Effect of Phenylbutyrate on Skeletal Muscle Atrophy induced by Hindlimb Unloading in Wistar Rat

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Abstract

Background: Mechanical unloading in the hindlimb negatively affects skeletal muscle and also alter the gut microbiota that recently been recognized as a major contributor(s) in disrupted metabolism and physiology. Present study aimed to investigate the potential role of phenylbutyrate on skeletal muscle atrophy and colon induced by hindlimb unloading (HLU0 in rats.

Methods: All rats were allocated into three groups *viz* control, hindlimb unloaded (HLU) (for 2 weeks,) and HLU+PB (for 300mg/kg orally for 14 days). All rats were sacrificed, and serum, colon tissue, and gastrocnemius (GN) muscles were collected for further endpoints. Various motor coordination activities, serum creatine level; colon, and GN muscle oxidative stress levels, and histopathology were performed.

Results: The findings showed that phenylbutyrate treatment significantly improved muscle coordination in hind limb unloading rats. Further, results showed the restoration of the levels of creatine, oxidative stress, and antioxidants. Furthermore, histological results confirmed that phenylbutyrate treatment significantly improved the GN muscle and colonic histological architecture in HLU rats.

Conclusions: These findings suggested that phenylbutyrate ameliorates muscle coordination, colon, and skeletal muscle cellular architecture in the HLU-induced rat model. Phenylbutyrate significantly restored the antioxidant and oxidative stress levels in HLU-treated rats. In conclusion, phenylbutyrate may be an effective intervention against colon and skeletal muscle loss in the hind limb unloading model.

Keywords: Skeletal muscle atrophy, phenylbutyrate, hindlimb unloading, colon, oxidative stress

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Introduction

Mechanical unloading of skeletal muscles leads to progressive loss of muscle mass and force over and negatively affects several physiological functions of the body, including liver and gut microorganisms. Study shows that dysbiosis, as well as the loss of microbiota, causes significant changes in skeletal muscle metabolism (Grosicki et al., 2018). This illness is relevant in a variety of settings, ranging from prolonged bed rest in chronic pathology patients to microgravity during space flights (Fitts et al., 2010). Several interventions have been used to reduce disuse muscle atrophy such as supplements, passive muscle contraction, electrical stimulation, and exercise) (Cava et al., 2017). However, a handful of therapies and pharmacological interventions are there to increase muscle mass and gut microbiota during prolonged inactivity. The hindlimb unloading (HLU) experimental rodent model reproduces several aspects of mechanical unloading, such as muscle atrophy and gut microbiota, and is an excellent model for testing potential pharmacological interventions (Grosicki et al., 2018). However, no effective drug therapy exists to prevent/treat muscle atrophy and lot of research are going on to explore the molecular mechanism(s) linking inactivity to muscle wasting. Harmful gut microbiota showed reduced muscle mass and indications of muscle atrophy through

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increased ubiquitin-proteasome, autophagy-lysosome, and proteolytic pathways and decreased IGF/PI3K/Akt pathways. Activation of AMPK in microbiota muscle further suggests that the AMPK-FoxO3-atrogenes cascade could be one possible signaling pathway to the atrophy observed in gut microbiota muscle tissues (Bindels & Delzenne, 2013). To establish direct causality between muscle and gut microbiota detriment, muscle mass and force must be assessed in disease models. Recent research has shown that HDAC inhibitors have the potential to treat muscle atrophy in the gut microbiota to a certain extent, but there have been no conclusive findings in this lineage to date. Phenylbutyrate acid (PBA) is a short-chain fatty acid that is converted/oxidized in the human body by -oxidation into phenyl acetate (PAA) (Reddy et al., 2019). The transactivation of the Foxo transcription factors including acetylation is modulated by various posttranslational modifications. Enhanced p300/CBP HAT directly oppressed Foxo 3 function and inhibited its nuclear localization in reaction to catabolic stimuli such as nutrient depletion or treated with dexamethasone. Furthermore, a recent experimental data/study suggested that class I HDACs stimulate Foxo activity during muscle atrophy and that HDAC inhibitors would prevent muscle disuse by down regulating Foxo-regulating genes involved in autophagy and the proteasome (Osseni et al., 2022). Keeping these findings in mind, we propose that the HDAC inhibitor phenylbutyrate could alleviate the colon and skeletal muscle atrophy in the hind limb unl model.

