The Protective Effects of Beta-Hydroxy-Beta-Methylbutyrate on Skeletal Muscle in Glucocorticoid Treated Mice

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March 2025



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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

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Dr. Mahita Kadmiel

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Dr. Lauren French

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Abstract

Glucocorticoids are widely used in clinical settings but are associated with significant muscle wasting due to increased proteolysis and suppressed protein synthesis. Dexamethasone (Dex), a synthetic glucocorticoid, is commonly used to model glucocorticoid-induced muscle atrophy. Beta-Hydroxy Beta-Methylbutyrate (HMB), a leucine-derived metabolite, has been proposed as a potential countermeasure to muscle loss; however, its efficacy in attenuating Dex-induced muscle atrophy remains unclear. In this study, male C57BL/6J mice were assigned to one of four groups: Control, Dex, HMB, or Dex + HMB. Body weight, food intake, and water consumption were recorded daily for 21 days, and skeletal muscle masses (soleus and gastrocnemius) were assessed postmortem. Dex administration resulted in significant reductions in body weight ($p = 0.0434$), tail mass ($p = 0.0001$), and muscle mass ($p = 0.0029$), confirming its catabolic effects. HMB supplementation alone had no significant effect on body weight but increased water consumption ($p = 0.0438$). Notably, HMB failed to mitigate muscle atrophy in Dex-treated mice, as muscle masses in the Dex and Dex + HMB groups were comparable ($p = 0.0029$ for soleus). These findings indicate that HMB supplementation does not prevent glucocorticoid-induced muscle loss, suggesting limited efficacy as a therapeutic intervention in this context. Further studies are warranted to explore alternative strategies for preserving skeletal muscle during glucocorticoid therapy.

Introduction

Chronic inflammation is a persistent immune response that fails to resolve over time and has been linked to a wide array of chronic diseases. It affects nearly 35% of Americans, contributing to a significant public health burden (Pease, 2024). While inflammation is a natural and necessary response to injury, infection, or harmful stimuli, its prolonged presence can have detrimental effects on tissues and organs. Acute inflammation is typically short-lived, characterized by redness, swelling, heat, and pain, and resolves once the underlying issue is addressed. However, in chronic inflammation, the immune system remains in a heightened state of activity, leading to prolonged tissue damage and systemic dysfunction (Oray et al., 2016). This prolonged inflammatory state has been implicated in numerous diseases, including autoimmune disorders such as rheumatoid arthritis and psoriasis, as well as inflammatory bowel diseases like Crohn's disease (Bonnet & Walsh, 2005; Costa et al., 2007). Chronic inflammation is also closely associated with metabolic disorders, including type 2 diabetes and obesity, where excessive inflammatory signaling disrupts insulin sensitivity and metabolic homeostasis (Hotamisligil, 2006). Moreover, persistent inflammation has been linked to neurodegenerative diseases such as Alzheimer's disease, where inflammatory cytokines contribute to neuronal damage (Heneka et al., 2015). Additionally, inflammation plays a pivotal role in cancer progression, as inflammatory mediators can promote tumor growth, angiogenesis, and metastasis (Coussens & Werb, 2002).

One of the primary pharmacological interventions for chronic inflammation is the use of glucocorticoids, a class of steroid hormones that exert potent anti-inflammatory and immunosuppressive effects. These drugs, including dexamethasone and prednisone, are

commonly prescribed for conditions such as asthma, rheumatoid arthritis, and inflammatory bowel diseases (Vandewalle et al., 2018). Glucocorticoids function by binding to glucocorticoid receptors (GR), which are widely expressed throughout the body and play a crucial role in regulating inflammation, cell proliferation, cognition, metabolic homeostasis, and even psychological well-being (Rhen & Cidlowski, 2005). By activating these receptors, glucocorticoids suppress the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), thereby dampening the immune response (Barnes, 2011). Despite their efficacy, long-term glucocorticoid use is associated with a broad spectrum of adverse effects that impact multiple organ systems. These side effects include musculoskeletal complications such as osteoporosis, muscle atrophy, and impaired wound healing, as well as metabolic disturbances like hyperglycemia and insulin resistance (Oray et al., 2016). Neurological and psychiatric effects, including mood swings, anxiety, depression, and cognitive impairments, have also been reported, likely due to glucocorticoids' influence on neurotransmitter systems and brain structure (McEwen et al., 2015). Cardiovascular risks, including hypertension and increased susceptibility to atherosclerosis, further complicate long-term glucocorticoid therapy (Van der Goes et al., 2014).

One of the most concerning side effects of glucocorticoid therapy is its impact on skeletal muscle. Muscle wasting, or glucocorticoid-induced myopathy, occurs due to a combination of increased protein degradation and suppressed protein synthesis. Glucocorticoids promote muscle atrophy by upregulating the ubiquitin-proteasome system, a key pathway responsible for protein degradation, leading to the breakdown of structural and contractile proteins in muscle cells (Schäcke et al., 2002). Additionally, glucocorticoid receptors interact with lysine-specific demethylase 1 (LSD1), a co-regulator that enhances the repression of anabolic target genes,

further contributing to muscle loss (Cai et al., 2024). These mechanisms collectively result in reduced muscle mass, decreased strength, and impaired physical function, particularly in patients undergoing long-term glucocorticoid treatment (Schakman et al., 2013). Given these detrimental effects, there is growing interest in identifying potential interventions that could mitigate glucocorticoid-induced muscle wasting. Beta-Hydroxy Beta-Methylbutyrate (HMB), a metabolite of the essential amino acid leucine, has gained attention for its potential muscle-preserving properties. HMB has been shown to enhance protein synthesis via the mammalian target of rapamycin (mTOR) signaling pathway while simultaneously reducing muscle protein breakdown through inhibition of the ubiquitin-proteasome pathway (Wang et al., 2016). Studies have demonstrated that HMB supplementation can help preserve lean muscle mass in individuals experiencing muscle-wasting conditions, including sarcopenia and cachexia (Holecek et al., 2009). However, its effectiveness in counteracting glucocorticoid-induced muscle atrophy remains unclear.

