

The effect of diet on improved endurance in male C57BL/6 mice

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Category: Muscle or Integrative

Key Points:

- Diets enriched in vegetables and fruits stimulates mitochondrial biogenesis
- The responses are regulated through AMPK and SIRT1 expression
- The study shows that the diet enhances endurance in a mouse model
- This results help us better understand the mechanisms associated with diet induced endurance training and the benefits of nutritional intake

Abbreviations: GF, GrandFusion; OS, oxidative stress; ROS, reactive oxygen species; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive species; SOD, superoxide dismutase; BDNF, brain derived neurotrophic factor; GFAP, glial fibrillary acidic protein; AAT, anti-aging therapies.

Abstract

In the current study, the impact of a diet enriched in fruits and vegetables (GrandFusion®) on exercise endurance was examined in a mouse model. GrandFusion (GF) diets increased mitochondrial DNA, enzyme activity and stimulated mitochondrial mRNA synthesis *in vivo*. GF diets increased both mRNA expression of factors involved in mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α), mitochondrial transcription factor A (Tfam), estrogen-related receptor alpha (ERR α), nuclear respiratory factor 1 (NRF-1), cytochrome c oxidase IV (COXIV) and ATP synthase (ATPsyn). Mice treated with GF diets showed an increase in running endurance, rotarod perseverance and grip strength when compared to regular diet controls. In addition, GF diets increased the protein expression of phosphorylated AMP-activated protein kinase (AMPK), sirtuin 1 (SIRT1), PGC-1 α and peroxisome proliferator-activated receptor delta (PPAR- δ) over exercise related changes. Finally, GF reduced the expression of phosphorylated ribosomal protein S6 kinase 1 (p-S6K1) which demonstrated a decreased in autophagy. These results demonstrate that GF diets enhance exercise endurance mediated via mitochondrial biogenesis and function.

Abbreviations. GF, GrandFusion; AMPK, AMP-activated protein kinase; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1 α ; ERR α , estrogen related receptor α ; NRF-1, nuclear regulatory factor 1; Tfam, mitochondrial transcription factor A; SIRT1, sirtuin 1; PPAR δ , peroxisome proliferator-activated receptor δ

Introduction

The maintenance of physical activity throughout life is important for a healthy existence and long-term viability (Kokkinos, 2012). Exercise is dependent upon the generation ATP which is produced by mitochondria in the cells (Menshikova et al., 2006). An increase in mitochondrial number and performance is critical for the physical performance and overall well-being (Mankowski et al., 2015). Studies have shown that exercise increases mitochondrial biogenesis and oxidative capacity, specifically in skeletal muscle (Holloway, 2017).

The skeletal muscle is responsible for the skeletal movement and is composed of slow (type I) and fast (type II) muscle fiber (Talbot and Mayes, 2016). Type I and type IIa fibers are undergo oxidative metabolism, are rich in mitochondria and are fatigue resistant (Deane et al., 2017). Slow-twitch muscle fibers generate less power and strength than fast-twitch fibers, but they have can sustain activity for longer (Sung et al., 2017). Fast-twitch muscle fibers generate far more power and strength, but they fatigue much faster and require more time for recovery. The slow-twitch fibers metabolically adaptive and under specific conditions can improve mitochondrial function and outcomes (Southern et al., 2017).

GrandFusion® (GF) are mixtures of fruits and vegetables, highly enriched in vitamins that are able to limit the extent of cerebral ischemia injury and reverse several parameters of stroke, such as inflammation and oxidative stress and behavioral changes (Yu et al., 2016). Additionally, GF extracts have been shown to improved memory and learning in aged rats mediated by antioxidant enzymes and signaling pathways (Yu et al., 2017). Previous studies have shown that GF has anti-inflammatory, anti-oxidant, neuroprotective and neurogenic properties (Yu et al., 2016; Yu et al., 2017).

In the present study, we sought to determine the impact of diets rich in vegetables and fruits on the physiological and biochemical benefits on stamina and endurance. Mice were fed diets enriched in fruits and vegetables for 20 weeks and then examined. The results revealed that these diets enhanced physical performance in a number of different behavioral assays by altering muscle parameters. Muscle weight, muscle strength, mitochondrial qualities, and exercise endurance were examined in mice chronically exposed to the diets. Finally, we have demonstrated that the supplemented diets enhanced mitochondrial biogenesis and enhanced endurance through the activation of AMPK, SIRT1, PGC-1 α and PPAR δ . These data suggest that these diets can influence the pronounced changes seen in aging to slow the process and improve outcome.