Material and methods

Drug and Chemicals

Phenylbutyrate was purchased from Sigma-Aldrich (St. Louis, MO), USA. All chemicals were procured from Himedia and Sigma untill unless specified. A creatine kinase kit was purchased from Proton Biochemicals India Pvt. Ltd., India. All the solvents and chemicals were of analytical grade with 99% purity, and house-distilled water was used for experimenting.

Animals

Male albino Wistar rats (80–120 g) were used for this study. All rats were acclimatized to laboratory conditions for two weeks before the start of the experiment. The animals were housed in cages under controlled conditions (23°C, 12 hrs, light/dark cycle), with free access to a standard pellet diet and water *ad libitum*. The experiment was performed according to the CPCSEA guidelines for laboratory animals and ethics.

Experimental design

All animals were randomly divided into 3 groups (n = 6). The groups were then divided as follows: Group 1: served as a control with 0.5% carboxy methyl cellulose, Group 2: Hindlimb unlaoding (HLU) for 14 days, Group 3: HLU+PB (phenylbutyrate) (300 mg/kg for 14 days once a day). Animals were sacrificed by cervical dislocation at the end of the experiment, and Gastrocnemius (GN) muscle was collected instantly and stored at -20°C for further endpoints. The serum was used for creatine measurement. Colon and GN muscles were preserved in a 10% formalin solution.

Hindlimb unloading induced muscle atrophy

In brief, all wistar rats' hindlimbs were unloaded using a physical method in which Wistar rats' hindlimbs were hanged with the help of hard molded wire and a cage. The forelimbs of the rats were free to move, feed, and have water provided from time to time according to the experimental procedure. The two-hour relaxation period once a day was also provided for all experimental rats. This model was standardized according to the extent of muscle atrophy (Azeem et al., 2021).

Evaluation of body weight and GN muscle

Rat body weight and GN muscle were weighed on the experiment's first and last days.

Behavioral parameters

Estimation of muscle forefeet and hindlimb by footprint

The gait cycles of the respective groups of animals (control, HLU, and HLU+ PB) were assessed using the footprint test at the end of the experimental protocol. The rats' forefeet and hinds were painted with non-toxic black (control), red (HLU), and green (HLU+PB) paints to get the footprints. Following that, the rats were free to walk around on white paper. The average distance moved forward between steps was used to compute stride length. The average distance between the left and right hind footprints was used to calculate the width of the

hind base and the width of the front base. To assess step alternation homogeneity, the distance between the left-to-left and right-to-right front footprint and hind footprint overlapping was measured. For assessment, a set of six steps was chosen, eliminating impressions created at the start and finish of the run (Patel et al., 2012).

Estimation of creatine kinase level

The creatine kinase level was measured in the rat's serum to check for skeletal muscle damage during muscle atrophy, which is indicated by creatine leakage from muscle to the bloodstream. The level of creatine was analyzed with the help of a commercially available kit (Proton Biochemicals India Pvt. Ltd., India, which used a modified Jaffe's method). In brief, blood samples were collected from rats and taken out in microcentrifuge tubes without any anticoagulant, and the tubes were kept in a standing position for 20–30 minutes. As blood gets clots, samples were centrifuged at 1500 g for 10 minutes, and the supernatants (serum) were collected and used for this. Absorbance was measured at 505 nm, and the calculation was done according to the literature (Nishimura et al., 2017).

Estimation of total and myofibrillar protein concentration

Total and myofibrillar proteins from rat GN skeletal muscle were previously discussed (Koopman et al., 2010). In summary, 50 mg of muscle was homogenized in 50 ml using a homogenizer in a cold solution containing 8.5 percent sucrose, 50 mM KCl, 5 mM EGTA, and 100 mM MgCl2. The total protein concentration was determined using an aliquot of the crude muscle homogenate (100 µl). The homogenate was centrifuged for 15 minutes at a speed of 2500 x g at a temperature of 4°C. The pellet was then resuspended in a solution that had a pH of 6.8 and contained 5 mM EGTA, 100 mM KCl, 5 mM MgCl2, and 0.05 percent Triton X-100. The supernatant was discarded first. The suspension was centrifuged at 2500g for 10 minutes at 4˚C, with the supernatant discarded. This procedure was performed twice more. The remaining pellet was centrifuged at 2500g for ten minutes at a temperature of 4°C after being washed in a solution with a pH of 6.8 that included 5 mM EGTA and 100 mM KCl. This process was then performed twice more. Following this step, the myofibrillar pellet was resuspended in a solution that included 5 mM tris-hydroxymethyl aminomethane and 150 mM KCl (pH 7.4). This solution was used to assess the concentration of myofibrillar protein (mg/g total protein). The technique developed by Lowry was applied to calculate the total protein and myofibrillar protein concentrations. Albumin from bovine serum (BSA) was used as the standard (Nishimura et al., 2017).