In this study, I investigated the musculoskeletal effects of glucocorticoids and assess whether HMB supplementation can mitigate muscle degradation in dexamethasone-treated mice. By evaluating changes in body mass, muscle composition, and metabolic parameters, this research seeks to provide insights into potential therapeutic strategies for individuals undergoing long-term glucocorticoid treatment.

Methods

Treating Mice

Animal experiments were approved by the Animal Research Committee (ARC) of Allegheny College. 16 male C57BL/6J mice were purchased from The Jackson Laboratory, these mice were 4 weeks old when we received them. This experiment was 3 weeks long, with weights of the mice taken before treatment and every day after for 3 weeks. These mice were under a 12 hr light-dark cycle and single-housed. The mice were fed (normal chow) and water ad libitum. Bedding was changed once a week and water bottles were checked and replenished once a day, including weekends. This experiment used 16 mice total (n=4 per treatment group). Mice were chosen for this study because they are a good model to study human physiology and they have similar muscles to humans, and are cost effective (Waltz, 2005). There were 4 groups of 4 mice: Group 1 was the control, Group 2 was the Dex treated group receiving 1 mg/kg of Dexamethasone diluted in water (Quinn et al., 2018). Group 3 was the HMB treated group receiving 0.2% of Beta-Hydroxy-Beta-Methylbutyrate diluted in water. Group 4 will be the HMB and Dex treated group receiving 0.2% of Beta-Hydroxy-Beta-Methylbutyrate and 1 mg/kg of Dexamethasone diluted in water. Between the hours of 10:00am and 1:00pm everyday, the mice were weighed. Along with the mice being weighed, their food and water were also weighed, to do this we measured the total weight of the food or water the day prior and subtracted the new weight the next day. This gave us the total grams and milliliters of food and water they consumed each day. After the 3 weeks the mice were euthanized by carbon dioxide inhalation and cervical dislocation to ensure the mice were dead. Left leg muscles, soleus and gastrocnemius, were extracted by an independent study student, Ethan Heberle, for their senior project and the right soleus and gastrocnemius muscles were processed for cryopreservation and sectioned for histological studies.

Cryopreservations and Staining

After the soleus muscle was removed it was submerged in 4% paraformaldehyde (PFA), prepared from 10% paraformaldehyde and IHC-PBS, and placed in a 4^{o}C chamber for 24 hours. After the tissue incubated in the 4% PFA for 24 hours, it was transferred to 30% sucrose solution for another 24 hours. This step of cryoprotection prevents the formation of large ice crystals forming when the tissue is frozen. The tissue was individually submerged in TBS TFM-5 Tissue Freezing Medium and frozen at -20^{o}C. However, due to time constraints, the muscles from each group were cut out and put into 1 singular mold and cryosectioned using a cryostat. These sections were mounted on positively charged microscope slides and returned to the -20^{o}C freezer until stained. The slides were then removed from the freezer to sit out for 30 minutes to thaw and then were soaked in 4% PFA for 10 minutes to fix the muscle tissue. The waste was then discarded in a special waste container and the cells were washed with immunohistochemistry phosphate-buffered saline (IHC-PBS) 3 times for 5 minutes each. They were then permeabilized in 0.2% Triton X-100 made in IHC-PBS for 30 minutes. The cells were then blocked in a blocking solution (Blocking Solution: 1X IHC-PBS, 5% normal goat serum, 0.2% Triton X-100) for 30 minutes. The blocking solution was removed and the primary antibody (Ki-67) made in 1X IHC-PBS was added. The slides were then incubated at 4^{o}C overnight in an incubated chamber, and the Ki-67 stain was removed and washed with IHC-PBS 3 times for 5 minutes each. A combination of Hoechst (stains DNA), Phalloidin (stains actin filaments), and Goat anti-Mouse 488 (secondary antibody to Ki-67) was added to the tissues and left for 1 hour to stain. The slides were then washed in PBS 3 times 5 minutes each. Last, the slides were washed once more with DI water and a coverslip was mounted using fluorogel mounting medium. All slides were then stored in 4^{o}C until they were viewed under the fluorescent microscope.

Fluorescent Imaging

Photos were taken under the fluorescent microscope 3 different filters were used for each of the stains, blue highlighted muscle cell nuclei, red highlighted actin filaments, and the green highlighted cell proliferation. A total of 13 pictures were taken per group. The 3 pictures per muscle tissue were then merged using ImageJ and compared group to group for cell proliferation.

