ± 0.213 mmol/L, 6.720 ± 0.260 mmol/L, and 4.580 ± 0.271 mmol/L, respectively (Fig. 1A).

Subsequently, we evaluated 5-day mortality, serum inflammatory cytokine levels on Day 5, lean tissue mass, grip strength, and tibialis anterior muscle histopathology (Fig. 1B-F). Although no statistically significant difference in mortality was observed among the CLP model groups ($p = 0.070$), the high-dose BHB group exhibited a trend toward increased mortality (Fig. 1B). Compared with the NS group, serum levels of the pro-inflammatory cytokine IL-6 on Day 5 were significantly reduced in all BHB-treated groups (high-dose: 342.163 ± 79.512 pg/mL; medium-dose: 295.247 ± 62.460 pg/mL; low-dose: 288.475 ± 53.353 pg/mL vs. NS: 587.430 ± 97.993 pg/mL, all $p < 0.0001$). In contrast, no significant difference was observed in the anti-inflammatory cytokine IL-10 among the model groups ($p = 0.510$) (Fig. 1C, D).

With respect to lean tissue, only the medium-dose BHB group exhibited significantly greater lean tissue mass compared with the NS group (17.330 ± 0.578 g vs. 15.732 ± 0.672 g, $p = 0.004$) (Fig. 1E). Moreover, the lean tissue-to-body weight ratio in the medium-dose BHB group was significantly higher than that in the high-dose BHB group (0.844 ± 0.029 vs. 0.794 ± 0.026 , $p = 0.025$) and the NS group (0.844 ± 0.029 vs. 0.793 ± 0.019 , $p = 0.022$) (Fig. 1F). Grip strength in all CLP model groups was significantly lower than that in the sham group, but no significant differences were detected among the model groups (Fig. 1G).

H&E staining of the tibialis anterior muscle revealed that, compared with the sham group, all CLP model groups displayed loosely arranged muscle fibers, widened intercellular spaces, and a significantly reduced cross-sectional area (CSA) (Fig. 1H, I). Among the CLP model groups, the medium-dose BHB group exhibited a significantly greater muscle fiber CSA than the low-dose BHB group (1866.733 ± 145.457 μm^2 vs. 1446.000 ± 210.799 μm^2 , $p = 0.038$) and the NS group (1866.733 ± 145.457 μm^2 vs. 1362.400 ± 80.518 μm^2 , $p = 0.012$) (Fig. 1I).

Figure 1. Dose optimization of exogenous BHB supplementation in septic mice

1. (A) Dynamic changes in blood β -hydroxybutyrate (BHB) levels following subcutaneous injection of different doses of BHB or normal saline (NS)(n = 5). (B) Five-day survival curves of septic mice receiving different doses of BHB or NS (sham group n = 5; other groups n = 15). (C) Serum levels of pro-inflammatory cytokine IL-6 on Day 5 post-modeling(n = 5). (D) Serum levels of anti-inflammatory cytokine IL-10 on Day 5 post-modeling(n = 5). (E) Lean tissue mass on Day 5 post-modeling(n = 5). (F) Lean tissue-to-body weight ratio on Day 5 post-modeling(n = 5). (G) Forelimb grip strength on Day 5 post-modeling(n = 5). (H) Representative H&E-stained cross-sections of the tibialis anterior muscle (scale bars: 50 μ m). (I) Quantitative analysis of muscle fiber cross-sectional area (CSA)(n = 3). Data are presented as mean $\pm$ SD. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; ns: not significant.

1. 2.2 BHB Upregulates Early Expression Of PAX7, MYOD1, And Myogenin In The Tibialis Anterior Muscle Of Septic Mice, Promotes Satellite Cell Proliferation And Differentiation, And Regulates Skeletal Muscle Regeneration And Repair

Over the 10-day observation period, the BHB group exhibited consistently higher body weight than the NS group (main effect of group, p = 0.040). Both groups showed an initial decline followed by a subsequent increase in body weight, reaching their nadirs on Day 3 (BHB: 21.600 ± 0.322 g; NS: 20.650 ± 0.855 g) (Fig. 2A). No significant difference in mortality was observed between the two groups within 10 days (p = 0.663) (Fig. 2B).

Analysis of lean tissue mass revealed no significant time $\times$ group interaction (p = 0.078), but the main effect of group was significant (p < 0.001), with the BHB group maintaining greater overall lean tissue mass than the NS group (Fig. 2C). Consistently, the lean tissue-to-body weight ratio was also significantly higher in the BHB group (main effect of group, p = 0.049) (Fig. 2D). With respect to grip strength, neither the main effects of time or group nor their interaction were statistically significant (all p > 0.05) (Fig. 2E).

Figure 2. Temporal effects of BHB supplementation on muscle regeneration in septic mice

(A) Changes in body weight over the 10-day observation period(n = 6). (B) Ten-day survival curves of BHB- and NS-treated septic mice(n = 12). (C) Lean tissue mass on Days 1, 3, 5, and 10 post-modeling(n = 6). (D) Lean tissue-to-body weight ratio on Days 1, 3, 5, and 10 post-modeling(n = 6). (E) Forelimb grip strength on Days 3, 5, and 10 post-modeling(n = 6). Data are presented as mean $\pm$ SD. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; ns: not significant.

Tibialis anterior muscle CSA analysis revealed a significant time $\times$ group interaction (p = 0.005). Simple effects analysis demonstrated that the BHB group had a significantly larger muscle fiber CSA than the NS group on Day 5 (1815.3 ± 64.1 μ m² vs. 1437.0 ± 62.0 μ m², p = 0.002). However, following Bonferroni correction (adjusted significance threshold, p < 0.013), no significant differences were detected on Day 1 (2012.000 ± 35.511 μ m² vs. 1936.000 ± 95.687 μ m², p = 0.267), Day 3 (1751.667 ± 68.879 μ m² vs.

1584.667 ± 56.977 μm², $p = 0.032$), or Day 10 (2125.000 ± 77.149 μm² vs. 2022.667 ± 61.978 μm², $p = 0.148$) (Fig. 3A, B).