Methods

Animal experiments

Ten-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, U)SA) were housed in a controlled environment (25°C \pm 2°C, 55% \pm 5% relative humidity with a 12-h light/12-h dark cycle) at the Medical University of South Carolina animal facility. Throughout the experiment, the mice were allowed free access to food and water. After 1 week of acclimatization, the mice were randomly divided into three groups: Group 1 received a normal chow diet (Con); Group 2 received a 2% GrandFusion (GF1, NF-216 - Fruit and Veggie #1 Blend), with the ND; and Group 3 received a 2% GrandFusion diet (GF2, NF-316 - Fruit #2 Blend) with ND provided by NutriFusion, LLC (Naples, FL, USA) www.nutrifusion.com. Food intakes and body weights were measured once a week throughout the experiment. At the end of

the 20-week oral administration period, the exercise endurance of all mice was measured on a treadmill (Omnitech Electronics, Inc., Columbus, OH, USA) before the mice were sacrificed using CO₂ gas. Their soleus and gastrocnemius muscles were removed and frozen in liquid nitrogen and stored at -70°C. This study adhered to the Guide for the Care and Use of Laboratory Animals developed by the Office of Laboratory Animal Welfare.

Endurance testing

Grip power reflecting the total power of four limbs of mice was measured using a standard grip strength meter for mice (San Diego Instruments, San Diego, CA, USA). The mice were put on a metal mesh and pulled horizontally. The power of traction when the mice released the mesh was defined as the grip power. Measurements were repeated three times, and the data were averaged. Running distance was measured on a mouse treadmill (Omnitech Electronics, Inc., Columbus, OH, USA) according to a previously described protocol (Mitsuishi et al., 2013; Kim et al., 2016). Mice were forced to run on the motor-driven treadmill until they were completely exhausted, which was defined as the point at which they remained on the electrical shocker plate for more than 30 s. The treadmill was set at a 10% incline, and the speed was 18 cm/s at the beginning and was increased by 3 cm/s every 2 min, following 3 days of acclimation running at 18 cm/s for 5 min. The average running time until exhaustion for wild-type mice on normal chow was ~55 min. The distance traveled and the time were recorded. Fine motor coordination and stamina: rats were placed on an accelerating rotarod (San Diego Instruments, San Diego, CA) consisting of a slowly accelerating (+2 rpm/30 s; 20 rpm max) rotating dowel (7 cm diameter) and latency to fall was recorded (max 400 seconds).

Reverse transcription-polymerase chain reaction

Total RNA was isolated from the soleus using TRIzol reagent (ThermoFisher-Invitrogen, Carlsbad, CA, USA) and then converted to cDNA with Reverse Transcription Premix (ThermoFisher-Applied Biosystems, Waltham, MA, USA). To quantify mRNA expression, the cDNA was amplified with SuperScript VILO PCR PreMix (ThermoFisher-Applied Biosystems, Waltham, MA, USA) and primer pairs (IDT, Coralville, IA, USA). PCR was performed using a iCycler PCR System (Bio-Rad, Hercules, CA, USA). PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by on a DigiDoc-it Imaging System (UVP, Upland, CA, USA). β -Actin was used as an internal control. The primers were as follows: PGC-1 α – Forward GTCCTTCCTCCATGCCTGAC, Reverse GACTGCGGTTGTGTATGGGA; ERR α – Forward GAGGTGGACCCTTTGCCTTT, Reverse GGCTAACACCCTATGCTGGG; NRF-1 - Forward CTTCATGGAGGAGCACGGAG, Reverse ATGAGGCCGTTTCCGTTTCT; Tfam - Forward GAGCGTGCTAAAAGCACTGG, Reverse CCACAGGGCTGCAATTTTCC; COXIV - Forward GGGCCTCGTTAGTGCAGCAGG, Reverse GGGCTCCCAGAAAAGGTTGCCT; ATPsyn - Forward TGGGGACCAGGGCAGCCATT, Reverse AGGGCTTGCTGCCACACAT; β -Actin Forward GCTCCGGCATGTGCAA, Reverse AGGATCTTCATGAGGTAGT.

Western blot analysis

Skeletal muscle tissues were homogenized and lysed by RIPA lysis buffer (Boston Bioscience, Boston, MA, USA) with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysate protein concentrations were determined by the Bradford assay. The primary antibodies used were phosphor-AMPK, AMPK, SIRT1, β -actin (1:1000; Cell Signaling,

Beverly, MA, USA), PGC-1 α , PPAR δ , phosphor-S6K1 and S6K1(1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Bound antibodies were detected using horseradish peroxidase-linked secondary antibodies (1:5000; Molecular Probes/Invitrogen, Eugene, OR, USA) for 2 h. Proteins were detected with the enhanced chemiluminescence (ECL) detection system (GE Life Sciences/Amersham Biosciences, Pittsburgh, PA, USA) and visualized using the DigiDoc-it Imaging System (UVP, Upland, CA, USA). β -actin was used as an internal control.

Examination of mitochondrial amount and enzyme activity in muscle.