Results

To determine whether or not HMB has a preventive effect on Dexamethasone treated mice, the mice were weighed everyday for 21 days and their groups average weight was recorded (**Figure 1)**. The average weight of the control group went up from ~20.5(g) day 1 ~23.5(g) day 22. This was to be expected due to the mice being 4 weeks old and not fully grown, since the average weight of a C57BL/6J mouse is 25.4(g). The average weight of the Dex group went from ~21(g) to ~20.2(g). Which proved that Dexamethasone worked in decreasing the overall mass. The average weight of the HMB group went from ~19.5(g) to ~24.5(g). Confirming that HMB had no negative effect on overall mass. The average weight of the Dex + HMB group went from ~20.1(g) to ~19.4(g). The decrease in weight was expected to be seen in the Dex group due to its muscle degrading factors, however, within the Dex + HMB group the average weight decreased similarly to the Dex only group. To analyze the collected data, we grouped the samples based on the presence or absence of Dex and HMB. The groups were categorized as Dex present (Dex and Dex + HMB) vs. Dex absent (Control and HMB) and HMB present (HMB and Dex + HMB) vs. HMB absent (Dex and Control). After running a mixed model on JMP the Dex present group had

a statistically significant difference in body mass compared to the Dex absent groups (p = 0.0434). While with the same mixed model on JMP, the HMB present group did not have a statistically significant difference in body mass compared to the HMB absent (p = 0.4287). These results showed that HMB did not have a preventative effect when it came to overall weight in glucocorticoid treated mice.

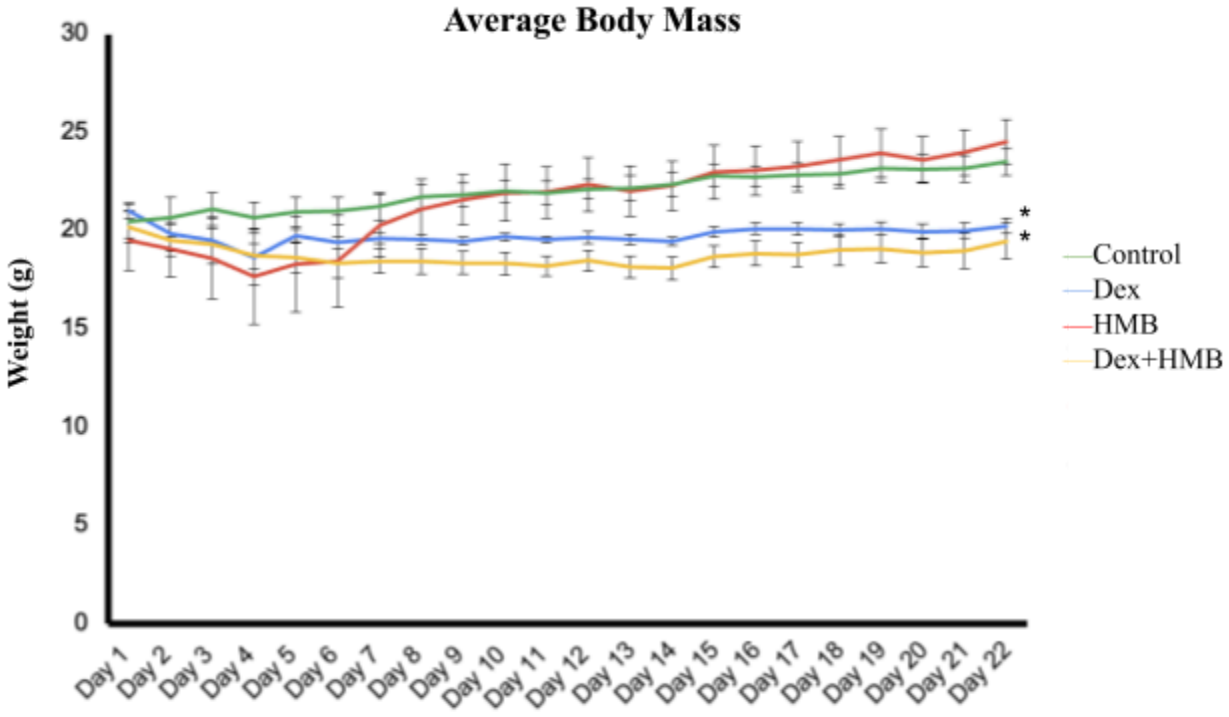


Figure 1: Average weight mice throughout trials. The X-axis shows the days, the Y-axis is the mice average weight in grams. (Control-green, Dex-blue, HMB-red, Dex+HMB-yellow) Four weights were gather per group and averaged together and the error bars were created using the standard error of mean. (n=4)

(*) shows a statistically significant difference in body mass in the Dex present groups from the Dex absent groups

Food consumption from each mouse was weighed everyday for 21 days to see if HMB had an effect on appetite. As shown in **Figure 2** the average food consumption per day of the Control group was ~4.3(g). The average food consumption per day for the HMB group was

~4.2(g), this is similar to the Control groups average showing no appetite suppression or enhancement. The average food consumption per day for the Dex group was ~4.6(g), which is higher than the Control groups and the HMB groups. The average food consumption per day of the Dex + HMB group was ~3.9(g), this is a decrease of average food consumption compared to the other groups. We ran a mixed model and found there was a significant effect of day ($p = 0.001$). Along with day there was also a significant interaction between Dex treatment and HMB treatment, meaning the effect of Dex depends on whether HMB is present and the effect of HMB depends on whether Dex is present. The addition of HMB to Dex or Dex to HMB lead to a decrease of average food intake. The Dex or HMB groups did not have a significant difference showing that alone they didn't have an effect on appetite.

