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Oral administration of ginseng berry concentrate improves lactate metabolism and increases endurance performance in mice

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In the present study, to determine the efficacy of oral supplementation of ginseng berry extracts in augmenting exercise performance and exercise-associated metabolism, male mice were given orally 200 and 400 mg/kg of body weight (BW) of GBC for nine weeks. Although there are no differences in pre-exercise blood lactate levels among (1) the control group that received neither exercise nor GBC, (2) the group that performed only twice-weekly endurance exercise, and (3) and (4) the groups that combined twice-weekly endurance exercise with either 200 or 400 mg/kg GBC, statistically significant reductions in post-exercise blood lactate levels were observed in the groups that combined twice-weekly endurance exercise with oral administration of either 200 or 400 mg/kg GBC. Histological analysis showed no muscle hypertrophy, but transcriptome analysis revealed changes in gene sets related to lactate metabolism and mitochondrial function. GBC intake increased nicotinamide adenine dinucleotide levels in the gastrocnemius, possibly enhancing the mitochondrial electron transport system and lactate metabolism. Further molecular mechanisms are needed to confirm this hypothesis. [BMB Reports 2023; 56(6): 353-358]

INTRODUCTION

Based on an analysis of the National Institutes of Health's PubMed database through the past decade, it has been found that an average of 892 publications pertaining to ginseng have been published annually. The significant volume of research in this area highlights the considerable interest and efforts invested in investigating the potential health benefits of ginseng. This suggests that there are a lot of studies showing the health benefits of ginseng (1-3). While scientific evidence supporting the health benefits of ginseng berry supplementation is considerably limited compared to ginseng root, several studies suggest that it may have potential benefits for a variety of health conditions (4-6).

This study assessed the efficacy of the oral supplementation of ginseng berry extract in augmenting exercise performance when combined with regular endurance exercise, in comparison to exercise alone. The study involved a comparative analysis of the performance metrics, including endurance performance, post-exercise blood lactate, ammonia, LDH, and MDA, of three distinct groups: an untreated control, a treadmill exercise-only cohort, and two combined exercise and ginseng berry extract intake groups. The study lasted nine weeks, with one group receiving ginseng berry extract orally seven times per week, while all groups exercised twice weekly on a treadmill. The findings may reveal the impact of ginseng berry supplementation on exercise performance and metabolism.

RESULTS

Oral administration of ginseng berry concentrate (GBC) reduced post-exercise blood lactate levels and improved endurance performance

To evaluate the effect of GBC oral administration on lactate metabolism during exercise, 200 and 400 mg/kg of BW of GBC were orally administered for nine weeks. First, male mice with similar ages and BWs were randomly assigned to four groups: (1) control (Ctrl), (2) exercise only (Ex), (3) 200 mg/kg (GBC 200 mg/kg), and (4) 400 mg/kg GBC (GBC 400 mg/kg) group. At the 7-week oral administration, blood lactate levels were evaluated before and after treadmill running (Fig. 1A). Body weight (Fig. 1B) and food intake (Fig. 1C) were monitored in all groups. The Ex, GBC 200 mg/kg, and GBC 400 mg/kg groups underwent two sessions of endurance exercise per week (Fig.

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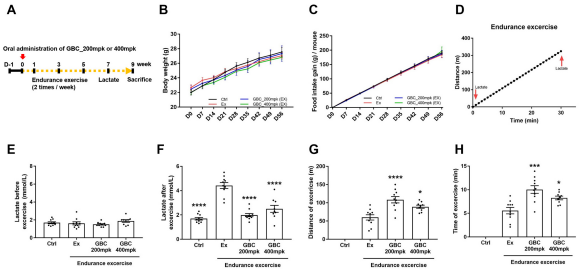

Fig. 1. Oral administration of ginseng berry concentrate (GBC) enhanced lactate elimination in the mice. (A) A diagram outlining the sequence of the experiment. (B, C) Curves showing the body weight evolution (n = 9-10, from day 0 to 56 of the oral administration, (B) and the cumulative food intake (n = 9-10, from day 0 to 56 of the oral administration (C). (D) A diagram showing the protocol of the endurance test. (E, F) Bar plots presenting blood lactate levels before (E) and after (F) an endurance treadmill run (n = 9-10, at 7 weeks of the oral administration). (G, H) Oral administration of ginseng berry concentrate improved the endurance performance. The travel distance and time presented in G and H, respectively (n = 9-10, at 7 weeks of the oral administration). All values in (B-G) are represented as mean ± standard deviation, and the P-values were determined by one-way ANOVA followed by Tukey's test. P-values of < 0.05 (*), < 0.001 (***), and < 0.0001 (****) were considered statistically significant. All P-values are generated by comparing from the Ex-group.