Tissue samples from gastrocnemius and soleus muscles were obtained at the ages of 20 wk for physiological and molecular biological analysis. The mitochondria were isolated from tissue by a density gradient centrifugation method (ThermoFisher, Waltham, MA, USA). Tissue samples were homogenized in mitochondria isolation buffer to rupture cells and centrifuged at 600 *g* for 10 min to remove debris and large cellular organelles. The supernatant was collected and centrifuged at 12,000 X *g* for 15 min to isolate mitochondria. The pellet was collected and resuspended, and the centrifugation steps were repeated once more before recovery of the isolated mitochondria in the final pellet. Activity of cytochrome *c* oxidase (COX) was quantified by mixing isolated mitochondria and ferrocytochrome *c*, and absorption at 550 nm was measured to determine COX activity of the samples (Invitrogen, Camarillo, CA, USA). Activity of β -hydroxyacyl-CoA dehydrogenase (β -HAD) was determined by a previously established method (3). Activity of citrate synthase (CS) was measured (Sigma-Aldrich, St. Louis, MO).

Analysis of mitochondrial DNA content

Total RNA was isolated from skeletal muscle tissue using TRIzol reagent (Invitrogen, Eugene, OR, USA), and converted to cDNA. The results were expressed as relative number of mitochondrial genomes per diploid nuclei. The following primers (IDT, Coralville, WA, USA) were used: mtDNA-specific 16s rRNA, forward CCGCAAGGGAAAGATGAAAGAC, reverse TCGTTTGGTTTCGGGGTTTC, and nuclear genome-specific hexokinase 2 gene, forward GCCAGCCTCTCCTGATTTTAGTGT, reverse GGGAACACAAAAGACCTCTTCTGG. The ratio of mtDNA and genomic DNA was determined by measuring relative density of the band.

Protein determination

Protein was measured by the Bradford ELISA method using bovine serum albumin as standard.

Statistical analysis

The results were expressed as the mean \pm standard deviation (SD). The statistical significance of the results in the RNA, behavioral studies, physiological and biochemical data were analyzed using a T-test or one-way analysis of variance (ANOVA) followed by Fisher's post hoc test. Repeated-measures ANOVA were computed on the monitoring data and the significance of the difference among groups were evaluated by Fisher's post hoc test.

Results

Ten week old mice were fed diets supplemented with GrandFusion diets (2%) for 20 weeks. The diets were as follows: Group 2 received a 2% GrandFusion (GF1, NF-216 - Fruit and Veggie #1 Blend), with the ND; and Group 3 received a 2% GrandFusion diet (GF2, NF-316 - Fruit #2 Blend). These are same diets that were used in previous studies (Yu et al., 2016; Yu et al., 2017). The animals were examined for food intake and body weight every week for the twenty weeks of feeding. As seen in Figure 1A, the mice on all diets maintained a constant intake of food over the course of the study. In addition, consistent with the food intake, all of the mice showed a similar gain in weight over the twenty weeks.

To determine the impact of the diets on exercise endurance and activity, the mice were subjected to several paradigms (Figure 2). In Figure 2A, the mice on both the GF1 and GF2 supplemented diets showed a 1.5fold increase in distance traveled compared to the control diet mice (Figure 2A). In addition, the GF1 and GF2 mice demonstrated a 1.7 and 1.8 fold increase in time on the treadmill (Figure 2B). When the mice were subjected to the rotarod testing, the mice showed a 1.2 fold increase in the time to fall for both the GF1 and GF2 supplemented diets when compared to the control diet (Figure 2C). When the GF1 and GF2 were compared to the control diet for grip strength, there was a 1.45 (GF1) and a 1.40 (GF2) fold increase. Finally, the both the soleus (SM) and gastrocnemius (GM) muscles were removed from the mice following the 10 twenty week treatment, and the GF treated mice showed 1.33 (SM) and 1.45 (GM) fold increase for the GF1 diet and 1.32 (SM) and 1.55 (GM) for the GF2 diet. These results show that the GF supplemented diets significantly increased exercise endurance in the mice compared to the control diet animals.

To determine role of the skeletal muscle cell mitochondria on the increased endurance in the mice treated with GF diets, at the end of the study, the skeletal muscle was removed and examined for mitochondria DNA content (mtDNA, Figure 3A). As seen in the figure, there was an increase of 1.76 (GF1) and 1.85 (GF2) fold over the control diet fed mice. Figure 3B shows in the changes in mitochondrial enzymes, protein was isolated and subjected to enzymatic analysis. When mice were treated with the GF diets, there were significant increases in cytochrome c oxidase (COX), β -hydroxyacyl-CoA dehydrogenase (β -HAD) and citrate synthase (CS) (Figure 3B). As seen in the figure, COX was increased by 2.0 (GF1) and 1.9 (GF2); β -HAD was increased by 2.5 (GF1) and 2.9 (GF2); and CS was increased by 1.5 (GF1) and 1.6 (GF2) fold over control mice. Finally, we analyzed several mitochondrial mRNAs to establish the role of GF supplementation on mitochondrial function (Figure 3C). The increases in mRNA expression were: PGC-1 α – 2.3 (GF1) and 2.8 (GF2); Tfam – 1.8 (GF1) and 2.3 (GF2); ERR α – 2.0 (GF1) and 2.3 (GF2); NRF-1 – 1.8 (GF1) and 1.85 (GF2); COXIV – 1.8 (GF1) and 2.6 (GF2); ATPsyn – 2.0 (GF1) and 1.7 (GF2). These results demonstrate an increase in mitochondrial activity and function with the treatment of GF diets.