Immunohistochemical staining of satellite cell regeneration markers on Days 3 and 5 further showed that for PAX7⁺ and MYOD1⁺ cell counts, the time × group interaction was not significant ($p = 0.660$ and $p = 0.270$), but the main effect of group ($p < 0.001$ and $p = 0.0215$) and time ($p = 0.039$ and $p = 0.008$) were both significant (Fig. 3C, D, F, G).

Compared with NS, BHB treatment significantly increased the overall PAX7⁺ cell counts (marginal mean: 735.167 ± 98.652 cells/mm² vs. 490.167 ± 91.648 cells/mm², $p < 0.001$) and MYOD1⁺ cell counts (marginal mean: 561.833 ± 126.879 cells/mm² vs. 449.333 ± 66.147 cells/mm², $p = 0.0215$). While PAX7⁺ and MYOD1⁺ cell counts decreased significantly from Day 3 to Day 5 (marginal mean: 669.333 ± 155.154 cells/mm² vs. 556.000 ± 149.557 cells/mm², $p = 0.039$ and 575.333 ± 103.427 cells/mm² vs. 435.833 ± 75.460 cells/mm², $p = 0.008$) (Fig. 3F, G). Additionally, the counts of Myogenin⁺ cells were significantly higher in the BHB group than in the NS group on Day 5 (477.330 ± 25.541 cells/mm² vs. 239.330 ± 22.368 cells/mm², $p < 0.001$) (Fig. 3E, H).

Figure 3. Temporal effects of BHB supplementation on muscle satellite cell dynamics in septic mice.

1. (A) Representative H&E-stained cross-sections of the tibialis anterior muscle (scale bars: 50 μm) on Days 1, 3, 5, and 10 post-modeling ($n = 3$). (B) Quantitative analysis of muscle CSA on Days 1, 3, 5, and 10 post-modeling ($n = 3$). (C) Representative immunohistochemical staining for PAX7 (scale bars: 50 μm). (D) Representative immunohistochemical staining for MYOD1 (scale bars: 50 μm). (E) Representative immunohistochemical staining for Myogenin (scale bars: 50 μm). (F) Quantitative analysis of PAX7⁺ cells on Days 3 and 5 post-modeling ($n = 3$). (G) Quantitative analysis of MYOD1⁺ cells on Days 3 and 5 post-modeling ($n = 3$). (H) Quantitative analysis of Myogenin⁺ cells on Days 3 and 5 post-modeling ($n = 3$). Data are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns: not significant.

1. 2.3 BHB Activates The Wnt Pathway To Enhance Early PAX7 Expression And Drive The Transition From MYOD1⁺ To Myogenin⁺ Satellite Cells, Temporally Regulating Early Skeletal Muscle Regeneration And Repair In Septic Mice

The above results demonstrate that BHB significantly upregulates the expression of PAX7, MYOD1, and Myogenin, suggesting that it may exert its pro-regenerative effects by targeting upstream regulatory pathways governing satellite cell proliferation and differentiation. The Wnt signaling pathway is a key cascade controlling satellite cell fate determination. Canonical Wnt/β-catenin signaling can directly activate the transcription of *Myf5* and *MyoD* to promote satellite cell proliferation and lineage commitment, while non-canonical Wnt signaling also participates in the fine-tuning of myogenic differentiation^[24–26]. Based on this, we hypothesize that BHB, via Wnt pathway activation, exerts a phased effect on the transition of satellite cells from the activated state (PAX7⁺/MYOD1⁺) toward

terminal differentiation (Myogenin⁺), thereby contributing to the early skeletal muscle regeneration process in septic mice.

To test this hypothesis, we administered the Wnt inhibitor IWP-2 in septic mice to create the BHB + IWP-2 and NS + IWP-2 groups. All groups received daily interventions for 5 consecutive days and were compared with the Day 5 BHB and NS groups from Experiment 2.

On Day 5 post-intervention, lean tissue mass in the BHB group was significantly greater than that in the NS group ($18.060 \pm 0.620\text{g}$ vs. $16.488 \pm 0.678\text{g}$, $p = 0.011$), the BHB + IWP-2 group ($18.060 \pm 0.620\text{g}$ vs. $15.922 \pm 0.496\text{g}$, $p < 0.001$), and the NS + IWP-2 group ($18.060 \pm 0.620\text{g}$ vs. $15.792 \pm 1.100\text{g}$, $p < 0.001$). No significant difference was detected between the BHB + IWP-2 and NS + IWP-2 groups ($p > 0.05$) (Fig. 4A). The lean tissue-to-body weight ratio in each group showed similar trends (Fig. 4B). These findings indicate that the protective effect of BHB on lean tissue was completely abolished by IWP-2.

With respect to muscle regeneration markers, the number of PAX7⁺ cells was significantly higher in the BHB group than in the NS group ($668.000 \pm 94.694\text{ cells/mm}^2$ vs. $444.000 \pm 96.535\text{ cells/mm}^2$, $p = 0.036$), the BHB + IWP-2 group ($668.000 \pm 94.694\text{ cells/mm}^2$ vs. $278.333 \pm 54.096\text{ cells/mm}^2$, $p = 0.001$), and the NS + IWP-2 group ($668.000 \pm 94.694\text{ cells/mm}^2$ vs. $307.000 \pm 26.627\text{ cells/mm}^2$, $p = 0.002$), whereas no significant difference was observed between the BHB + IWP-2 and NS + IWP-2 groups ($p > 0.05$) (Fig. 4C, F). No significant differences in MYOD1⁺ cell counts were detected among any of the groups (all $p > 0.05$) (Fig. 4D, G). The number of Myogenin⁺ cells in the BHB group was significantly higher than that in the NS group ($477.330 \pm 25.541\text{ cells/mm}^2$ vs. $239.330 \pm 22.368\text{ cells/mm}^2$, $p < 0.001$), the BHB + IWP-2 group ($477.330 \pm 25.541\text{ cells/mm}^2$ vs. $289.000 \pm 52.115\text{ cells/mm}^2$, $p < 0.001$), and the NS + IWP-2 group ($477.330 \pm 25.541\text{ cells/mm}^2$ vs. $177.670 \pm 20.306\text{ cells/mm}^2$, $p < 0.001$). Notably, the number of Myogenin⁺ cells in the BHB + IWP-2 group remained significantly higher than that in the NS + IWP-2 group ($289.000 \pm 52.115\text{ cells/mm}^2$ vs. $177.670 \pm 20.306\text{ cells/mm}^2$, $p = 0.022$) (Fig. 4E, H). Overall, these results suggest that BHB may upregulate PAX7 and Myogenin expression through activation of the Wnt signaling pathway, and may thereby promote skeletal muscle regeneration and lean tissue preservation in septic mice.