1D), while the GBC 200 mg/kg and GBC 400 mg/kg groups received oral GBC every day. There were no group differences in BW gain and food intake (Fig. 1B, C). Nor were there any group differences in basal blood lactate levels (Fig. 1E). Post-exercise basal blood lactate levels significantly increased, compared to the control group (Ctrl) that did not exercise (Fig. 1F). Blood lactate levels in both the 200 and 400 mg/kg groups significantly decreased, compared to the Ex-group. There were no differences between the GBC treatment groups. Consistently, all groups that received GBC orally exhibited increased running distance and duration on the treadmill (Fig. 1G, H), implying that a nine-week oral administration of GBC enhances cellular metabolism by generating and eliminating lactate, which might lead to improved exercise performance in mice.

GBC administration orally for nine weeks did not induce muscular hypertrophy

We used hematoxylin and eosin (H&E) and laminin staining to examine the effects of nine weeks of continuous endurance training and GBC supplementation on muscle tissue. In terms of muscle injury or regeneration, there were no obvious abnormalities between the various groups in H&E staining (Fig. 2A). Myofiber size (the mean and distribution of the cross-sectional area) was compared using laminin staining, but no statistically significant differences were found between the groups (Fig. 2B-D), showing that a nine-week oral administration of GBC did not induce muscle hypertrophy.

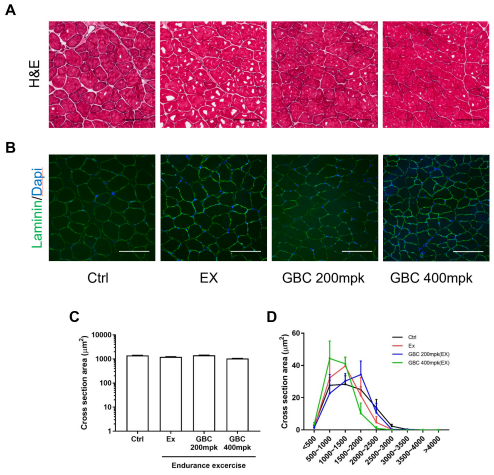

Fig. 2. Histological analysis of Skeletal muscle. (A) Hematoxylin & Eosin Staining. (B-D) Cross section analysis determined by Laminin staining (n = 300, B). The Mean (C) and distribution (D) of the cross-sectional area. All values in (C) are represented as mean ± standard deviation, and the P-values were determined by one-way ANOVA followed by Tukey's test.

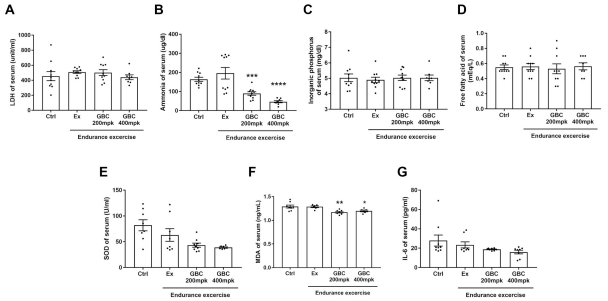

Fig. 3. Blood biochemistry and cytokines estimating toxicity, oxidative stress and inflammation. (A-D) Bar plots showing lactate dehydrogenase (LDH) (A), ammonia (B), inorganic phosphorus (C), free fatty acid in serum. (E-G) Bar plots showing superoxide dismutase (SOD), malondialdehyde (MDA), and interleukin 6 (IL-6) in serum. Ctrl = 8-10, Ex = 6-10, GBC 200 mpk = 7-10, GBC 400 mpk = 6-8. All values are represented as mean ± standard deviation, and the P-values were determined by one-way ANOVA followed by Tukey's test. P-values of <0.01 (**), < 0.001 (***), and < 0.0001 (****) were considered statistically significant.

Oral administration of GBC showed no signs of toxicity, oxidative stress, or inflammation, while lowering blood ammonia levels

To investigate the systemic effects of GBC oral supplementation for nine weeks, the levels of lactate dehydrogenase (LDH) indicating any damage to the heart, muscle, or liver (Fig. 3A), ammonia indicating dysfunction of the liver and kidney (Fig. 3B), inorganic phosphorus indicating kidney disease or hyper-

parathyroidism (Fig. 3C), free fatty acids reflecting abnormal lipid metabolism (Fig. 3D), superoxide dismutase (SOD) marking oxidative stress (Fig. 3E), malondialdehyde (MDA) (Fig. 3F), and interleukin 6 (IL-6) representing inflammation, were evaluated in the blood (Fig. 3G).