The GF supplemented diets stimulate exercise signaling in the skeletal muscle cells. AMPK, SIRT1, PGC-1 α , and PPAR δ are critical sensors and regulators of energy expenditure and biogenesis in the mitochondria (). GF diets increased the expression of phosphorylated AMPK, SIRT1, PGC-1 α and PPAR δ *in vivo* compared to the control mice (Figure 4). The increases in protein expression were: pAMPK – 4.7 (GF1) and 5.1 (GF2); SIRT1 – 4.4 (GF1) and 5.0 (GF2); PGC-1 α – 4.4 (GF1) and 4.1 (GF2); and PPAR δ – 4.7 (GF1) and 4.85 (GF2). Finally, we examined the expression of S6K1 and its phosphorylated form in the presence of control of GF supplemented diets (Figure 5). Treatment with GF diets resulted in a decrease in

the p-S6K1 (-60%, GF1) and (-67%, GF2) compared to the control diets. These data support the hypothesis that the GF supplemented diets enhance mitochondrial biogenesis and function while suppressing autophagy (Jaspers et al., 2017).

Discussion

In the present study, we examined the control of mitochondrial function in skeletal muscle by diets rich in vegetables and fruits and found that long-term feeding of these diets for 20 weeks increased skeletal muscle mitochondria and mitochondrial function which enhanced the exercise endurance.

Previous studies have shown that a number of factors are important in the regulation of exercise endurance mediated through mitochondrial function (Scarpulla et al., 2012; Goron et al., 2017; Ziaaldini et al., 2017). Research has implicated PGC-1 α in controlling mitochondrial biogenesis and links processes associated with energy metabolism (Marin et al., 2017). Because of this relationship, PGC-1 α is known to cooperate with NRF-1 to activate the expression of Tfam. Increased expression of Tfam has been shown to mediate the replication of mitochondrial DNA, activate transcription, and orchestrate oxidative phosphorylation in skeletal muscle (Carey and Kingwell, 2009). Additionally, PGC-1 α stimulates the expression of ERR α that is important for the regulation of fatty acid oxidation and the articulation of enzymes in the oxidative phosphorylation pathway (Sano et al., 2008). The GF supplemented diets can stimulate the expression of PGC-1 α mRNA as well as ERR α , NRF-1, and Tfam (Ost et al., 2016; Park et al., 2016). The data further supports the hypothesis that nutritional supplements can increase the total mitochondrial supply by stimulation of mitochondrial biogenesis (Sun et al., 2017). Therefore, we can further speculate that nutritional enhancements can adjust endurance during exercise by intensifying mitochondrial behavior (He et al., 2016). Additionally, studies have demonstrated that mitochondrial production and performance decrease in the aging population, genetic defects in mitochondrial function and that alteration in mitochondrial activity is associated with age-related disorders (Finck et al., 2007; Golestaneh et al., 2016; Dong et al.,

2017). Thus, the impact of nutritional supplements on the specific roles of mitochondrial may transcend many different physiological states ()

As indicated above, AMPK, SIRT1, PGC-1 α , ERR α , NRF-1 and PPAR δ all perform important responsibilities in energy metabolism (Carey and Kingwell, 2009). In addition, they are all enhanced by exercise and various diets that (Carey and Kingwell, 2009; Kim et al., 2016). Recent studies have shown that overexpression and deletion of these factors contribute to the regulation of exercise endurance, and that stimulation of these entities is critical to the improve physical endurance (Wang et al., 2004; Thomson et al., 2007; Matsakas et al., 2010). Transgenic PGC-1 α mice have been shown to regulate and coordinate factors that are involved in skeletal muscle function and hypertrophy (Valeron, 2014; Ruas et al., 2012). PGC-1 α increases the expression of genes associated with red fibers, mitochondrial function, fatty acid oxidation, and branched chain amino acid (BCAA) degradation (Halling et al., 2016; Eisele et al., 2015). In addition, studies have shown that the purine nucleotide pathway, malate-aspartate shuttle, and creatine metabolism, are elevated by PGC-1 α , further characterizing its role in exercise metabolism. The metabolic sensor and regulator, SIRT1 overexpression in skeletal muscle demonstrated fiber shift from fast-to-slow twitch, increased levels of PGC-1 α , oxidative metabolism and mitochondrial biogenesis (Chalkiadaki et al., 2014; Hatazawa et al., 2015). This study demonstrated that GF supplementation activated, both mRNA and protein of AMPK, SIRT1, PGC-1 α , ERR α , NRF-1, and PPAR δ in the signal cascade of exercise.