(A) Lean tissue mass on Day 5 post-modeling in the BHB, NS, BHB + IWP-2, and NS + IWP-2 groups (n = 6). (B) Lean tissue-to-body weight ratio on Day 5 post-modeling in the BHB, NS, BHB + IWP-2, and NS + IWP-2 groups (n = 6). (C) Representative immunohistochemical staining for PAX7 (scale bars: 50 μm). (D) Representative immunohistochemical staining for MYOD1 (scale bars: 50 μm). (E) Representative immunohistochemical staining for Myogenin (scale bars: 50 μm). (F) Quantitative analysis of PAX7⁺ cells on Day 5 post-modeling (n = 3). (G) Quantitative analysis of MYOD1⁺ cells on Day 5 post-modeling (n = 3). (H) Quantitative analysis of Myogenin⁺ cells on Day 5 post-modeling (n = 3). Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns: not significant.

3 Methods

3.1 Animals

Specific pathogen-free (SPF) male C57BL/6 mice (8–10 weeks old, body weight 22–25 g) were purchased from Hangzhou Qizhen Experimental Animal Technology Co., Ltd. (Hangzhou, China). All mice were housed in the Animal Experiment Center of Soochow University under controlled environmental conditions (temperature: 22–24°C; humidity: 50 ± 10%; 12-hour light/dark cycle) with free access to food and water. After one week of acclimatization, experiments were initiated. Body weight was recorded daily throughout the experimental period. On the final day of the experiment, mice were euthanized by cervical dislocation, and the tibialis anterior muscles were harvested, snap-frozen in liquid nitrogen, and stored at – 80°C until further analysis. All animal procedures were approved by the Institutional Animal Care and Use Committee of Soochow University (approval no. 202510A0012) and were performed in accordance with the relevant guidelines and regulations. All methods were carried out in compliance with the ARRIVE guidelines. All animals were euthanized in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020).

3.2 Sepsis Model

After one week of acclimatization, mice were fasted for 12 hours prior to surgery. Sepsis was induced in the CLP model group using the cecal ligation and puncture (CLP) procedure. Briefly, mice were anesthetized by intraperitoneal injection of 1.25% tribromoethanol (0.2 mL/10 g body weight). Following abdominal skin disinfection, a midline laparotomy (approximately 1 cm) was performed. The cecum was gently exteriorized and ligated at the distal half position using 4 – 0 silk suture. A single through-and-through puncture was made in the ligated cecum with a 20-gauge needle, and a small amount of fecal content was extruded into the peritoneal cavity. The cecum was then returned to the abdominal cavity, and the incision was closed in layers. Buprenorphine (0.1 mg/kg) was injected subcutaneously once for postoperative pain control. All surgical mice received the corresponding intervention subcutaneously or intraperitoneally immediately after surgery. Mice in the sham group underwent laparotomy under anesthesia without cecal ligation or puncture.

3.3 Experimental Design

Experiment 1

Mice were divided into a CLP model group and a sham group. CLP mice were further randomized to receive subcutaneous injections of different doses of BHB (CAS No.:150-83-4; Shanghai Aladdin Biochemical Technology Co., Ltd.; Shanghai, China) or normal saline. The final groups were as follows: high-dose BHB (3 g/kg), medium-dose BHB (2 g/kg), low-dose BHB (1 g/kg), normal saline (NS), and sham. The sham group comprised 5 mice, whereas all other groups comprised 15 mice each. Immediately after CLP surgery, mice in the BHB groups received subcutaneous injections of the corresponding BHB dose, and the NS group received 1 ml sterile normal saline. All treatments were administered once daily at the same time for 5 consecutive days. On Day 5 post-surgery, 5 surviving mice from each group were euthanized.

Experiment 2

A total of 50 mice were initially enrolled in each group to compensate for the anticipated mortality associated with the CLP procedure. From each group, 12 mice were randomly assigned to the survival observation cohort and followed for 10 days without euthanasia. The remaining 38 mice were allocated for tissue collection at four predefined post-operative time points (Days 1, 3, 5, and 10). At each time point, 6 surviving mice were randomly selected from this subset and euthanized for sample harvest. Any mouse that died prior to its scheduled time point was excluded from further analysis.

Experiment 3

Mice were randomly assigned to two groups: BHB + IWP-2 and NS + IWP-2 (n = 15 per group). Mice received subcutaneous injections of 2 g/kg BHB or an equivalent volume of sterile normal saline, concurrently with intraperitoneal injection of the Wnt pathway inhibitor IWP-2 (5 mg/kg) (CAS No.:686770-61-6; Shandong SparkJade Biotechnology Co., Ltd.; Jinan, Shandong, China), which was dissolved in DMSO. All treatments were administered once daily at the same time for 5 consecutive days. On Day 5 post-surgery, five surviving mice from each group were euthanized.

3.4 Measurement of Inflammatory Cytokine Levels by ELISA

Serum levels of the inflammatory cytokines IL-6 and IL-10 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Jianglai Biotechnology, Shanghai, China).

3.5 Body Composition Analysis

Mice were guided into a cylindrical restrainer and placed into the minispec analyzer probe (LF50, Bruker, Karlsruhe, Germany). Data were automatically acquired by the instrument. Upon completion of the scan, the mice were removed from the restrainer.

