

The effect of Bu Zhong Yi Qi decoction on simulated weightlessness-induced muscle atrophy and its mechanisms

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Received August 10, 2016; Accepted May 11, 2017

DOI: 10.3892/mmr.2017.7287

Abstract. Microgravity has been previously demonstrated to induce skeletal muscle atrophy, loss of muscle force and disorders in myogenesis and metabolism. Current pharmacological strategies exhibit poor efficacy. Bu Zhong Yi Qi decoction (BZ) is a well-known traditional Chinese medicine decoction used for myasthenia gravis. In the present study, its effect on unloading induced muscle atrophy was investigated. The mousetail suspension model was used to simulate weightlessness induced muscle atrophy. The results indicated that BZ could significantly protect muscles from simulated weightlessness-induced atrophy. To elucidate the underlying mechanisms, drugCIPHER-CS methods were introduced to predict its potential targets, significantly enriched pathways and biological processes. The results demonstrated that the calcium signaling pathway, citrate cycle, biosynthetic and lipid metabolic process are affected by BZ. Among the targets, nuclear receptor corepressor 1 (NCoR1) is one of the most important proteins involved in myogenesis and metabolism. The results indicated that BZ significantly downregulated NCoR 1 expression, and further induced muscle differentiation and metabolism by regulating NCoR1-associated gene expression *in vivo* and *in vitro*. In summary, the present study

indicated that may be effective in combating weightlessness-induced muscle atrophy. Combined with bioinformatics, the underlying mechanism for this decoction was investigated, which provided an improved understanding of this decoction.

Introduction

Prolonged space flight can cause rapid and severe atrophy in skeletal muscle due to the weightlessness experienced. It has been demonstrated that real and simulated spaceflight results in a loss of muscle mass and reductions in muscle strength and endurance in humans and rodents (1-4). Satellite cells are skeletal muscular stem cells that are the primary source for muscle regeneration. Satellite cell dysfunction, representing impaired proliferation capacity or increased apoptosis, is suggested to contribute to the decreasing muscle regeneration in atrophy (5,6). Oxidative stress can be one of the triggers of muscle atrophy (7), while alterations of mitochondrial biogenesis and dynamics have been observed in prolonged unloading of muscle (8,9). Countermeasures against these causes may be a possible resolution for weightlessness-induced muscle atrophy. Effective prevention of weightlessness-induced muscle atrophy required for space missions of increasing durations. Chinese medicine has been used previously in countermeasures for muscle-associated diseases. In traditional Chinese medicine (TCM), it is believed that the spleen governs the muscle, and is closely associated with *qi* (10). In TCM it is thought that in space, as the earth *qi* is reduced, the spleen is relatively weak. Bu Zhong Yi Qi decoction (BZ) predominantly consists of Radix Ginseng, Radix Angelicae and has been reported to improve spleen function and is used in treatment of myasthenia gravis (11). In the present study, the effects of BZ on weightlessness-induced muscle atrophy and its mechanisms were investigated. BZ is additionally commonly used in clinical practice for chronic fatigue and chronic atrophic gastritis, and is widely produced (12). Large scale epidemiological studies in clinical trials have been conducted, suggesting that BZ has a clear effect on improving muscle function (11). In addition, investigations have been conducted into the bioactivities of monomers present in BZ.

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Key words: traditional Chinese medicine, protective effects, simulated weightlessness-induced muscle atrophy, network pharmacology

Hexadecanoic acid from BZ was reported to induce bone marrow mesenchymal stem cell proliferation depending on the retinoic acid receptor pathway and upregulated the mRNA levels of insulin-like growth factor-1 (13). However, individual pharmacological effects of the monomers present do not fully account for the effects of this formula in general, and the pharmacology of BZ remains unclear. Bioinformatic methods provide predictions of drug-target interactions, identifying underlying molecular mechanisms. The drugCIPHER-CS method, which was developed by Zhao and Li (14), offers an effective method to understand the complicated multitargeted mechanisms of TCM herbal formulas. The two basic principles of this method are that drugs with similar chemical structures are likely to bind functional-associated proteins, and functional associations between the proteins can be measured by their distance in the protein interaction network (15). It has been demonstrated as a reliable method to obtain potential targets for decoctions (16). In addition, it is possible to analyze the significantly enriched pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway analysis and Gene Ontology (GO) analysis to identify the biological processes involved in these targets. In the present study, it was identified that BZ was able to protect from simulated weightlessness-induced loss of muscle mass, with a reduction in the gastrocnemius (GAS) cross sectional area and a decrease in twitch force. The results of the bioinformatics analysis identified the main biological pathways and processes involved the effect of BZ. Experimental verification is required in order to further reveal the underlying mechanisms.