Our previous studies have demonstrated the impact of nutritional supplements in the improved recovery from neurological disorders as well as attenuating age-related declines (Yu et al., 2016; Yu el at., 2017). We showed that supplementation of GF diets prior to cerebral ischemic injury in mice, attenuated the damage in the brain following the injury (Yu et al., 2016).

In addition, the diets reduced the levels of oxidative stress, inflammation and induced neurogenesis. In the aged rat model, the diets reduced oxidative stress and inflammation and improved physical performance in the aged animals (Yu et al., 2017). The presence of vegetables and fruits containing phytonutrients are essential for prevention or reducing the risk of disease as well as tempering the outcomes following injury or disease.

In summary, this study demonstrated that long-term treatment of animals to diets enriched with vegetable or fruit extracts increased muscle mitochondria via activation of AMPK pathways. In addition, we realized that the diets increased muscle mass and function that correlated with exercise endurance. These data suggest that the increase in mitochondria in the muscle is instigated by the diets which contain antioxidants, anti-inflammatory agents, nutrients and factors that provide the increase in exercise endurance. Therefore, since the muscle mitochondria are dynamic elements that help to enhance physical implementation, approaches that stimulate and invigorate mitochondrial viability are necessitated to continue the process of exercise endurance.

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Additional information**Competing interests**

Dr. Stephen Perry is a technical and science consultant for NutriFusion, LLC.

Author contributions

J.Y. and M.S.K. provided the study concept and the design. J.Y., H.Z., S.T. and M.S.K. acquired the data. S.P. and M.S.K. provided the analysis and interpretation of the data. M.S.K. drafted the manuscript. All authors critically reviewed the manuscript for important intellectual content. J.Y. and M.S.K. supervised the study.

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Figure Legends

Figure 1. Effects of GF diets on food intake and body weight. (A) Food intake changes of 10 weeks. (B) Body weight changes in mice on various diets over 10 weeks. Mice were fed a normal diet or diets supplemented with 2% GF. Each point represents mean \pm SD (n=10 per time point).

Figure 2. Effects of GF diets on exercise endurance and skeletal muscle mass in C57BL/6 mice. (A) Running distance and (B) time of normal mice and mice fed a normal diet enriched with GF supplements. (C) Time to fall in a rotarod test (D) grip strength and (E) the ratio of skeletal muscle mass (soleus and gastrocnemius muscle)/body weight in normal and GF supplemented mice. Data are expressed as the mean \pm SD (n=10, *P<0.01 compared to control group).

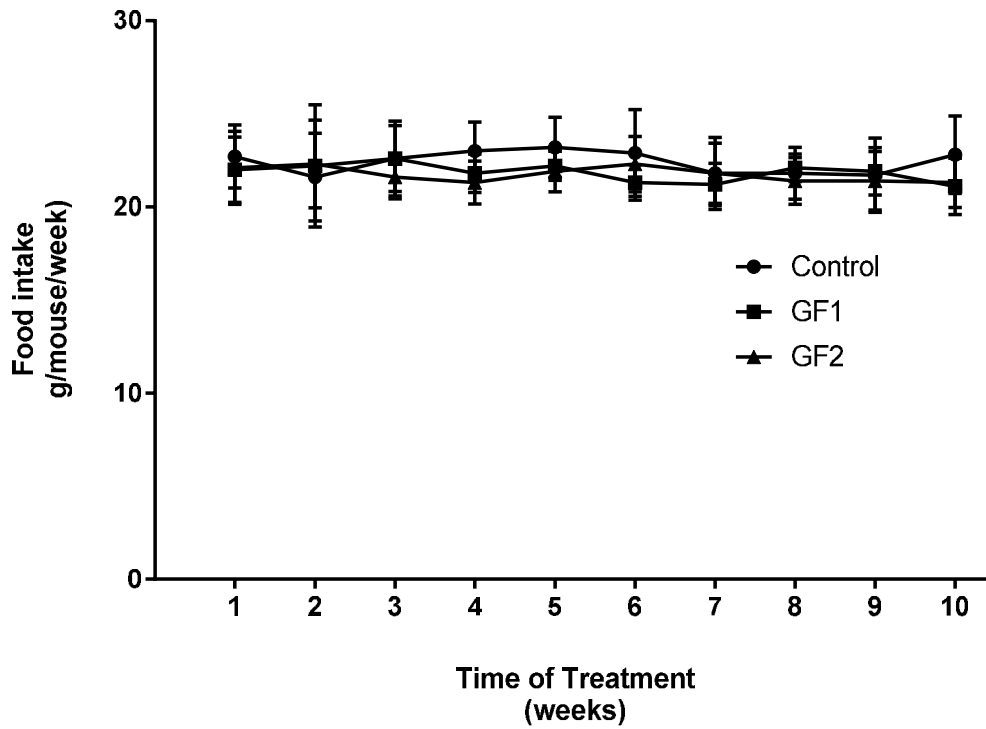
Figure 3. Effect of GF diets on muscle mitochondrial biogenesis in C57BL/6 mice. (A) The amount of mitochondrial DNA (mtDNA) in mice fed a control diet or a diet enriched in GF as determined by the mtDNA/genomic DNA ratio. (B) The activity of mitochondrial enzymes COX, β -HAD and CS were evaluated as a percent of control. (C) The relative mRNA levels of PGC-1 α , Tfam, ERR α , NRF-1, COXIV and ATPsyn in control and GF fed mice. Total RNA was isolated from soleus skeletal muscle tissue and expression of mitochondrial-related genes was estimated by quantitative PCR analysis. The results are expressed as the mean \pm SD (n=10, *P<0.01 compared to the control group).