Estimation of oxidative stress and antioxidant level in HLU rat model

Estimation of lipid peroxidation by malondialdehyde

The measurement of malondialdehyde (MDA) content by thiobarbituric acid (TBA) directly determines the calculation of the non-enzymatic oxidative state in GN and colom tissue homogenized for lipid peroxidation (Gupta et al., 2020). In summary, 30 percent trichloroacetic acid (TCA), 0.8 percent TBA, and one milliliter of homogenized tissue were well-mixed, then incubated for ten minutes at room temperature. The resulting reaction mixture was then heated at 80 degrees C for half an hour while being diluted with 120 microlitres of water. After the mixture had been allowed to cool, it was centrifuged at a speed of 5000 x g for 15 minutes, and the absorbance of the supernatant was measured at 540 nm. The results have been reported as nmoles of MDA-TBA complex generated per mg protein.

Estimation of protein peroxidation by protein carbonyl content

The concentration of protein carbonyl content (PC) evaluation 2, 4-Dinitrophenylhydrazin (DNPH) directly determines the estimate of the non-enzymatic oxidative state in GN tissue homogenized for protein peroxidation (Gupta et al., 2020). In summary, 10 percent TCA and 150 µl homogenized tissue (Gastrocnemius and colon) were combined, and the mixture was left to incubate at room temperature for ten minutes. After centrifuging the reaction mixture at 13000g for two minutes, the supernatant was obtained. After 30 minutes of incubation with 0.2 percent DNPH, the cell pellets were subjected to continuous vortexing with 100 percent TCA for the next 5 minutes before being centrifuged at 13000g for the same amount of time. Again, the supernatant was collected, and

cell pellets were washed in a 1:1 v/v ethanol: ethyl acetate solution. Cell pellets were dissolved in 6M guanidine HCl, and supernatant absorbance at 360nm was measured.

Estimation of reduced glutathione activity by Ellman's reagent

The amount of glutathione (GSH) was determined by reducing 5, 5'-dithiobis-(2-nitrobenzoic acid) (also known as DTNB or Ellman's reagent) by the thiol group of GSH, which resulted in a yellow-colored GS-TNB complex that was read by an ELISA reader that was active at 405nm (Gupta et al., 2020). In summary, 50 percent TCA and 100µl tissue homogenized were combined and incubated for 10 minutes at room temperature was promptly precipitated. After centrifugation at 5000g for 10 minutes, the precipitates were removed. In a reaction mixture comprising 0.6mM DTNB and 0.2M sodium phosphate buffer, free -SH groups in the supernatant were measured (pH 8.0). The absorbance was determined at a wavelength of 405 nanometers, and the results were expressed in nanomoles of GSH per milligram of protein.

Estimation of superoxide dismutase activity by pyrogallol activity

The superoxide dismutase (SOD) activity was determined by inhibiting the reduction of pyrogallol activity by SOD present in the sample (Gupta et al., 2020). In summary, 0.5 M tris buffer with a pH of 8.0 and containing 50mM tris HCl, 1mM EDTA was mixed with 2900µl of tris buffer, and 100µl of tissue homogenate before being incubated for 5 minutes at room temperature. To stop the reaction, pyrogallol was added. The change in absorbance after adding pyrogallol for an interval of 3 minutes and absorbance was measured at 420nm. SOD activity was evaluated in millimoles of reduced pyrogallol/min/mg protein.

Estimation of cellular toxicity by histological analysis

The GN muscles and colon were isolated and put in 10% formaldehyde for fixation. The muscle and colon fixed in paraffin were sliced to a thickness of 5 micrometers and then stained with Hematoxylin and Eosin (H&E) according to a standard protocol (Takada et al., 2013).