3.6 Grip Strength Test

Forelimb grip strength was measured daily using a grip strength meter (SA415, SANS Biotechnology, Nanjing, Jiangsu, China). Each mouse was tested three times with a 5-minute interval between trials, and the average of the maximum grip strength values was recorded as the forelimb grip strength. All measurements were performed by the same investigator throughout the experiment.

3.7 Hematoxylin and Eosin Staining

Immediately after excision, the tibialis anterior muscle specimens were labeled, immediately immersed in tissue fixative, and processed for routine paraffin embedding. Sections were cut at a thickness of 3 μ m and stained with hematoxylin and eosin (H&E). Stained sections were examined under a light microscope, and muscle fiber cross-sectional area (CSA) was quantified using Fiji across multiple fields of view.

3.8 Immunohistochemistry Staining

Paraffin-embedded tibialis anterior muscle sections were deparaffinized and subjected to antigen retrieval in citrate buffer (pH 6.0) using a pressure cooker. After cooling at room temperature for 2 hours, sections were rinsed with TBST wash buffer. Endogenous peroxidase activity was blocked by applying 3% hydrogen peroxide to the tissue sections for 5 minutes, followed by three washes with TBST. The sections were then incubated with diluted primary antibody (anti-PAX7, Absin Bioscience, Shanghai, China; anti-MYOD1, Proteintech, Wuhan, China; or anti-Myogenin, Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature. Following three TBST washes, sections were incubated with the corresponding secondary antibody for 30 minutes at room temperature and rinsed again with TBST. Freshly prepared DAB chromogen solution was applied, and positive cell staining (brownish-yellow or brownish-black signal) was monitored under a microscope.

3.9 Measurement of Blood β -Hydroxybutyrate Concentration

Blood β -hydroxybutyrate (BHB) concentration was measured using a Precision Xtra blood ketone meter with BHB test strips (Abbott Laboratories, Chicago, IL, USA). Tail vein blood samples were collected before injection (0 min) and at 30, 60, 90, and 120 min post-injection.

3.10 Statistical Analysis

All data were analyzed using SPSS 27.0.1 and GraphPad Prism 10.4.1. Data are presented as mean $\pm$ standard deviation (*SD*). Between-group differences were assessed using independent samples t-test, one-way analysis of variance (ANOVA), or two-way ANOVA, depending on the data distribution characteristics. The Bonferroni correction was applied for multiple comparisons to control the type I error rate. A p-value < 0.05 was considered statistically significant.

4 Discussion

In this study, we systematically evaluated the effects of exogenous BHB on skeletal muscle regeneration using a CLP-induced sepsis mouse model. Our core findings suggest that BHB improves muscle fiber cross-sectional area and lean tissue mass, possibly through Wnt signaling pathway activation, and may be involved in the entire regenerative process—from satellite cell activation to terminal differentiation.

However, these structural gains did not translate into concomitant functional improvement within the 10-day observation window. This dissociation between morphological and functional recovery not only defines the temporal efficacy window of BHB but also provides important insights into the multi-phasic nature of ICUAW recovery, wherein myogenesis and neuromuscular reintegration follow distinct timelines.

Within 24–72 hours after CLP, mice exhibit an acute critical phase characterized by the cytokine storm and hypermetabolism, leading to rapid muscle protein degradation via the ubiquitin-proteasome

system^[27–29]. By Day 5, systemic inflammation has subsided from peak levels, marking the transition from a catabolic state to a repair and reconstruction window—a critical juncture for evaluating interventions that attenuate muscle wasting and promote recovery^[30, 31]. All CLP groups exhibited significantly reduced muscle CSA, lean tissue mass, and grip strength compared with the sham group, faithfully recapitulating ICUAW hallmarks. Moderate-dose BHB (2 g/kg) effectively attenuated lean tissue loss and reduced serum IL-6 levels without the mortality trend observed in the high-dose group, consistent with evidence that supraphysiological ketone body levels may be detrimental^[32, 33]. This informed our dose selection for subsequent experiments.

In Experiment 2, temporal analysis across Days 1, 3, 5, and 10 revealed that BHB-treated mice maintained significantly higher body weight and lean tissue mass than those in the NS group throughout the observation period. Histologically, the BHB group exhibited significantly larger muscle fiber CSA on Day 5, yet this difference disappeared by Day 10. Rather than interpreting this convergence as a waning of BHB efficacy, we attribute this pattern to the intrinsically multiphasic nature of muscle regeneration. Prior to Day 5, regeneration is primarily driven by satellite cell activation and proliferation—processes that are precisely targeted by BHB through activation of the Wnt pathway. After Day 5, regeneration transitions toward myofiber maturation, reinnervation, and functional establishment, processes less directly influenced by Wnt signaling^[34, 35]. As the acute inflammatory storm subsides, endogenous repair mechanisms may gradually operate, narrowing the intergroup gap. Thus, BHB appears to accelerate the regenerative timeline rather than indefinitely augmenting the final regenerative capacity. This temporal compression is therapeutically valuable, as shortening the duration of structural deficit may reduce the window of vulnerability to secondary complications.

Immunohistochemical analyses on Days 3 and 5 offered insights into the underlying mechanisms. The BHB group exhibited a more abundant PAX7⁺ satellite cell pool at both time points, suggesting enhanced regenerative reserve. By Day 5, the BHB group showed significantly increased Myogenin⁺ cell density, indicating that more cells might have entered terminal differentiation. Satellite cells, residing beneath the myofiber basal lamina, maintain quiescence through PAX7 expression. Upon injury, they upregulate MYOD1, initiate proliferation, and subsequently upregulate Myogenin to complete terminal differentiation and fuse into nascent myotubes. In sepsis, TNF- α inhibits myoblast differentiation via NF- κ B signaling; excessive nitric oxide and microvascular dysfunction disrupt the satellite cell niche; and metabolic derangements constrain satellite cell activation at the energetic level^[36, 37]. BHB intervention appears to have counteracted these inhibitory factors by promoting the PAX7⁺→MYOD1⁺→Myogenin⁺ cascade, thereby accelerating structural recovery.