Figure 4. The effect of GF diets on the exercise signaling pathways. (A) The protein expression of exercise-associated markers such as p-AMPK, AMPK, SIRT1, PGC-1 α and PPAR δ was evaluated by Western blot analysis. Total protein was isolated from skeletal muscle and examined. (B) The protein expression levels in (A) were plotted for statistical analysis. The relative protein levels were determined. The results are expressed as mean \pm SD (n=10, *P<0.01 compared to the control group).

Figure 5. GF diet suppresses TORC1 activity blocking autophagy. (A) Mice fed control or GF diets were evaluated for p-S6K1 and S6K1 activity. Protein isolated from skeletal muscle was examined by Western blot analysis. (B) Evaluation of data from A. The results are expressed as mean \pm SD (n=10, *P<0.01 compared to the control group).

Figure 1.

A.



B.

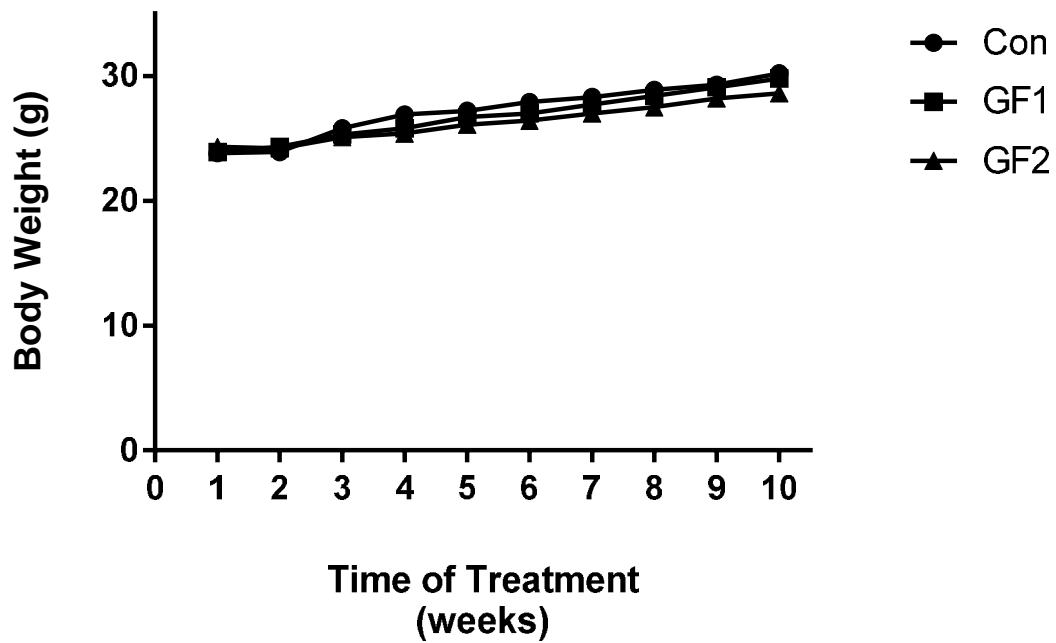
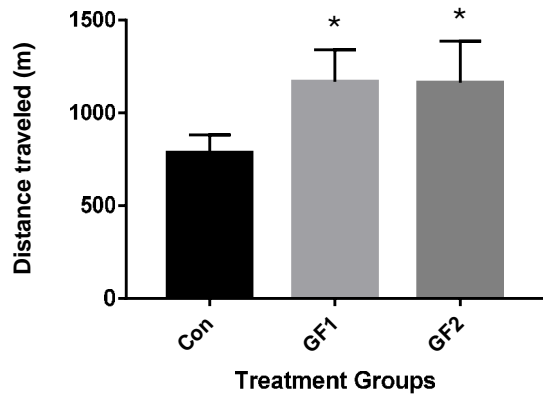
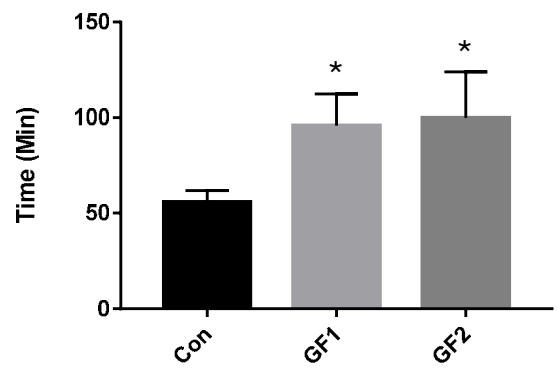


Figure 2.