Statistical Analysis

The data were provided as mean ± SEM for each group. The Graph Prism pad (version 8.01) statistical tool was utilized for the statistical analysis. The significance of the difference between different group comparisons was determined using a one-way analysis of variance (ANOVA). If the ANOVA revealed significant differences the Bonferroni test was used for *post hoc* analysis. The values of P<0.05 were considered statistically significant.

Results

Effect of phenylbutyrate on body weight variation and gastrocnemius (GN) muscle in HLU model

At the end of the study (14 days), HLU and HLU+PB groups showed a significant decrease in body weight as compared to the control group. Further results showed that phenylbutyrate treatment significantly increased the body weight and GN muscle weights in the HLU group (Fig 1 and 2).

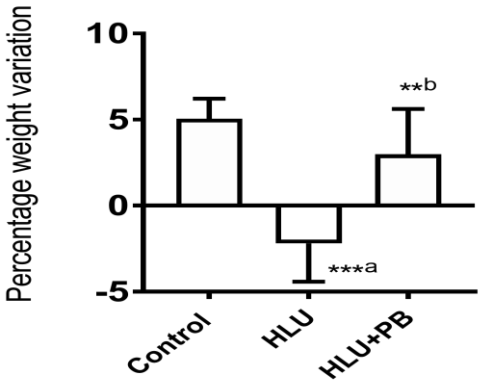


Fig. 1: Effect of phenylbutyrate on body weight variation in HLU model. All the values are expressed as mean ±SEM (n=5). **P<0.01 and ***P<0.001, a control *vs* HLU and b HLU *vs* HLU+PB.

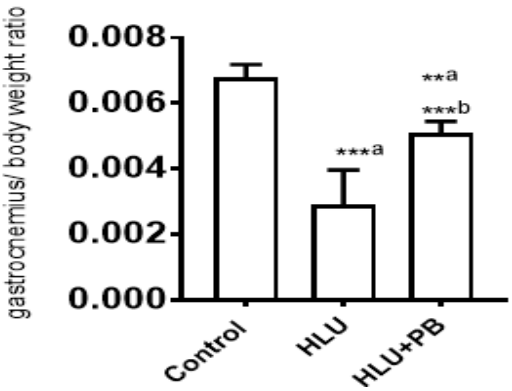


Fig 2: Effect of Phenylbutyrate in gastrocnemius muscle mass of rat in HLU model. All the values are expressed as mean ±SEM (n=5) ***p<0.0001 and **p<0.001, [a] control *vs* HLU, and [b]HLU *vs* HLU+PB.

Effect of Phenylbutyrate on creatine level in HLU model

The hindlimb unloaded group showed that there was decreased creatine level resulted in significant muscle damage as compared to the control. Phenylbutyrate treatment restored this level in the HLU model (Fig. 3).

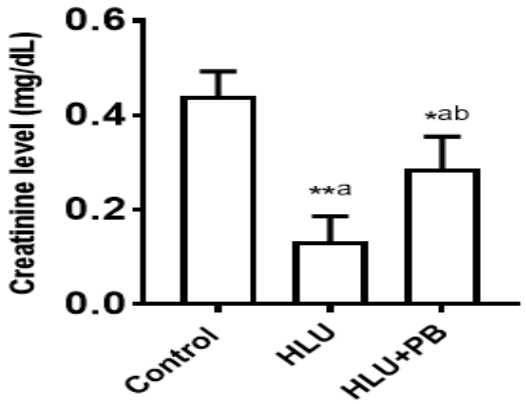


Fig 3: Effect of phenylbutyrate on creatinine level in HLU rat model. All the values are expressed as mean ±SEM (n=5). **p<0.001, [a] control *vs* HLU, and [b]HLU *vs* HLU+PB.