Materials and methods

Animal studies. Male Kunming (KM) mice (n=24; age, 8 weeks old; weight, ~25 g; Beijing Vital River Laboratory Animal Technology Co, Ltd., Beijing, China) were divided into control (Ctrl), hindlimb-suspended (HS) and BZ-fed-hindlimb-suspended (BZ) groups (n=8 mice/group). All mice were maintained in a temperature (23°C) and humidity (50-70%)-controlled specific pathogen free (SPF) animal facility with a 12 h light/dark cycle and were given free access to food and water. HS and BZ mice were suspended by attaching a harness to the proximal end of the tail. The hindlimb suspension model was adopted to simulate unloading-induced muscle atrophy, as previously described (17). The mice were suspended at a 30° angle to the floor to prevent contact with any supportive surfaces, with only the forelimbs maintained in contact with the grid floor, which allowed the mice to move and access food and water freely. HS and BZ mice were suspended for 14 days. BZ mice were fed with BZ during this period and 7 days previously, while HS and Ctrl mice were fed with water. No necrosis of hindlimb or unexpected death of animals was observed during the experimental procedure. At the end of suspension, mice were euthanized for muscle isolation by intraperitoneal injection with 1.5% pentobarbital sodium (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The experimental procedures were approved by the Animal Care and Use Committee of China Astronaut Research and Training Center (Beijing, China), and all animal studies were performed according to the approved guidelines for the care and use of live animals.

Drug preparation and dose consideration. According to its origin, *Treatise on the Spleen and Stomach* (18), the BZ consists of eight Chinese herbs, at a ratio of 5:5:3:5:2:4:2:3, as follows: *Radix Astragalus membranaceus* (Huangqi); *Radix Panax ginseng* (Renshen); *Radix Angelica Sinensis* (Danggui); *Glycyrrhiza uralensis* (Gancao); *Cimicifuga foetida* (Shengma); *Herba Bupleurum chinense* (Chaihu); *Citrus sinensis Osbeck* (Chenpi); and *Atractylodes macrocephala* (Baizhu), respectively. Herbs were purchased from Tongren Tang Pharmacy (Beijing, China). These 8 types of herbs, at a total weight of 89 g, were extracted twice in 890 ml boiling water for 1 h. A liquor solution was formed after extraction. The liquid was then concentrated to obtain a paste, which was collected and water was added to a total volume of 100 ml, so that the liquid contained 0.89 g herbal medicine/ml. A brown medical liquor was finally obtained with pH of 7.4. Based on the recommended human daily dosage of 89 g/day, according to the equivalent conversion between humans and mice by body weight, a dosage of 5.93 mg/g was used in the current study.

Drug-containing serum preparation. BZ was fed to male Wistar rats for 5 days (n=5; weight, 293-301 g; supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd.). Rats were maintained in a temperature (23°C) and humidity (50-70%)-controlled SPF animal facility with a 12 h light/dark cycle and were given free access to food and water. The dosage of BZ used was 11.87 mg/g, which was twice the dosage for the mice during tail-suspension period as previously described. Serum was taken on the fifth day 2 h after the rats were fed with BZ.

Ex vivo assessment of skeletal muscle contractile properties. Following 14 days of hindlimb suspension, the GAS was isolated from distal tendons intact and nylon sutures were attached to the tendons. The other end of the string was attached to the MedLabBio-Signal Processing System (MedLab-U/4C501H; Nanjing Medeas Science and Technology Co., Ltd, Nanjing, China; <http://www.medeas.com.cn>). Muscles were adjusted perpendicularly to the operation table to obtain the maximal twitch force and bathed with PBS solution to remain wet. *Ex vivo* muscle stimulation and data analysis was performed using the Medlab system. Peak isometric twitch force was analyzed from isometric contraction, and isometric twitch contractions were stimulated in muscles at a frequency of every 50 Hz, with the stimulation current varying from 0 to 10 V.

Histological analysis. Cryosections of GAS were fixed in 4% paraformaldehyde in PBS for 10 min at 4°C and washed in PBS. After fixing of the samples with 15% goat serum (Sigma-Aldrich; Merck KGaA) at room temperature, the sections were incubated overnight at 4°C with mouse monoclonal anti-laminin primary antibodies (CWP223; 1:50; Beijing Kangwei Century Biotechnology Co., Ltd., Beijing, China). The primary antibody was detected by incubation at room temperature for 1 h with fluorescein-conjugated affinity-pure goat anti-mouse immunoglobulin G (TA130003; 1:200; OriGene Technologies, Inc., Rockville, MD, USA.) diluted in BSA. Three independent GAS cryosections and 8 fibers per section were counted for cross-sectional area (CSA) analysis.