No significant difference was observed in the levels of LDH in the blood between the groups (Fig. 1A). The level of blood ammonia was significantly decreased in the GBC-administrated groups, compared to the Ex-group (Fig. 1B). No significant differences were observed in the blood levels of inorganic phosphorus and free fatty acid between the groups (Fig. 1C, D). The levels of SOD, MDA, and IL-6 in the blood were decreased in both groups given GBC, although they did not reach statistical significance (Fig. 1E-G). Taken together, these blood biochemistry findings suggest that a nine-week oral administration of GBC did not cause any toxicity, such as tissue failure or dysfunction, oxidative stress, or inflammation.

GBC-fed mice displayed elevated gene sets linked to lactate metabolism and mitochondrial electron transport chain

To elucidate the molecular mechanisms underpinning changes in blood lactate levels induced by GBC oral administration, RNA-sequencing was performed. Gene set enrichment analysis (GSEA), an unbiased analysis, was then performed on the gastrocnemius transcriptomes obtained to reveal the gene sets and molecular pathways associated with differences in muscle transcriptomes between the GBC-administrated group, which showed improved lactate metabolism and endurance, and the Ex-group (Fig. 4A).

Interestingly, three gene sets were identified that could explain the decreased blood lactate levels in the GBC-administrated groups. These were the Gene Ontology Biological Process (GOBP)-lactate metabolic process (GO:0006089), GOBP-mitochondrial electron NADH to ubiquinone (GO:0006120), and Gene Ontology Cellular Component (GOCC)-inner mitochondrial membrane protein complex (GO:0098800), all of which were enriched in the groups receiving GBC orally for nine weeks along with exercise (Fig. 4B). The GOBP-lactate metabolic process had a normalized enrichment score (NES) of 1.69, a nominal (Nom) P-value, and a false discovery (FDR) rate Q value of 0.006 (Fig. 4C). The GOBP-mitochondrial electron NADH to ubiquinone showed a NES of 1.38, and Nom P and FDR Q values of 0.027. The GOBP-mitochondrial electron NADH to ubiquinone generated a NES of 1.42, a Nom P and an FDR Q values of 0.018. The heat map depicts the elevated expression levels of representative genes linked with lactate metabolism in GBC-treated muscles, as observed in the three aforementioned gene sets (Fig. 4D). Collectively, the GSEA results demonstrate that muscles from mice fed GBC have higher levels of gene sets associated with lactate metabolism and the mitochondrial electron transport chain.