Effect of Phenylbutyrate on myofibrillar protein content in HLU model

Hindlimb unloaded group showed that there was an increase in muscle proteolysis resulting in a significant loss of myofibrillar proteins as compared to the control. Phenylbutyrate treatment restored the protein content in the muscles of HLU (Fig. 4)

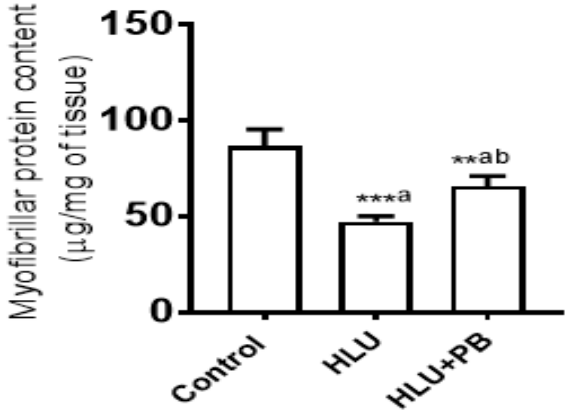


Fig 4: Effect of Phenylbutyrate on myofibrillar protein content in HLU model. All the values are expressed as mean ±SEM (n=5). ***p<0.0001 and **p<0.001, [a] control *vs* HLU, and [b]HLU *vs* HLU+PB.

Effect of Phenylbutyrate on lipid peroxidation (MDA) level in HLU model

Colon and GN muscle MDA level was significantly increased in the hindlimb unloading model compared with the control. Phenylbutyrate intervention significantly decreased the MDA level in the HLU model (Fig. 5A and B).

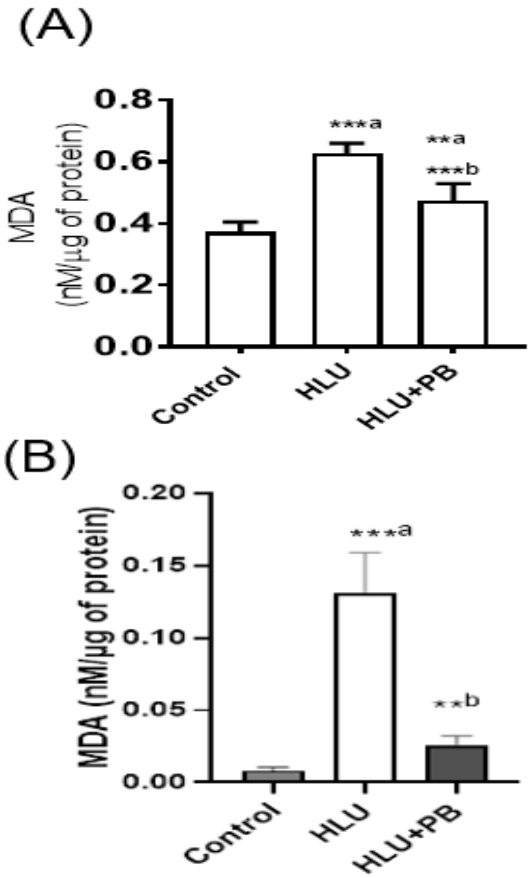
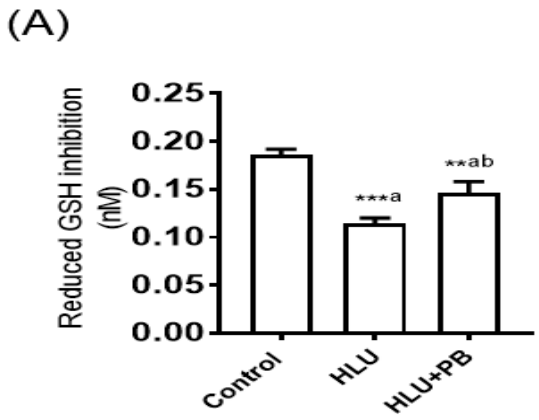


Fig 5: Effect of Phenylbutyrate on MDA level (A) GN muscle (B) Colon in HLU model. All the values are expressed as mean ±SEM (n=5). ***p<0.0001 and **p<0.001, [a] control *vs* HLU, and [b]HLU *vs* HLU+PB.

Effect of Phenylbutyrate on glutathione inhibition in HLU model

Results showed that in the HLU group there was a reduction in colon and muscle GSH inhibition when compared with control and restored by intervention with phenylbutyrate (Fig 6A and B).