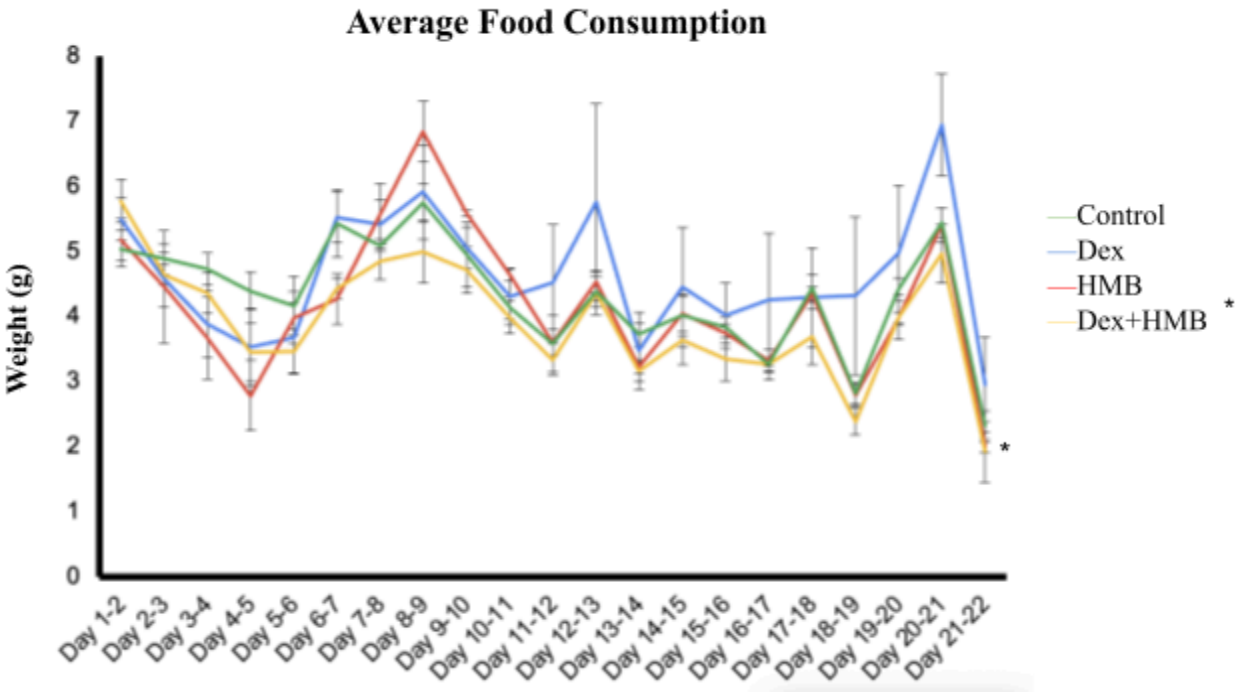


Figure 2: Average food consumption in treated mice throughout trials. The X-axis shows the days, the Y-axis is the food weight in grams. (Control-green, Dex-blue, HMB-red, Dex+HMB-yellow) Four weights were gather per group and averaged together and the error bars were created using the standard error of mean. (n=4)

(*) shows a statistically significant difference in food consumption

The water consumption from each mouse was weighed everyday for 21 consecutive days to determine if HMB had an effect on thirst As shown in **Figure 3**, the average water consumption per day of the Control group was ~4.8mL. The average water consumption per day of the Dex group was ~4.9mL, similar to the Control group. The HMB group's average daily water consumption was approximately 6.7 mL, including the day before we measured bottle leakage. When that day was excluded and leakage was accounted for the rest of the experiment, the average dropped slightly to 6.3 mL. In both cases, water consumption in the HMB group was over 1 mL higher than in the Control group. The average water consumption per day of the Dex + HMB group was ~4.5mL. There is a slight decrease in the Dex + HMB compared to the Control group. After running a mixed model on JMP the Dex present group had a statistically significant decrease in water consumption compared to the Dex absent groups ($p = 0.0063$). The HMB present group had a statistically significant increase in water consumption compared to the HMB absent group ($p = 0.0438$). There was also a significant interaction between Dex treatment and HMB treatment, meaning the effect of Dex depends on whether HMB is present and the effect of HMB depends on whether Dex is present ($p = 0.0016$).