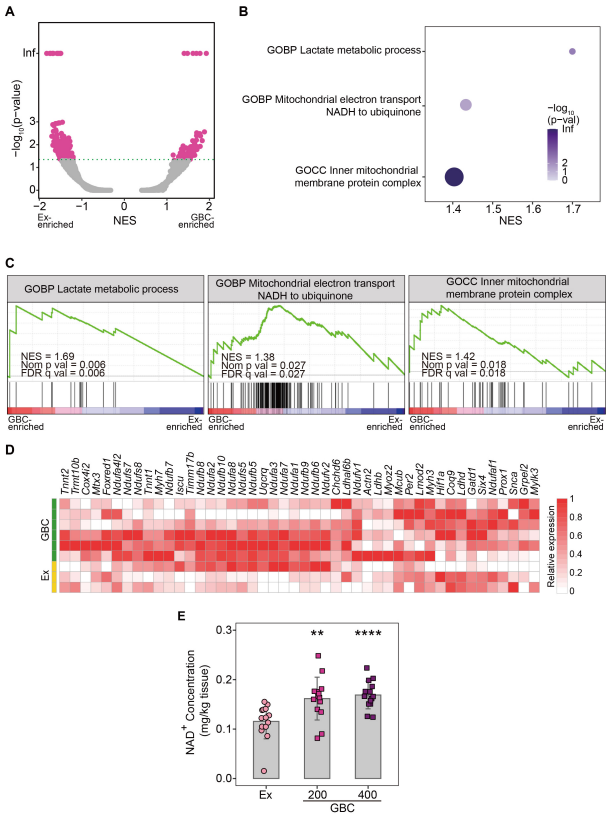

Fig. 4. Unbiased gene set enrichment analysis (GSEA) and muscular nicotinamide adenine dinucleotide (NAD^+) levels. (A) Volcano plot summarizing the results of unbiased GSEA with the normalized enrichment score (NES) and nominal P-value [$-\log_{10}$(P-val)]. Pink dots indicates statistically significantly altered gene sets. (B) Bubble plot highlighting representative gene sets linked to lactate metabolism. It indicates that for each gene set, the depth of the purple color indicates the nominal P-value, and the size of the node indicates the size. (C) Representative enrichment plot generated by GSEA related with lactate metabolism. NES, nominal (Nom) P-values, and false discovery rate (FDR) Q values are indicated. (D) Heatmap displaying representative genes of three genes, lactate metabolism, Mitochondrial electron transport NADH to ubiquinone, and Inner mitochondrial membrane protein complex. (E) NAD^+ levels in gastrocnemius. All values are represented as mean ± standard deviation, and the P-values were determined by one-way ANOVA followed by Tukey's test. P-values of < 0.01 (**), and < 0.0001 (****) were considered statistically significant.

Muscular nicotinamide adenine dinucleotide (NAD^+) levels were elevated in the mice given GBC

As is widely recognized (7-9), the NAD^+-Sirtuin pathway is a crucial regulator of mitochondrial function and biogenesis. NAD^+ boosters, which can increase cellular NAD^+ levels either by direct involvement in the NAD^+ anabolic and catabolic pathways or through the regulation of enzyme activity or expression of factors in the pathway, have been shown to enhance mitochondrial function and improve exercise performance (10-13). Notably, several NAD^+ boosters, such as nico-

tinamide riboside (NR), reduced form of NR (NRH), nicotinamide mononucleotide (NMN), and Nicotinic acid (NA, a.k.a. niacin), are available as dietary supplements (12, 14-16). To assess the potential of GBC as an NAD^+ booster, we investigated the effect of nine-week oral GBC administration on NAD^+ levels in the gastrocnemius of mice (Fig. 4E). As many natural products or natural product-derived small molecules that affect mitochondrial energy metabolism are known to modulate AMP-activated protein kinase (AMPK) activity, we evaluated phospho-AMPKα levels, reflecting AMPK activity. As expected, phospho-AMPKα levels were significantly increased in the gastrocnemius muscles of mice treated with GBC, compared to the untreated group (Supplementary Fig. 1 of the Supplementary Information (SI)).

In comparison to the Ex-group, the current study found a significant increase in NAD^+ in murine skeletal muscle after nine weeks of oral administration of 200 and 400 mg/kg GBC. The observed changes are in line with improved endurance performance and lactate metabolism, as well as the enrichment of gene sets associated with mitochondrial function and lactate metabolism. These findings suggest that GBC may generate beneficial effects on muscle physiology and metabolism. Further studies are warranted to explore the underlying mechanisms and potential nutraceutical applications of GBC as an NAD booster.

DISCUSSION

Our study demonstrated that the nine-week oral administration of GBC can improve endurance exercise performance by reducing blood lactate and ammonia levels. Notably, all groups receiving GBC exhibited a decrease in blood lactate levels, compared to the exercise only (Ex) group, although the effect was more pronounced in the GBC 200 mg/kg group, compared to the GBC 400 mg/kg group. While further investigation is required to identify the major active ingredients of GBC and confirm its concentration in the bloodstream (e.g., pharmacokinetics, etc.), it is plausible that off-target effects may occur at the 400 mg/kg concentration.

Histologic analysis showed that nine weeks of oral GBC did not produce any significant changes in the muscles. There was no evidence of muscle damage, fibrosis, or inflammation with H&E staining, nor were there any centralized nuclei produced during the muscle regeneration process. The type of exercise performed regularly was endurance running, so there was no effect on muscle fiber size. Consistent with histological analysis, blood biochemistry evaluating LDH, ammonia, inorganic phosphorus, free fatty acids, SOD, MDA, and IL-6 indicated that the nine-week oral administration of GBC did not generate any sign of systemic toxicity. High level of ammonia is a sign of hepatic and renal damage. It is known that exercise increases blood ammonia levels, which then decrease during the recovery process after exercise (17). Ammonia generated during exercise has also been proposed as a factor that may cause fatigue (18, 19). Therefore, demonstrating a relatively reduced level of blood ammonia following exercise of the same intensity may suggest enhancements in metabolic capacity and reduction in muscle fatigue. Furthermore, the observed reduction in blood SOD and MDA levels in the GBC-administrated groups implies that GBCs could potentially offer protection against free radicals within the tissues, or alternatively, they could be enhancing mitochondrial function, which may result in the decreased production of free radicals.