We hypothesized that BHB may promote satellite cell-mediated regeneration through Wnt pathway activation. The Wnt cascade is a central regulator of satellite cell fate, potentially driving cells from quiescence into activation and toward terminal differentiation^[38–40]. Experiment 3 provided key evidence: the protective effect of BHB on lean tissue mass and the increase in PAX7⁺ cells were completely abolished by IWP-2, suggesting that Wnt pathway activation is necessary for BHB's effect on

satellite cell pool expansion. The BHB-induced increase in Myogenin⁺ cells was significantly attenuated but not completely blocked by Wnt inhibition, suggesting that BHB may activate additional pro-differentiation signals that account for the remaining effect. The persistence of MYOD1⁺ cell increases despite Wnt inhibition further illustrates that BHB, as a pleiotropic molecule, may engage multiple signaling pathways in regulating muscle regeneration. These findings suggest that while Wnt signaling is a critical node for BHB's action on early satellite cell expansion, the full regenerative effect of BHB likely involves a broader signaling network that remains to be fully characterized.

Despite significant structural improvements, grip strength did not exhibit commensurate improvement, revealing an inherent temporal dissociation between structural reconstruction and functional recovery. We propose that this dissociation is not a failure of the intervention, but rather a fundamental reflection of the temporal architecture of neuromuscular recovery. Nascent myofibers require complex maturation—including myofibrillar assembly and excitation-contraction coupling refinement—before functioning as fully competent contractile units^[41–43]. Furthermore, motor neuron reinnervation substantially lags behind muscle fiber regeneration, and active Wnt signaling may exert limited influence on synapse formation^[44, 45]. Thus, the abundant Myogenin⁺ cells on Day 5 may not yet be fully innervated or functionally recruited. Methodologically, grip strength as a behavioral endpoint is susceptible to confounding by pain and motivation, exhibits greater variability, and requires larger effect sizes or longer observation periods to detect between-group differences compared with direct structural measurements^[46, 47]. While we acknowledge that grip strength is a single behavioral endpoint and may not fully capture all dimensions of neuromuscular function, it is the most widely accepted and clinically translatable measure of global limb strength in murine models. The persistent lack of improvement in this measure, despite robust structural gains, strongly suggests that functional reintegration lags behind myogenesis within the observational window, highlighting the need for longer-term studies combining motor neuron assessment. The persistent absence of functional gains strongly suggests that structural regeneration and functional restoration follow distinct temporal trajectories in the post-sepsis recovery process.

These findings carry important translational implications for the design of future ICUAW therapeutic strategies. First, the present data argue against the use of early functional endpoints (e.g., 5- or 10-day grip strength) as the sole criterion for evaluating muscle regenerative therapies, as structural benefits may precede functional translation by a substantial margin. Second, these observations support a sequential, phase-targeted approach to ICUAW management: metabolic modulators like BHB may be optimally deployed during the early structural repair phase to accelerate myogenesis, but achieving complete functional recovery will likely require additional or sequential interventions targeting NMJ stabilization, neural regeneration, or physical rehabilitation. Third, while the Wnt-dependent mechanism positions BHB as a promising candidate for phase-specific therapy, the risks of sustained Wnt activation—including potential off-target effects on fibrogenesis or osteogenesis—warrant long-term safety evaluation before clinical translation.

Several limitations of this study should be acknowledged. First, the 10-day observation period is insufficient to assess long-term functional recovery or the eventual convergence of structural and functional outcomes. Extended observation (e.g., 4–8 weeks) would be necessary to determine whether BHB-treated mice eventually achieve full functional recovery or whether the structural advantage dissipates without sustained benefit. Second, grip strength as a single functional endpoint has inherent limitations and may not fully capture the complexity of neuromuscular recovery. Complementary assessments such as in situ muscle contractility, electromyography, or NMJ morphometry would provide a more comprehensive functional evaluation. Third, the upstream regulatory mechanisms through which BHB activates the Wnt signaling pathway remain unexplored, representing a gap in the current mechanistic understanding. Finally, the short-term nature of the intervention precludes evaluation of the long-term safety and potential adverse effects of sustained BHB administration.

5 Conclusion

Exogenous BHB supplementation alleviates the inflammatory response, preserves lean mass, and accelerates the structural recovery of skeletal muscle in septic mice. Mechanistically, BHB appears to activate the Wnt signaling pathway, thereby promoting the expansion of PAX7⁺/MYOD1⁺ satellite cells and their transition into Myogenin⁺ differentiated cells, which temporally drives early myogenesis. BHB shows promise as a phase-specific metabolic intervention for the early structural phase of ICUAW, but achieving complete functional restoration may require sequential or combined approaches that also target neuromuscular junction remodeling and motor neuron recovery. Our findings provide a foundational framework for developing staged therapeutic algorithms in the management of sepsis-associated muscle weakness.

Abbreviations

ANOVA

analysis of variance

Akt

protein kinase B

BHB

β-hydroxybutyrate

CLP

cecal ligation and puncture

CSA

cross-sectional area

DAB

3,3'-diaminobenzidine

ELISA

enzyme-linked immunosorbent assay

H&E

hematoxylin and eosin
ICUAW
intensive care unit-acquired weakness
IGF-1
insulin-like growth factor 1
IL-1 β
interleukin-1 β
IL-6
interleukin-6
IL-10
interleukin-10
IWP-2
inhibitor of Wnt production-2
MAPK
mitogen-activated protein kinase
MuRF1
muscle RING finger 1
MYOD1
myogenic differentiation 1
NF- κ B
nuclear factor kappa B
NS
normal saline
PAX7
paired box 7
PI3K
phosphoinositide 3-kinase
SD
standard deviation
SPF
specific pathogen-free
TBST
Tris-buffered saline with Tween 20
TNF- α
tumor necrosis factor- α
UPS
ubiquitin-proteasome system

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of Soochow University (approval no. 202510A0012).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Conceptualization, X.W. and H.Y.; methodology, X.W.; software, X.W.; validation, H.Y. and W.Y.; data curation, X.W.; writing—original draft preparation, X.W.; writing—review and editing, H.Y.; visualization, X.W.; supervision, J.J.; project administration, Y.W.; funding acquisition, J. J. and Y. W. All authors have read and agreed to the published version of the manuscript.