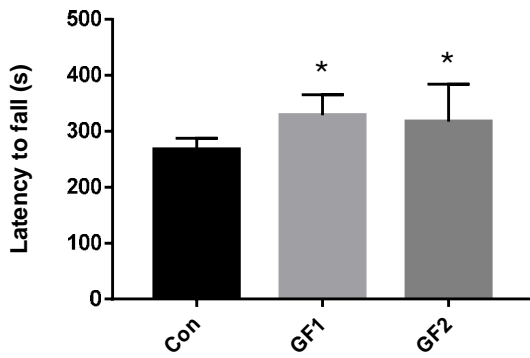
A.



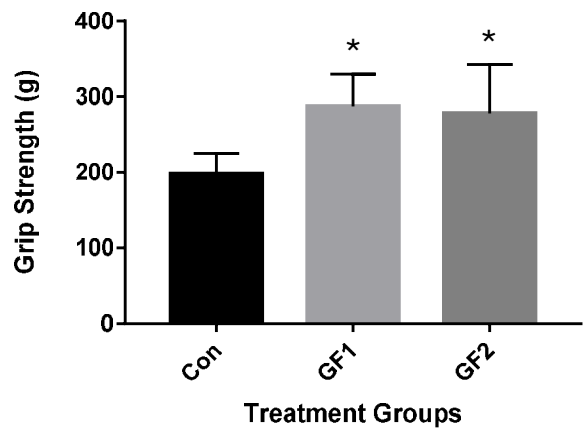
B.



C.



D.



E.

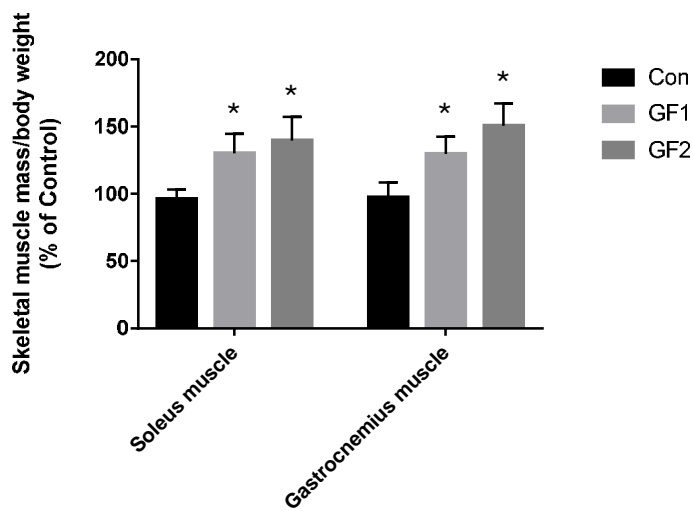
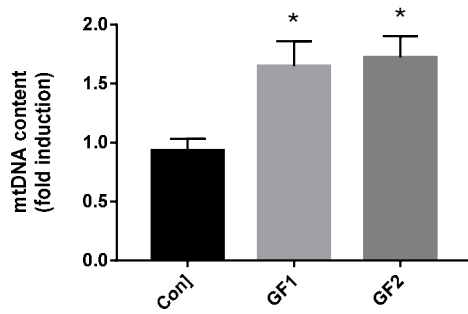
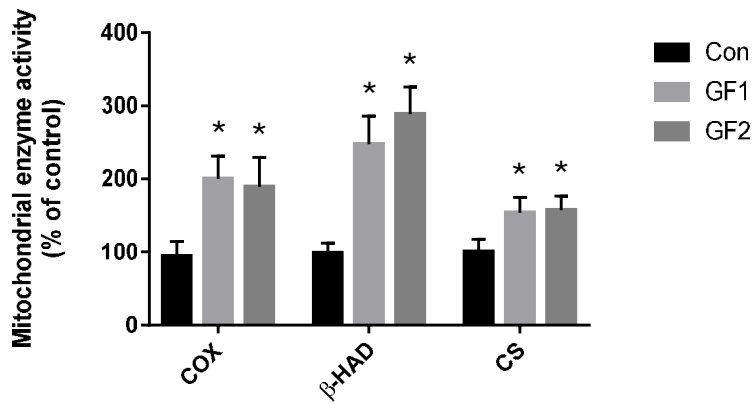


Figure 3.

A.



B.



C.