The only way to avoid lactate accumulation in the absence of a reduction in glycolysis rate is through pyruvate oxidation by mitochondria, particularly mitochondrial complex I. Thus, the three gene sets identified by GSEA may explain the reduced post-exercise blood lactate levels. Thus, oral administration of GBC for nine weeks resulted in significant changes in the expression of numerous genes in the muscle, particularly those related to mitochondrial energy and lactate metabolism, which may explain the observed phenotypes, such as the reduced blood lactate level, and improved endurance performance. However, the underlying molecular mechanisms by which GBC, a type of nutraceutical, induces these changes remain unclear. Further studies are needed to elucidate the precise mechanisms-of-action of GBC to fully understand its potential benefits for muscle physiology and metabolism. Like many other nutraceuticals (7, 20-23), GBC may activate the AMG/KG pathway and potentially activate the Sirtuin-PGC-1α pathway, as seen with resveratrol. Of note is the significant induction of expression changes in many genes in the same cellular biology function, rather than just a few related genes. Further investigations are needed to determine the RNA profile changes in cells directly treated with GBC or its major ingredients, such as ginsenoside Re or syringaresinol, especially with regard to mitochondrial genes. The fact that GBC or its primary ingredients can produce such effects, specifically the activation of most mitochondrial genes, implies that the drug may have a direct impact on important cell signaling pathways.

The results from our current study suggest that GBC may benefit metabolism, mitochondrial function, and muscle physiology. Further investigation is necessary to elucidate the underlying mechanisms and evaluate the potential nutraceutical applications of GBC as a NAD^+ booster. These findings have important implications for the development of novel therapies aimed at improving muscle function and overall metabolic health by boosting energy metabolism and reducing physical fatigue.

MATERIALS AND METHODS

Mouse studies

All animal experiments in this study were authorized by the Sungkyunkwan University School of Medicine's Institutional Animal Care and Use Committee and complied with the guidelines for animal experiments set by its Ethics Committee (protocol number: SKKUIACUC2021-10-28-1). Male C57BL/6 mice

aged nine weeks were obtained from DooYeol Biotech, Inc. (Seoul, Korea), and were maintained according to previous protocols (24). The mice were randomly grouped and co-housed in the same room under specific pathogen-free conditions. GBCs were administered orally daily for 9 weeks at doses of 200 or 400 mpk per group, as described below. Blood lactate measurements and endurance running on the treadmill were performed according to the instructions provided (24, 25). Phospho-AMPKα (Thr172) level and beta-actin were detected by using the antibodies purchased from Cell Signaling Technology (#2531) and Sigma-Aldrich (A2228), respectively.

Preparation of ginseng berry concentrate

The ginseng (Panax ginseng C. A. Meyer, Korea) berry concentrate (GBC) was processed by concentrating its juice to a level above 20 brix. After filtration, the concentrate was further concentrated to 70 brix and sterilized at a temperature of 95-100°C. The ginsenosides Rg1 (3.68 mg/g), Re (44.80 mg/g), Rf (0.65 mg/g), Rh1 (1.93 mg/g), Rg2 (5.44 mg/g), Rb1 (7.47 mg/g), Rc (12.82 mg/g), Rb2 (17.15 mg/g), Rd (28.16 mg/g), Rg3 (2.24 mg/g), and Rh2 (0.36 mg/g) present in the GBC were identified using HPLC analysis conducted with Waters equipment, including an auto-sampler, quaternary pump, and PDA detector. The GBC was obtained from the Amorepacific R&I Center.

Endurance test and measurement of lactate

The Columbus Exer-6 M treadmill was utilized to assess exercise performance. Mice were acclimated to the treadmill one day before the test by running at 5 meters per minute on a 5-degree incline for 5 minutes. Exercise performance testing was initiated at 10.2 m/min on a 5-degree incline and continued until exhaustion without altering the speed. Immediately after exercise cessation, blood was drawn from the tail of the mouse, and lactate was measured using a lactate meter (StatStrip Xpress® Lactate Systems, Nova Biomedical).

Blood biochemistry, histology, and immunohistochemistry

Blood biochemistry, histology and immunohistochemistry were conducted by OBEN Bio, a contract research organization (Suwon, Korea). Mouse blood was collected, and 50 µl of serum was obtained using a refrigerated centrifuge. The serum was analyzed using an AU480 Chemistry Analyzer (Beckman Coulter).