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene name	Forward primer	Reverse primer
NCoR1	5'-CTGGTCTTTCAGCCACCATT-3'	5'-CCTTCATTGGATCCTCCATC-3'
MEF2C	5'-TCCATCAGCCATTTCAACAA-3'	5'-GTTACAGAGCCGAGGTGGAG-3'
MEF2D	5'-CTTCGCGTAAACCGAGGATT-3'	5'-GGGACTAGTGATCAGTTCATGG-3'
Myoglobin	5'-CTGACGAAGGCCACTTTGCACCTCTG-3'	5'-GCACAAGATCCCGGTCAAGTACCTGGAG-3'
MCK	5'-TGAAGTCGCCCCGTCAGGCTGTTGAG-3'	5'-GATGTCATCCAGACTGGGGTGGACAACC-3'
Pax7	5'-CGATTAGCCGAGTGCTCAGA-3'	5'-GGAGGTCGGGTTCTGATTCC-3'
Myogenin	5'-GGTGCCAGTGAATGCAACT-3'	5'-GCTGTCCACGATGGACGTAA-3'
MyHC	5'-CCAAGGGCCTGAATGAGGAG-3'	5'-GCAAAGGCTCCAGGTCTGAG-3'
Citrate synthase	5'-GGAGCCAAGAACTCATCCTG-3'	5'-TCTGGCCTGCTCCTTAGGTA-3'
LCAD	5'-GTAGCTTATGAATGTGTGCAACTC-3'	5'-GTCTTGCGATCAGCTCTTTTCATTA-3'
VEGF-B	5'-AGCCACCAGAAGAAAGTGGT-3'	5'-GCTGGGCACTAGTTGTTTGA-3'
VEGF-A 121	5'-AACGATGAAGCCCTGGAGTG-3'	5'-TGAGAGGTCTGGTTCCCGA-3'
GAPDH	5'-TCACCACCATGGAGAAGGC-3'	5'-GCTAAGCAGTTGGTGGTGCA-3'
COX2	5'-CATCCCCTTCCTGCGAAGTT-3'	5'-TTTAAGTCCACTCCATGGCCC-3'
UCP2	5'-TGGCAGGTAGCACACAGG-3'	5'-CATCTGGTCTTGACGCAACTCT-3'

NCoR1, nuclear receptor corepressor 1; MEF2C, myocyte-specific enhancer factor 2C; MEF2D, myocyte-specific enhancer factor 2D; MCK, muscle creatine kinase; Pax7, paired Box 7; MyHC, myosin heavy chain; LCAD, long chain acyl-CoA dehydrogenase; VEGF-B, vascular endothelial growth factor B; VEGF-A 121, vascular endothelial growth factor A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COX2, cytochrome c oxidase subunit 2; UCP2, uncoupling protein 2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from GAS muscles using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions. A total of 3 μ g RNA was reverse-transcribed using the PrimeScript RT reagent kit (Perfect Real Time) with gDNA Eraser (Takara Bio, Inc., Otsu, Japan). Gene expression was assessed using qPCR with SYBR Premix Ex Taq II (TliRNaseH Plus; Takara Bio., Inc.) according to the manufacturer's protocol. qPCR values were normalized with U6 and the primers used are listed in Table I. The following thermal settings were used: 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Relative expression values were calculated using the comparative threshold cycle ($\Delta\Delta Cq$) method in accordance with previous guidelines (19,20).

Cell culture. C2C12 myoblasts were obtained from ATCC (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). The cells were differentiated in DMEM containing 2% horse serum [M&C Gene Technology (Beijing) Ltd., Beijing, China]. To compare the effect of serum from BZ-fed rats, we treated the cells with 2% serum from normal rat as a Ctrl.

Cell proliferation assay. C2C12 myoblasts were seeded into 96-well plates with growth medium for 12 h, then were cultured with differentiation medium (DMEM containing 2% horse serum) for 3 days. Cell proliferation was detected subsequent to incubation with 10% MTS (BioVision, Inc., Milpitas,

CA, USA) for 5-6 h under 485 nm UV light. The experiments were repeated three times.