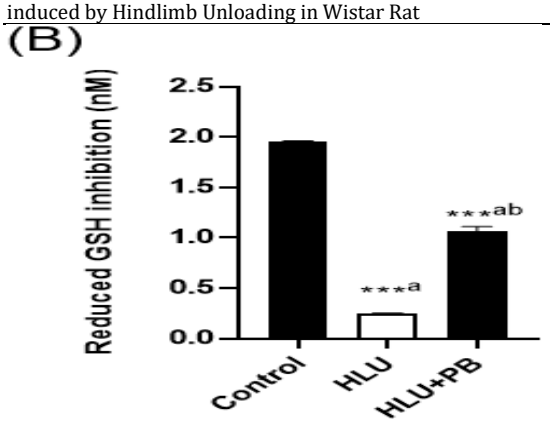


Fig 6: Effect of Phenylbutyrate on glutathione inhibition (A) GN muscle (B) Colon in HLU model. All the values are expressed as mean ±SEM (n=5). ***p<0.0001 and **p<0.001, [a] control *vs* HLU, and [b]HLU *vs* HLU+PB.

Effect of Phenylbutyrate on superoxide dismutase (SOD) level in HLU model

The hindlimb unloading group showed that significant decrease in skeletal muscle and colon SOD levels and was significantly restored after treatment with phenylbutyrate (Fig. 7A and B)

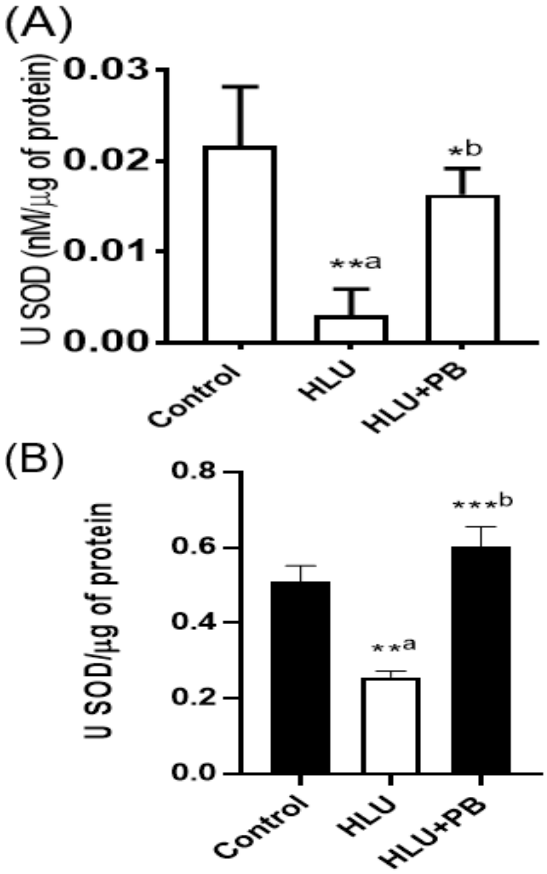


Fig 7: Effect of Phenylbutyrate on SOD level (A) GN muscle (B) Colon in HLU model. All the values are expressed as mean ±SEM (n=5). **p<0.001 and *p<0.005, [a] control *vs* HLU, and [b]HLU *vs* HLU+PB.

Effect of Phenylbutyrate on gait (footprint) in HLU model

Footprint analysis results showed that there was a significantly decreased in the number of gaits in both stride width and length in the HLU group as compared to the control. The intervention of Phenylbutyrate leads to improving stride length and width in the HLU group (Fig. 8).

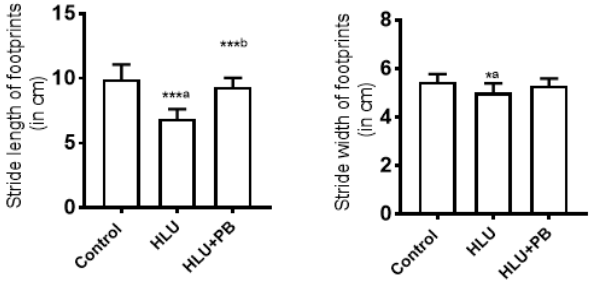


Fig. 8: Effect of Phenylbutyrate on Gait analysis in HLU model. All the values are expressed as mean ±SEM (n=5). **p<0.001 and *p<0.005, [a] control *vs* HLU, and [b]HLU *vs* HLU+PB.