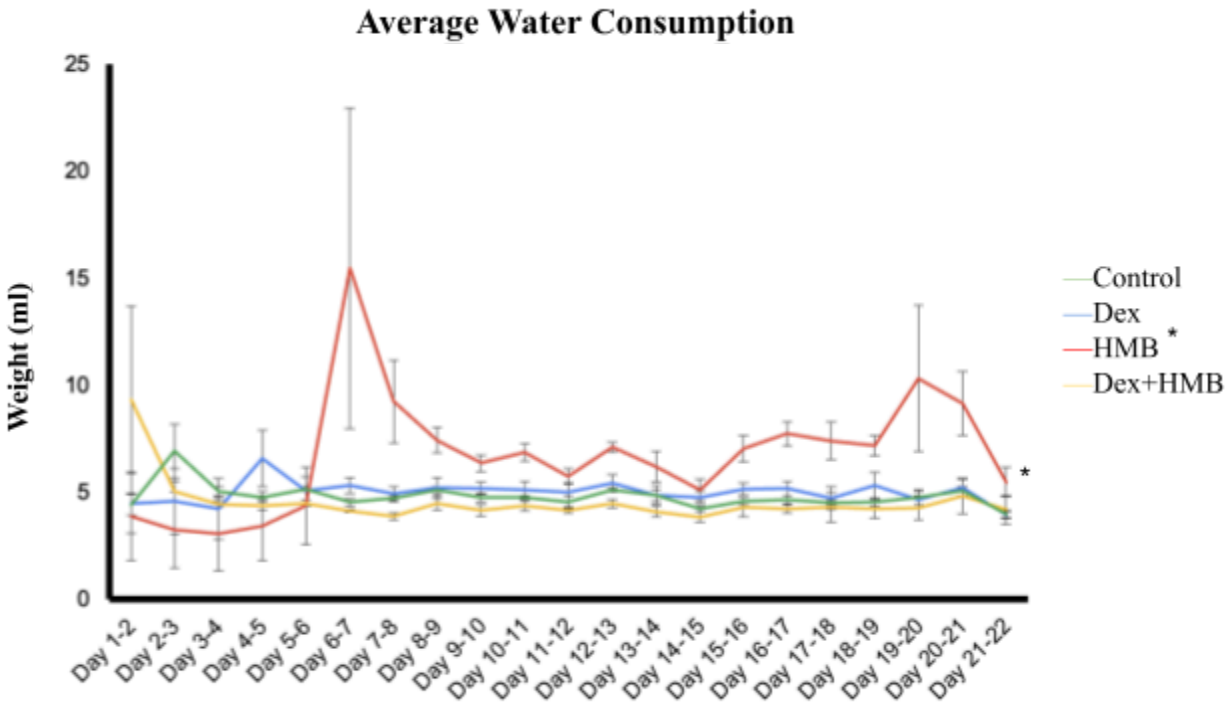


Figure 3: Average water consumption in treated mice throughout trials. The X-axis shows the days, the Y-axis is the water weight in milliliters. (Control-green, Dex-blue, HMB-red, Dex+HMB-yellow) Four weights were gather per group and averaged together and the error bars were created using the standard error of mean. (n=4)

(*) shows a statistically significant difference in water consumption

During the experiment the tails of the mice seemed to appear and feel different between the and hence were measured upon euthanasia and dissection. As shown in **Figure 4** the Control group's average tail weight was ~0.46(g). The Dex group's average tail weight was ~0.34(g), a decrease compared to the Control group. The HMB group's average tail weight was ~0.47(g). The Dex + HMB group's average tail weight was ~0.33(g), similar to the Dex only group and a severe decrease compared to the Control group. We performed a least squares fit model and the group significantly different from the groups not having Dex were the groups with Dex present, ($p = 0.0001$). The HMB did not show signs of preventative effects when it came to tail weight on

Dex treated mice. These results suggest that a short term oral treatment of Dex can decrease tail weight.

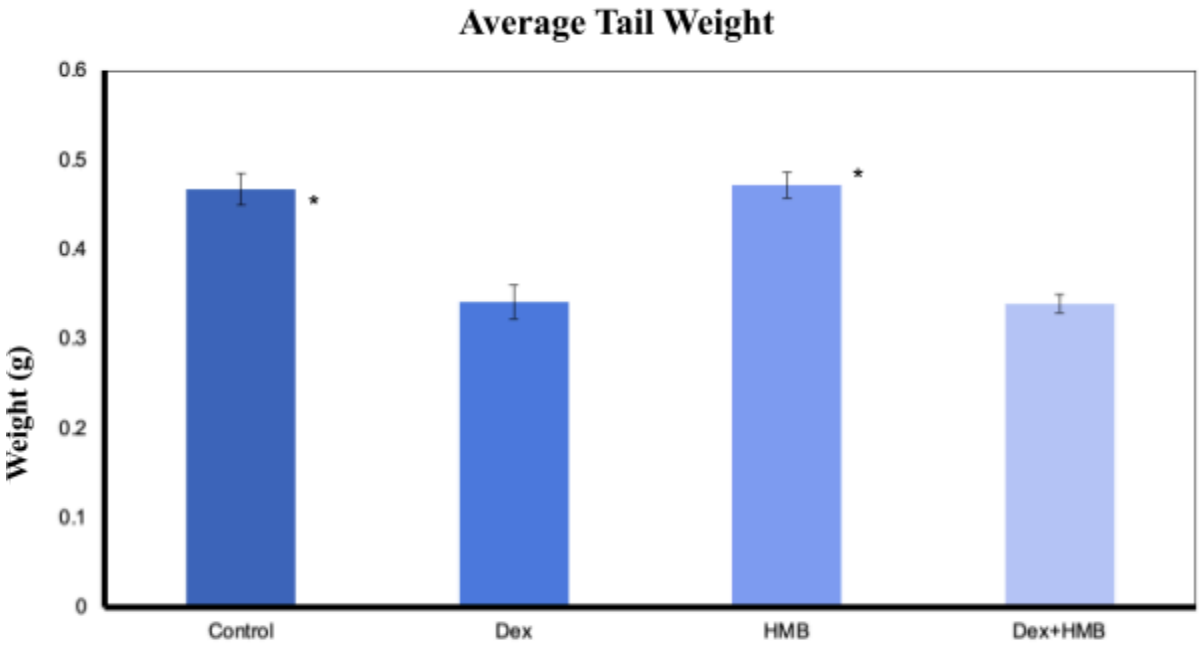


Figure 4: Average water consumption in treated mice throughout trials. The X-axis shows the days, the Y-axis is the water weight in milliliters. Four weights were gather per group and averaged together and the error bars were created using the standard error of mean. (n=4)