Drug-target prediction and analyses. Firstly, the composite compounds of BZ were identified from the literature. The drugCIPHER-CS method was applied to predict the potential drug-targets of the composite compounds. drugCIPHER-CS was developed by Zhao and Li (14), and can infer drug-targets in the genome-wide scale and achieves good prediction performance. Given a set of known drug (drug space)-target (target space) interactions, for a query drug and a candidate target-gene, drugCIPHER-CS will measure the likelihood of their interaction based on the correlation between the query drug's structure similarity with the drug space and the candidate gene's functional similarity with the target space. For each query compound, drugCIPHER-CS ranks its potential targets according to the order of the decreasing interaction possibility (14). In the current study, known drug-target interactions were obtained from the DrugBank database (version: May, 2011) (21), the chemical structure similarity is calculated based on compounds' MOLPRINT 2D descriptors and Tanimoto coefficient. The human protein interaction network was constructed by integrating the protein interaction data from: Human Protein Reference Database (HPRD; release 9.0, <http://www.hprd.org/>) (22), the Biological General Repository for Interaction Datasets (BioGRID; version 3.0.66, <https://thebiogrid.org/>) (23), the IntAct Molecular Interaction Database (IntAct; version 20100628; <http://www.ebi.ac.uk/intact/>) (24), the Molecular INteraction database (MINT; version 20100505; <http://mint.bio.uniroma2.it/>) (25), the Database of Interacting Proteins (DIP; version 20100614; <http://dip.doe-mbi.ucla.edu>) (26) and the Protein

Data Bank (PDB; <http://www.rcsb.org/pdb/home/home.do>) provided by Gibson and Goldberg (27).

After obtaining the potential targets of chemical components of BZ, hypergeometric cumulative distribution test is used to investigate the significantly enriched biological pathways and GO biological processes among these potential targets. A biological pathway or GO biological process was considered to be significantly enriched with drug-targets of BZ when the corresponding upper-tailed P-value of hypergeometric cumulative distribution test was <0.05 . In the present study, biological pathway data are from KEGG database (<http://www.genome.jp/kegg/>; last updated Oct 16, 2012) (28) and GO biological process annotation of human proteins downloaded from GO project website (version Sep 9, 2011; <http://www.geneontology.org/>) (29). The network topological analysis of targets is based on the integrated human protein interaction network as mentioned above.

Statistical analysis. Data are presented as the mean \pm standard deviation. Statistical significance was determined by the two-tailed t-test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Mice fed with BZ are resistant to simulated microgravity-induced muscle atrophy. To examine the role of BZ in skeletal muscle under HS, BZ was administrated to hindlimb-suspended mice for 14 days. Thereafter, the ratios of soleus (SOL) and GAS muscle weight to the whole-body weight were analyzed in the Ctrl, HS and BZ group mice. Hindlimb suspended mice exhibited over 30 and 12% reductions in relative SOL and GAS muscle mass, respectively, in comparison with Ctrl mice. However, hindlimb-suspended mice fed with BZ demonstrated ~ 20 and 10% increase in comparison with HS mice (Fig. 1A). This suggested that BZ partly protected hindlimb suspended mice from skeletal muscle atrophy. Immunostaining for laminin of GAS cross sectional area (CSA) delineated a decrease of GAS fiber size in the HS mice in comparison to Ctrl. In contrast, BZ muscle fiber size increased in comparison with HS (Fig. 1B). Morphometric analysis of GAS CSA highlighted a significant difference in myofiber size between HS and BZ muscles, confirming that BZ mice were resistant to muscle atrophy (Fig. 1B). GAS muscles were dissected and attached for assessment of isometric contractile properties. Representative twitch force traces of the muscles were presented (Fig. 1C). Isometric twitch force was significantly increased in the muscles from BZ fed mice compared with their hindlimb suspended counterparts (Fig. 1C).

Target prediction and analyses of the composite compounds of BZ. To elucidate the pharmacological mechanism of BZ, the potential drug-targets of the composite compounds of BZ were predicted and analyzed by bioinformatics analysis. First, by a comprehensive review of the literature, 238 non-redundant composite compounds were identified for 8 herbs of BZ. Subsequently, drugCIPHER-CS method was used to predict the potential targets of these compounds. For a query compound,