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Not applicable.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

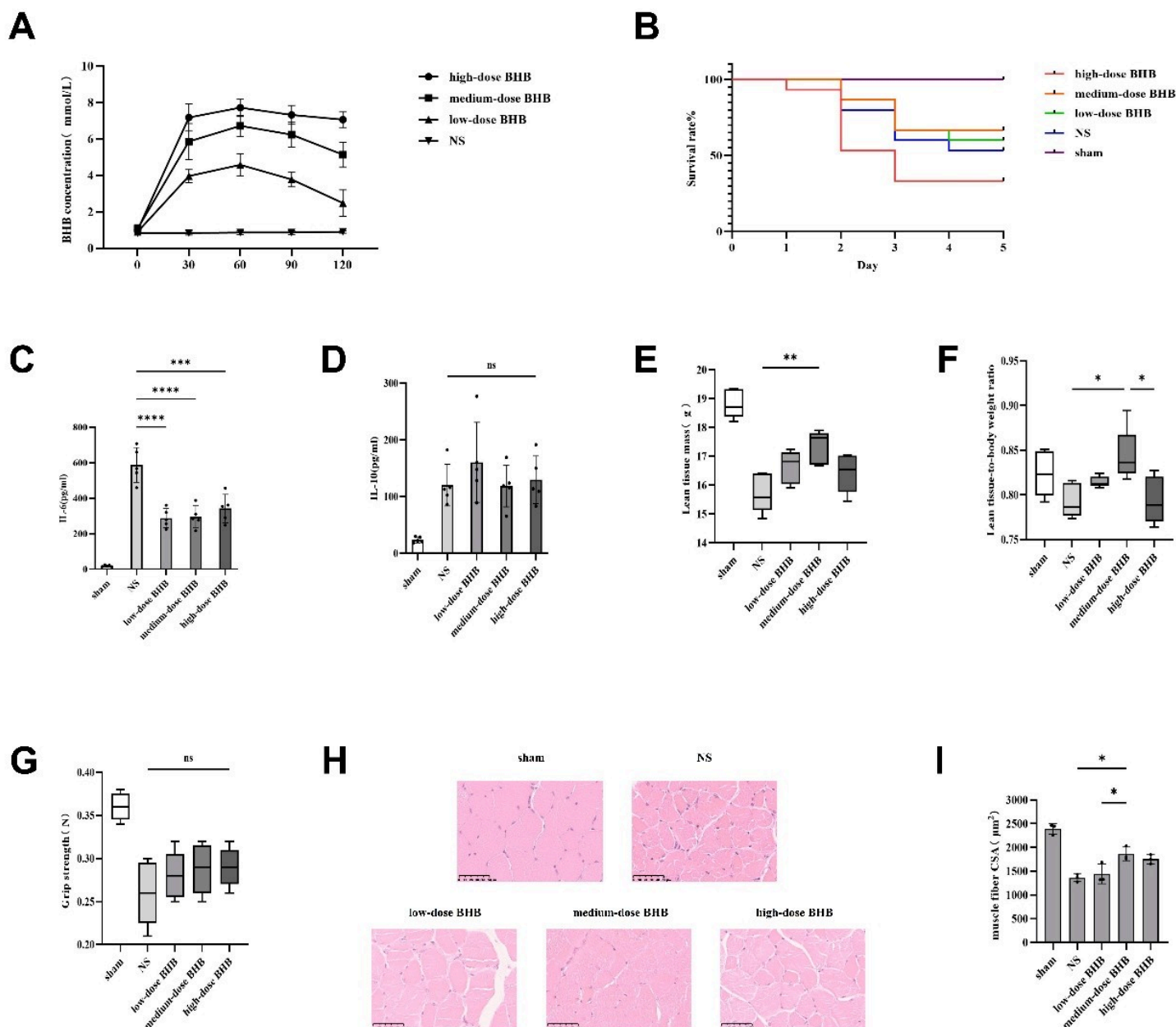


Figure 1

Dose optimization of exogenous BHB supplementation in septic mice

(A) Dynamic changes in blood β -hydroxybutyrate (BHB) levels following subcutaneous injection of different doses of BHB or normal saline (NS)(n=5). (B) Five-day survival curves of septic mice receiving different doses of BHB or NS (sham group n=5; other groups n=15). (C) Serum levels of pro-inflammatory cytokine IL-6 on Day 5 post-modeling(n=5). (D) Serum levels of anti-inflammatory cytokine IL-10 on Day 5 post-modeling(n=5). (E) Lean tissue mass on Day 5 post-modeling(n=5). (F) Lean tissue-to-body weight ratio on Day 5 post-modeling(n=5). (G) Forelimb grip strength on Day 5 post-modeling(n=5). (H) Representative H&E-stained cross-sections of the tibialis anterior muscle (scale bars: 50 μm). (I)

Quantitative analysis of muscle fiber cross-sectional area (CSA)(n=3). Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns: not significant.