Muscle tissue obtained from mice was embedded in Tissue-Tek OCT compound (SAKURA) and sectioned at 5 µm thickness using a cryo-cutter for histological analysis. Mayer's H & E (Sigma) staining was used to visualize the muscle tissue sections. For immunostaining of muscle tissue slides, a primary antibody against laminin (ab11575, 1:200; Abcam) was employed. The following steps were carried out: fixation with 4% PFA, permeabilization, antigen retrieval, blocking, incubation, and mounting. Images were captured using a Nikon ECLIPS TE-2000 U stereo microscope, and myofiber area was measured using ImageJ software (https://imagej.nih.gov/ij/).

Enzyme-linked immunosorbent assay (ELISA)

The quantification of superoxide dismutase (SOD), malondialdehyde (MDA), and interleukin 6 (IL-6) in serum was carried out as per the manufacturer's manual. Commercially available ELISA kits for SOD (MBS034842, MyBioSource), MDA (MBS 741034, MyBioSource), and IL-6 (M6000B, R&D System) were employed for measurements. Diluted serum and standard samples were added to the wells of 96-well plates and incubated, following which the plates were washed and detection antibodies were added. The plates were then washed again, and substrate was added. Finally, a stop solution was added, and the samples were analyzed with an ELISA plate reader to obtain respective concentrations. The sample diluents provided with each kit were used as recommended by the manufacturers.

RNA sequencing and gene set enrichment analysis

RNA-sequencing was performed by Theragen Bio (Seoul, Korea), which involved RNA isolation, library preparation, and production of FASTQ files. The quality of the raw sequencing data was assessed using FastQC. Read-sequence alignment was performed using the mouse reference genome (GRCm39), and the resulting transcripts per million measurements (TPM) were used. Log2 TPM values were generated to facilitate subsequent analyses, such as gene set enrichment analysis (GSEA) and co-expression analysis. GSEA was conducted using the GSEA Java desktop software developed by the Broad Institute available for Windows at https://www.gsea-msigdb.org as described previously (26).

Liquid chromatography-tandem mass spectrometry

Analysis of NAD^+ was performed using a Shimadzu LCMS-8050 liquid chromatography mass spectrometer (Shimadzu Corp., Kyoto, Japan). The column was an Intrada Amino Acid column (50 mm × 3.0 mm, 3 µm) (PeakMan, Seoul, Korea). Mobile phase condition for NAD^+ analysis was applied with gradient elution of 0.1% formic acid in ACN and ACN/100 mM ammonium formate in water = 20:80 (*v/v*), as A and B buffer solutions, respectively. Gradient elution of mobile phase B was initiated from 0% (2.5 min) at a flow rate of 0.3 ml/min and increased from 0 to 100% (6.5 min), and then held at 100% for 2 min. Mass spectrometry detection was performed in electrospray ionization positive mode, and multiple reaction monitoring mode was used for quantification of NAD^+.

Sample preparation for NAD^+ analysis in muscle tissue by LC-MS/MS

Muscle tissue samples (5 mg/ml) were homogenized in DW using a ultrasonicator (IKA-Werke GmbH & Co.KG, Staufen, Germany), and the homogenized samples were transferred to a Spin-X tube filter (0.45 µm) and centrifuged at 13,500 rpm for 3 min. Deproteinization was performed by adding acetonitrile (100 µl) to muscle tissue (5 mg) containing 3-deazauridine as IS (0.1 ng). After centrifugation, the supernatants were transferred to auto vials and 15 µl aliquot was injected into the LC-MS/MS system.

Statistical analysis

One-way analysis of variance (ANOVA) was utilized to compare normally distributed continuous variables with three or more treatment groups, followed by Tukey's post hoc test for pairwise comparisons. Statistical analyses of all datasets were performed and visualized with R (v 1.3.1073), with *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 defined as statistically significant.

AUTHOR CONTRIBUTIONS

E.-J.J., Y.J., S.W., T.T.N., M.J., M.-J.P., J.-H. J., S.J.I., and D.R. conceived and designed the project. E.-J.J., S.J.I., and D.R. wrote the manuscript with contributions from all other authors. Y.J., performed bioinformatic analysis and E.-J.J. performed *in vivo* analysis under the supervision of J.-H. J., S.J.I., and D.R. Metabolite extraction and NAD^+ quantification were conducted by M.J., and M.-J.P.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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