Effect of Phenylbutyrate on histology of gastrocnemius muscle in HLU model

For affirmation of the results, we decided to perform the histopathological analysis of the muscle tissue for all the groups. H& E staining of gastrocnemius muscle fibers revealed increased inter-fascicular spacing in the HLU group, whereas Phenylbutyrate treatment in the HLU group muscle showed substantial improvement (Fig. 9). The representative colon photomicrographs showed that there was an increase in thickness of external mucosa and submucosa layer in HLU group when compared to the control group. Further, there was destruction in villi and crypt lining in the HLU group exhibiting the inflammatory changes. While the intervention of phenylbutyrate improved the colon architecture of the HLU group (Fig. 9).

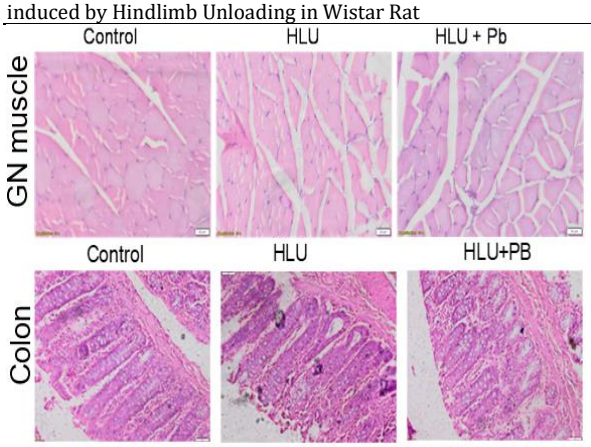


Fig. 9: Representative diagram showed the effect of Phenylbutyrate on GN muscle and colon histology in the HLU model

Discussion

Muscle atrophy has been identified as a major event causing decreased mobility and/or increased mortality in a variety of conditions, including sepsis, COPD, AIDS, cancer, diabetes, and others (Furrer & Handschin, 2019). Study showed that phenylbutyrate, an inhibitor of histone deacetylases demonstrates the neurogenic/neuroprotective effect in the ipsilateral (hypoxic–ischemic) model (Jaworska et al., 2019). The key findings of the present work are that phenylbutyrate significantly restored the muscle antioxidant status and myofibrillar protein levels and recovered colonic and muscle architectures in the HLU model.

Our results showed that the hindlimb unloading group significantly reduced muscle mass during hindlimb unloading, while treatment with phenylbutyrate improved the same. It has already been established that hindlimb unloading increases muscle permeability, which results in the leakage of creatine from muscle to the bloodstream. So we thought to check the level of serum creatinine in hindlimb unloading and found that there was a significant decrease in level as compared to the control group. Phenylbutyrate treatment restored the creatine level, which indicates evidence against the protection of muscle atrophy. Further, we checked the myofibrillar protein content in the gastrocnemius muscle, and the result showed a decline in basal muscle protein synthesis rate in the HLU condition. Intervention with phenylbutyrate treatment significantly improved the myofibrillar protein, showing that phenylbutyrate could be involved in an anabolic pathway. Furthermore, the study found that oxidative stress causes muscle atrophy by activating FOXO3, which increases protein degradation by upregulating the proteasome pathway (Guo et al., 2017). Next, we measured the different oxidative stress parameters in muscle tissue to determine the ameliorative effects of phenylbutyrate. Results showed that the hindlimb unloading group had a significant increase in muscle and colonic MDA levels and a decrease in SOD and GSH levels as compared to the control group. Phenylbutyrate restored oxidative stress and antioxidant levels and exhibited improved colonic and muscle condition in the HLU rat model. Further, we evaluated the footprint analysis, and our findings clearly showed that the HLU group has lesser physical activity and impaired foot strength as compared to the control group, and the intervention of phenylbutyrate improved the same. Further confirmation of the results was carried out with histology. H&E staining reveals a significant difference between the control and unloading groups, and phenylbutyrate treatment improved muscle and colon architecture.

Conclusion:

Phenylbutyrate showed protection against colon damage and skeletal muscle loss in the hindlimb unloading rat model. From a practical perspective, these experiments suggest that the HDAC inhibitor, phenylbutyrate could support in reducing the rate of muscle atrophy during hindlimb unloading. Further, a more research required to explore the potential treatments of HDAC inhibitors could reduce muscle atrophy specially in spaceflight missions and bed rest mechanical unloading.

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