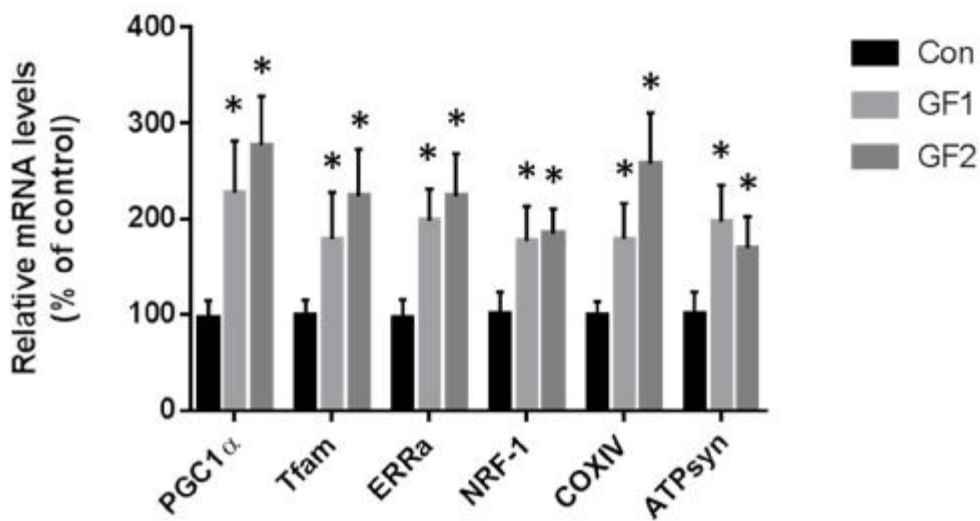


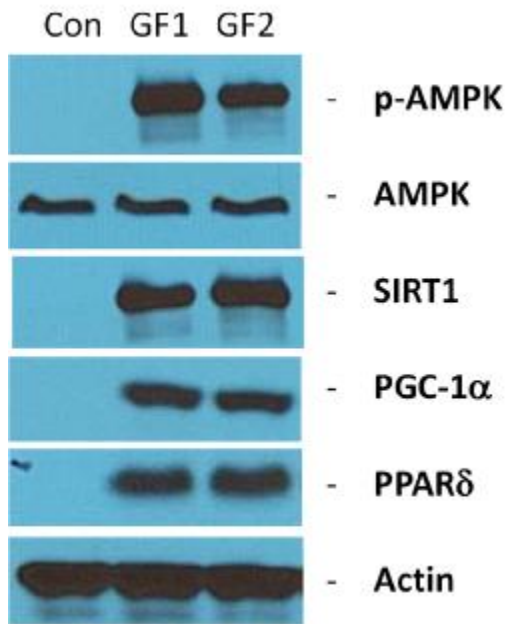
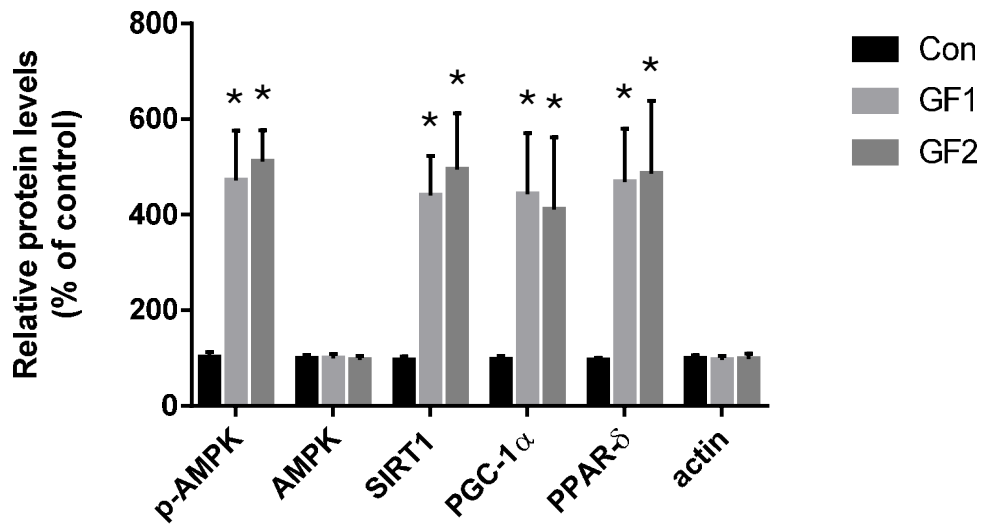
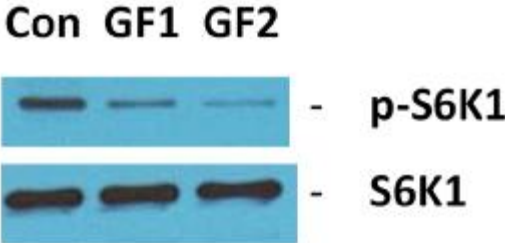
Figure 4.**A.****B.**

Figure 5.

A.



B.