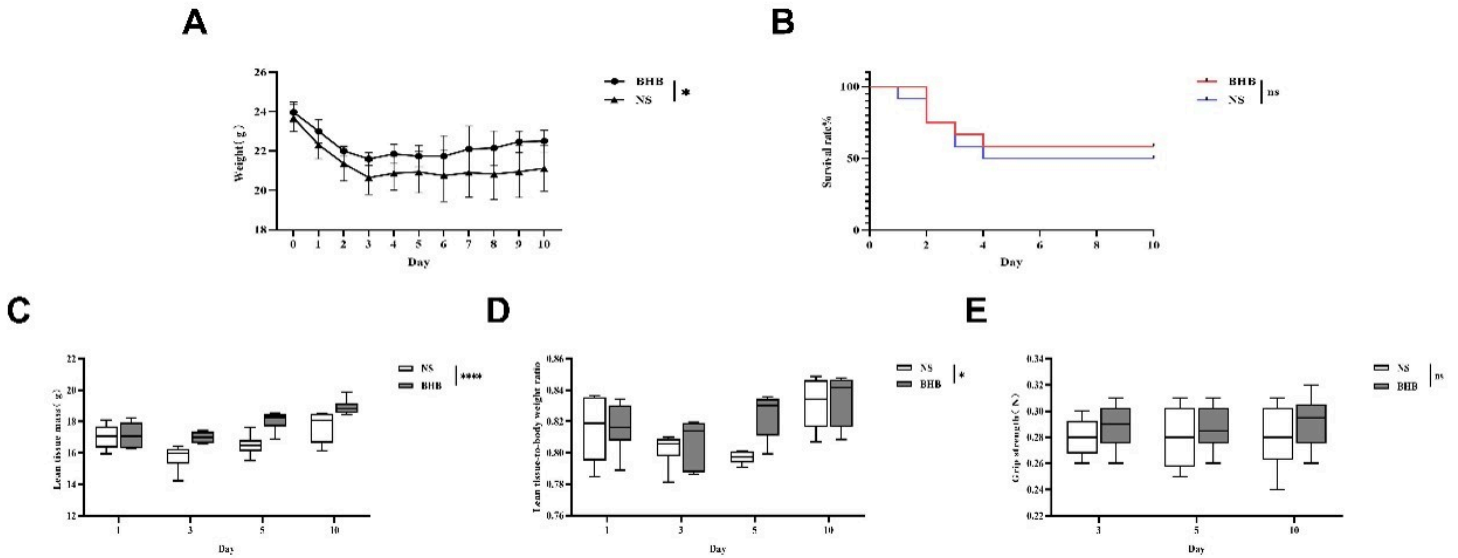


Figure 2

Temporal effects of BHB supplementation on muscle regeneration in septic mice

(A) Changes in body weight over the 10-day observation period(n=6). (B) Ten-day survival curves of BHB- and NS-treated septic mice(n=12). (C) Lean tissue mass on Days 1, 3, 5, and 10 post-modeling(n=6). (D) Lean tissue-to-body weight ratio on Days 1, 3, 5, and 10 post-modeling(n=6). (E) Forelimb grip strength on Days 3, 5, and 10 post-modeling(n=6). Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns: not significant.

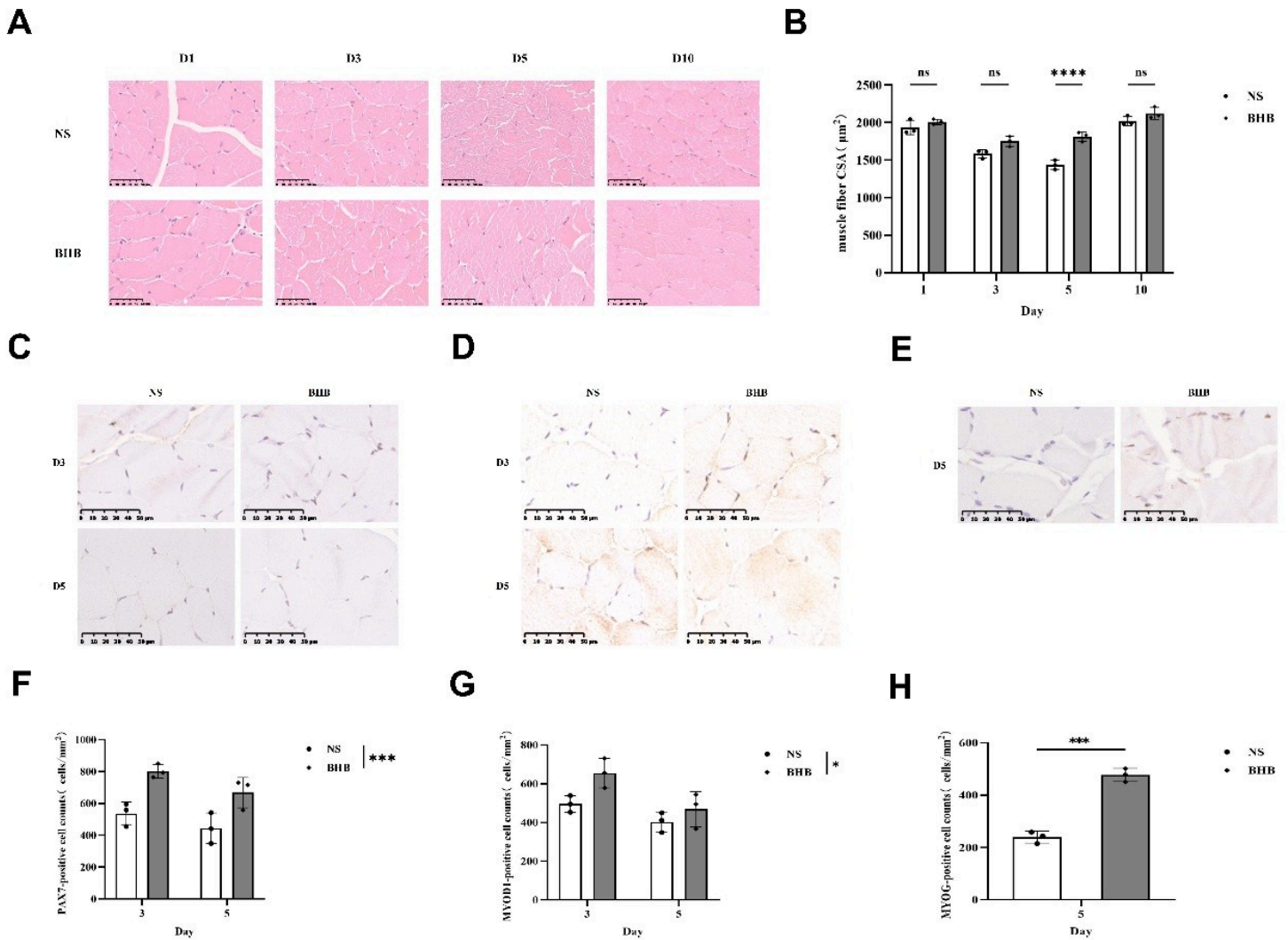


Figure 3

Temporal effects of BHB supplementation on muscle satellite cell dynamics in septic mice.