(*) shows a statistically significant difference in average tail weight

At the end of treatments the Soleus and Gastrocnemius muscles were removed and their weights were measured and shown in **Figure 5** and **Figure 6**. The Control group's average soleus muscle weighed ~0.007(g). The Dex group's average soleus weight was ~0.005(g), a decrease in soleus weight compared to the Control group. The HMB group's average soleus weight was ~0.007(g). The Dex + HMB group's average soleus weight was ~0.005(g), similar to the Dex group. When a least squares fit model was used based on whether Dex was present or absent, the Dex treatment group was the only group statistically significant in having lower weight of soleus and gastrocnemius muscles compared to those of the groups without Dex (p =

0.0029). This result suggests that our results are consistent with the previously established role of Dex in muscle atrophy.

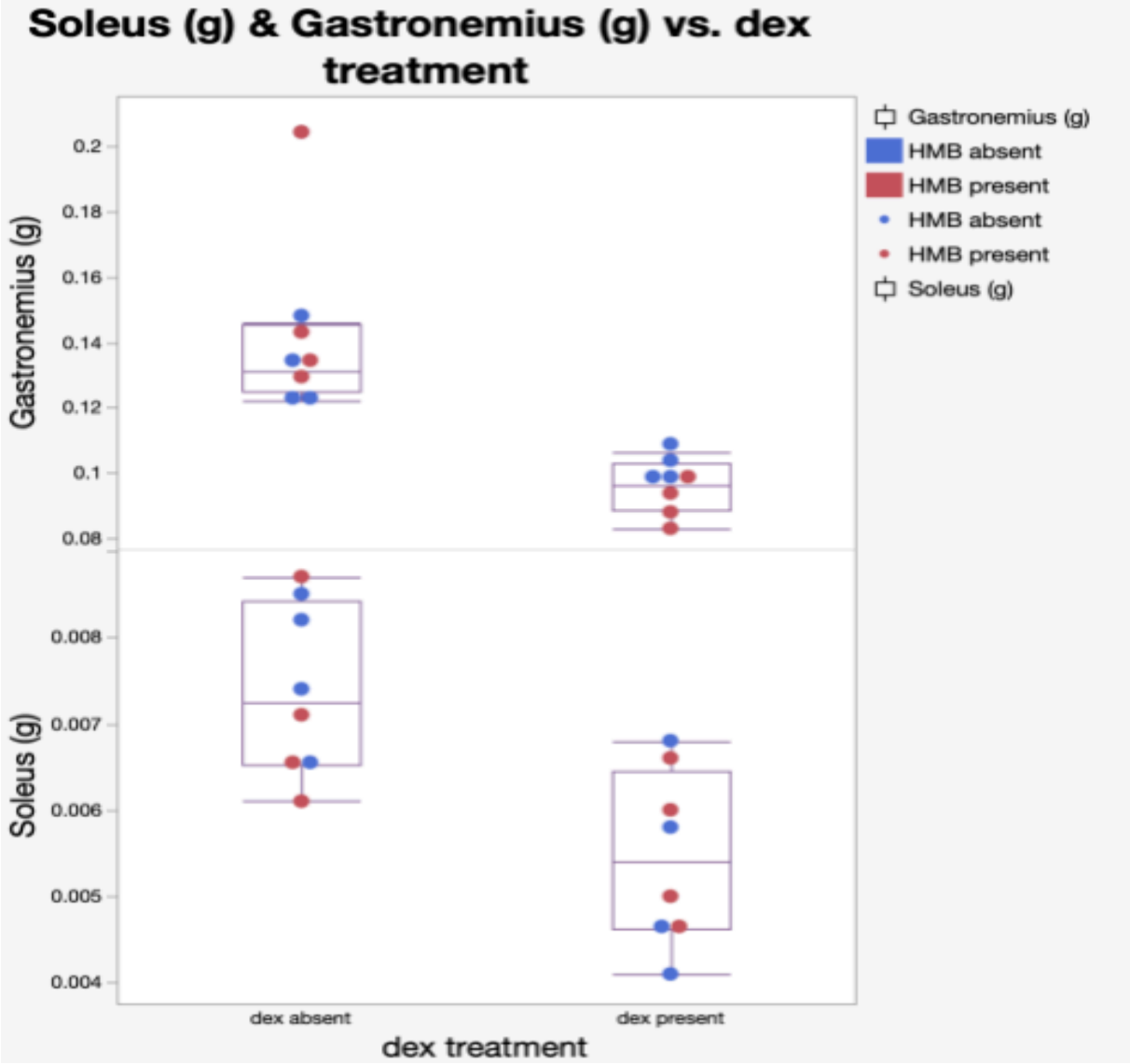


Figure 5 shows both Soleus and Gastrocnemius average muscle weights on the Y-axis and Dex present or Dex absent on the X-axis along with HMB present(red) HMB absent(blue). (n=4)

After the tissues were stained and imaged under the microscope **Figure 6 A**, each image was measured to see the amount of cell proliferation. Cell proliferation was determined by the

cell nuclei stain (DAPI) overlapping with the cell proliferating stain (Ki-67). Each groups total was then averaged and put in a graph **Figure 6 B**

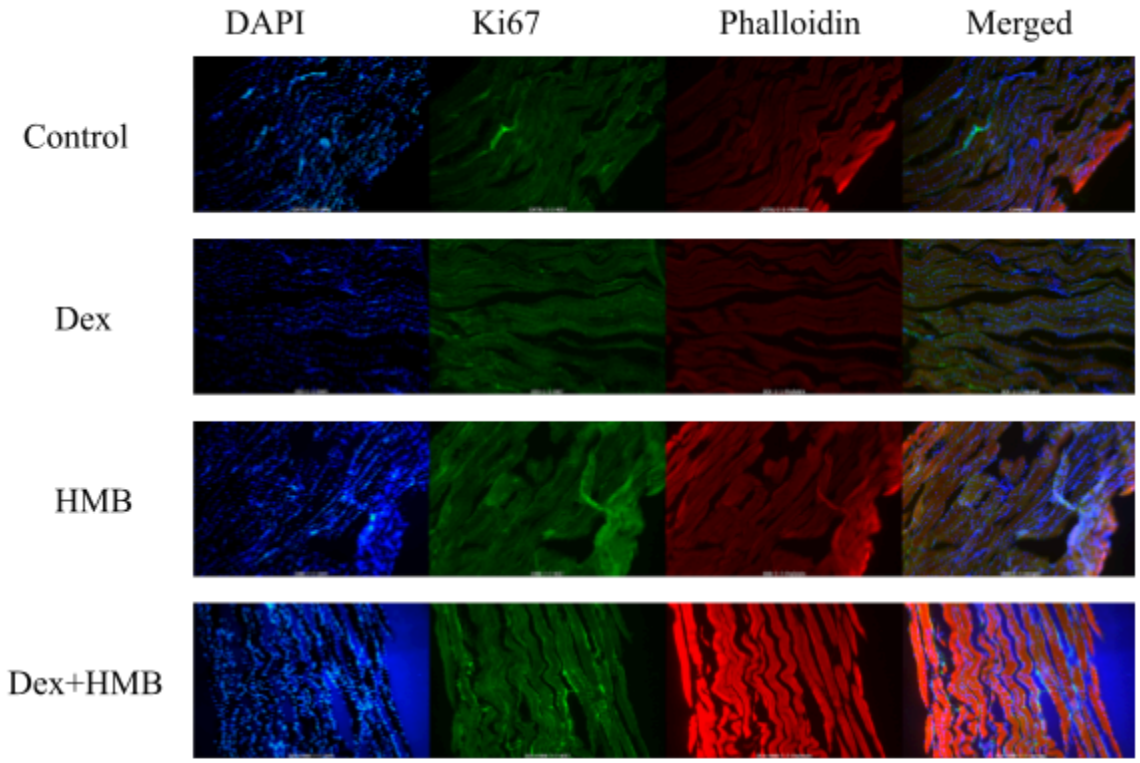


Figure 6 A shows the comparison of the muscle tissues in each of the groups, with a specific Ki-67 stain to look for cell proliferation. (DAPI (blue)-cell nuclei stain : Ki67 (green)-cell proliferation stain : Phalloidin (red)-actin filament stain : Merged-all three stains overlapped) (n= 4) 3 images were capture and 1 representative per group was chosen in the picture.