(A) Representative H&E-stained cross-sections of the tibialis anterior muscle (scale bars: 50μm) on Days 1, 3, 5, and 10 post-modeling(n=3). (B) Quantitative analysis of muscle CSA on Days 1, 3, 5, and 10 post-modeling(n=3). (C) Representative immunohistochemical staining for PAX7 (scale bars: 50 μm). (D) Representative immunohistochemical staining for MYOD1 (scale bars:50 μm). (E) Representative immunohistochemical staining for Myogenin (scale bars: 50 μm). (F) Quantitative analysis of PAX7⁺ cells on Days 3 and 5 post-modeling(n=3). (G) Quantitative analysis of MYOD1⁺ cells on Days 3 and 5 post-modeling(n=3). (H) Quantitative analysis of Myogenin⁺ cells on Days 3 and 5 post-modeling(n=3). Data are presented as mean ± SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001; ns: not significant.

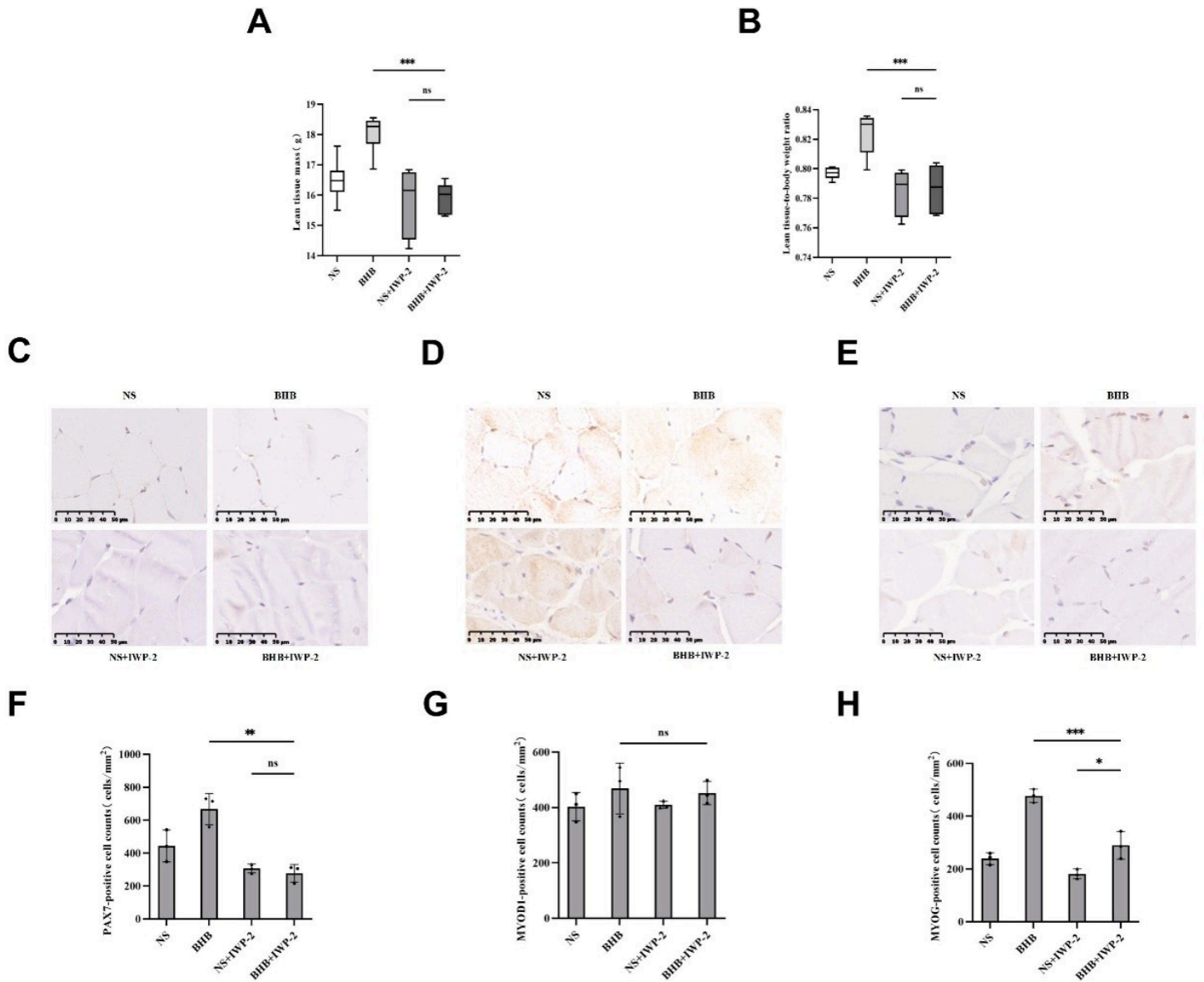


Figure 4

Wnt pathway inhibition abolishes BHB-mediated muscle protection and satellite cell regulation in septic mice.

(A) Lean tissue mass on Day 5 post-modeling in the BHB, NS, BHB+IWP-2, and NS+IWP-2 groups(n=6). (B) Lean tissue-to-body weight ratio on Day 5 post-modeling in the BHB, NS, BHB+IWP-2, and NS+IWP-2 groups(n=6). (C) Representative immunohistochemical staining for PAX7(scale bars: 50 μ m). (D) Representative immunohistochemical staining for MYOD1(scale bars: 50 μ m). (E) Representative immunohistochemical staining for Myogenin (scale bars: 50 μ m). (F) Quantitative analysis of PAX7⁺ cells on Day 5 post-modeling(n=3). (G) Quantitative analysis of MYOD1⁺ cells on Day 5 post-modeling(n=3). (H) Quantitative analysis of Myogenin⁺ cells on Day 5 post-modeling(n=3). Data are presented as mean $\pm$ SD. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; ns: not significant.