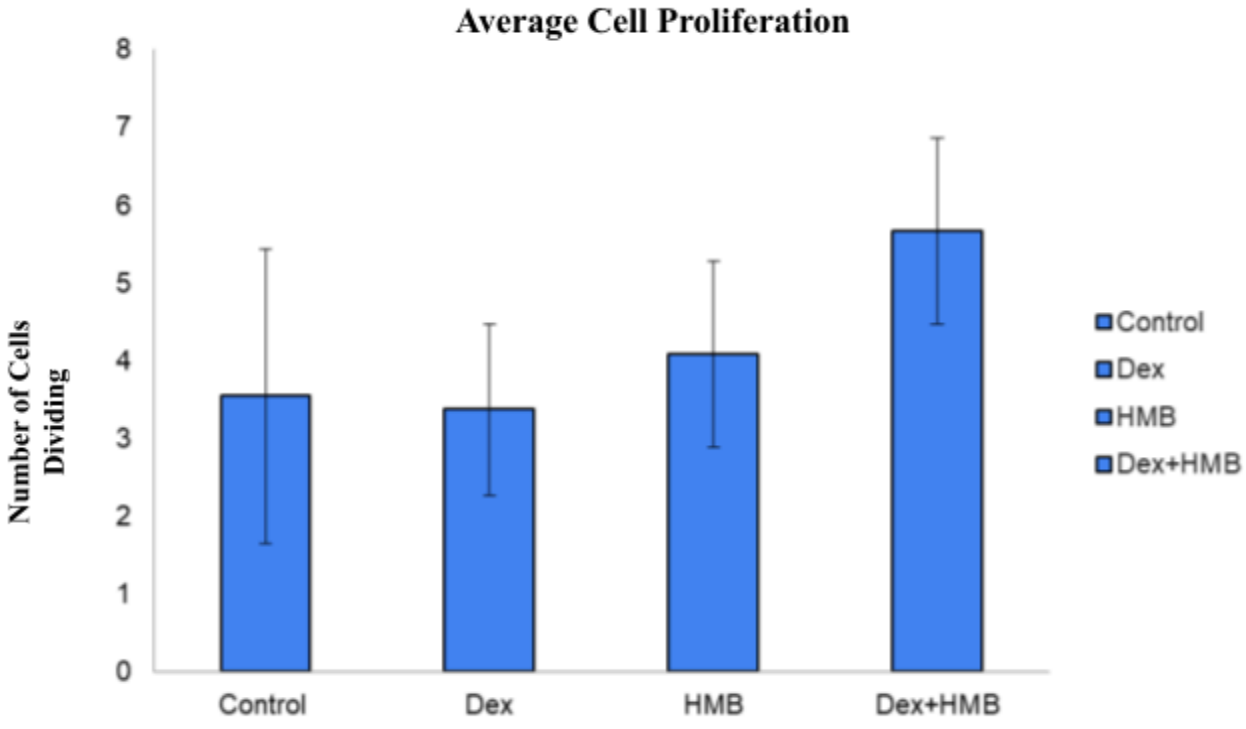


Figure 6 B shows

Discussion

The present study investigated the potential protective effects of β-hydroxy-β-methylbutyrate (HMB) against dexamethasone-induced muscle atrophy in mice (Aversa et al., 2012a; Girón et al., 2015; Hasselgren, 2014). Contrary to our expectations, HMB supplementation did not prevent the reduction in body weight or muscle mass associated with dexamethasone treatment. These findings diverge from several previous studies that have reported beneficial effects of HMB in mitigating muscle wasting.

In our study, dexamethasone-treated mice exhibited a decrease in average body weight from approximately 21 g to 20.2 g over the 21-day period, indicating muscle atrophy.

Co-administration of HMB did not counteract this effect, as the Dex + HMB group also showed a weight reduction from 20.1 g to 19.4 g. This lack of protective effect contrasts with findings by (Noh et al., 2014), who demonstrated that HMB supplementation attenuated dexamethasone-induced muscle atrophy in Sprague-Dawley rats by modulating muscle degradation pathways. Similarly, Aversa et al. reported that HMB prevented dexamethasone-induced myotube atrophy in vitro by inhibiting protein degradation and stimulating protein synthesis (Aversa et al., 2012b). The discrepancy between our results and previous studies may be attributed to differences in experimental design, species, dosage, and duration of treatment. For instance, previous studies utilized rat models, whereas our study employed mice, which may respond differently to HMB and dexamethasone. Additionally, variations in HMB dosage and administration methods could influence its efficacy.

Regarding food consumption, our findings indicated that dexamethasone treatment led to an increase in average daily food intake (~4.6 g) compared to controls (~4.3 g), while the Dex + HMB group consumed less (~3.9 g). These results suggest a complex interaction between dexamethasone and HMB on appetite regulation. Previous research has shown that dexamethasone can reduce food intake and weight gain in rats (Jahng et al., 2008). The observed increase in food intake in our dexamethasone-treated mice may reflect species-specific responses or differences in experimental conditions.

Water consumption was significantly higher in the HMB group (~6.3 mL/day) compared to controls (~4.8 mL/day), while the Dex + HMB group showed a slight decrease (~4.5 mL/day). The increase in water intake with HMB supplementation cannot be explained. I could not find specific studies that directly report increased thirst or water consumption as a result of HMB supplementation in rodents. The decrease in water consumption in the Dex + HMB group

suggests a potential interaction between dexamethasone and HMB affecting hydration status, warranting further investigation.

In conclusion, our study did not observe a protective effect of HMB against dexamethasone-induced muscle atrophy in mice. These findings highlight the need for further research to elucidate the conditions under which HMB supplementation may be effective in preventing muscle wasting, considering factors such as species differences, dosing regimens, and treatment durations.

There are several potential avenues for future research that could expand upon the findings of this study and provide a more comprehensive understanding of HMB's effects on dexamethasone-induced muscle atrophy. One important consideration is the dosage of HMB used in the experiment. The current study utilized a 0.2% HMB concentration, which did not produce a statistically significant effect in preventing weight loss or muscle degradation in dexamethasone-treated mice. Future studies could investigate the impact of a higher HMB concentration, such as 2%, 10 times the concentration I used, to determine whether an increased dosage yields more pronounced protective effects.

Additionally, alternative methods of HMB administration should be explored. In this study, HMB was provided through drinking water, which may have influenced the overall intake and bioavailability of the supplement. Future experiments could evaluate the efficacy of different administration methods, such as oral gavage via a concentrated liquid or pill form, to ensure consistent dosing and potentially enhance HMB absorption. This approach would also help control for variations in individual water consumption that could affect the total amount of HMB ingested. Additionally, investigating the effects of HMB administration via subcutaneous or

intraperitoneal injection could provide further insight into its bioavailability and direct physiological effects, bypassing potential issues related to digestion and metabolism. These alternative delivery methods could help determine the most effective way to administer HMB for optimal muscle preservation in dexamethasone-treated models.

Another crucial factor to consider in future research is the inclusion of both male and female mice. The present study focused solely on male mice, but previous research has indicated that sex differences can play a significant role in muscle metabolism, glucocorticoid sensitivity, and response to dietary supplements. Investigating whether HMB's effects differ between male and female mice could provide valuable insights into its potential applications across diverse populations.

Expanding on these variables will help clarify the potential of HMB as a therapeutic intervention for glucocorticoid-induced muscle atrophy and refine its application in clinical settings. Future research should also assess the long-term effects of HMB supplementation, its interaction with other dietary components, and its influence on additional physiological markers such as inflammatory cytokines and protein synthesis pathways.

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