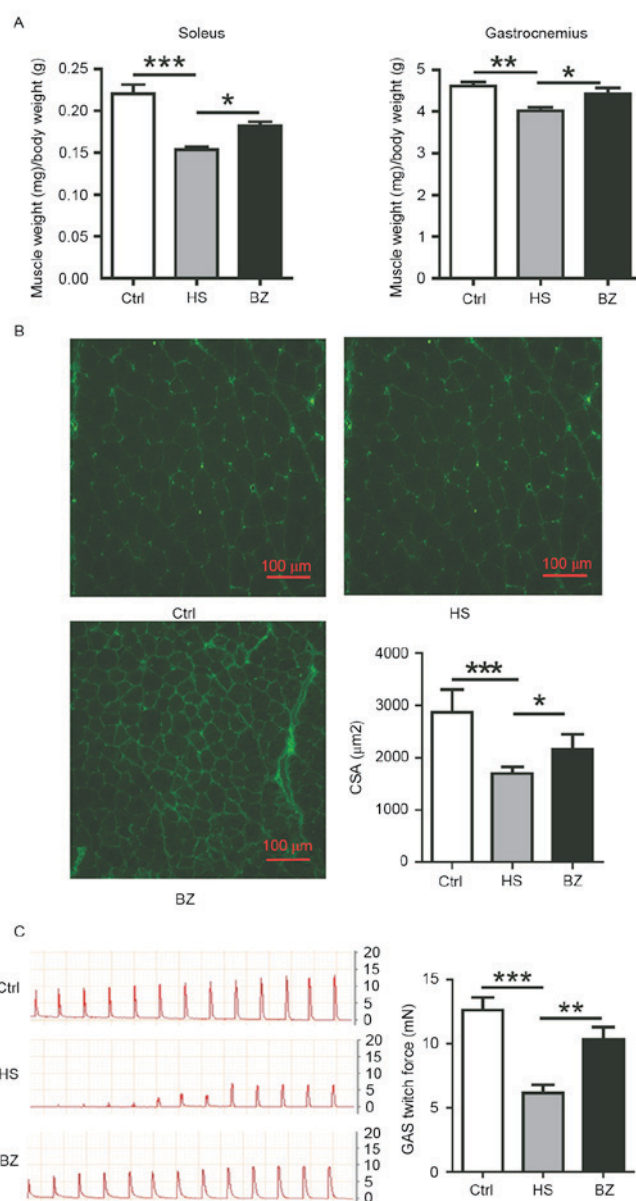


Figure 1. The effect of BZ on skeletal muscle atrophy induced by tail suspension in mice. KM mice in HS and BZ group were suspended for 14 days, during which time BZ group mice were treated with BZ. (A) The ratios of SOL and GAS muscle weight to body weight in the mice of Ctrl, tail suspended and BZ treatment group. Data are presented as mean \pm standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; $n = 8$ for each group. (B) Immunostaining for laminin of GAS muscle in Ctrl, tail suspension and BZ fed mice. Data are presented as the mean \pm standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. A total of 3 independent GAS cryosections and 8 fibers per section were counted for CSA analysis, $n = 8$ for each group. (C) *Ex vivo* contractile properties of Ctrl, tail suspension and BZ fed mice, twitch force of GAS muscle from mice. Data are presented as the mean \pm standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; $n = 8$ for each group. BZ, Bu Zhong Yi Qi decoction; KM, Kunming; HS, hindlimb suspended; SOL, soleus; GAS, gastrocnemius; Ctrl, control.

a score for each candidate target was given by measuring the compound-target interaction possibility. When the top 0.1% candidate targets were considered, 451 potential targets of BZ were obtained. Candidate genes were on average targeted by 5.8 composite compounds.

Furthermore, bioinformatics analysis was conducted for these candidate targets. It was identified that there were 17 significantly enriched KEGG biological pathways among

Table II. Significantly enriched KEGG biological pathways among top 0.1% candidate target genes of BZ composite compounds.

KEGG pathway number and name	P-value	Coverage
hsa04020-Calcium signaling pathway	5.06x10 ⁻⁵	0.095505618
hsa00020-Citrate cycle	4.67x10 ⁻⁴	0.1875
hsa04260-Cardiac muscle contraction	9.95x10 ⁻⁴	0.1125
hsa00590-Arachidonic acid metabolism	2.42x10 ⁻³	0.120689655
hsa00510-N-Glycan biosynthesis	3.32x10 ⁻³	0.130434783
hsa00640-Propanoate metabolism	3.77x10 ⁻³	0.151515152
hsa04080-Neuroactive ligand-receptor interaction	8.15x10 ⁻³	0.059602649
hsa00561-Glycerolipid metabolism	1.42x10 ⁻²	0.111111111
hsa00512-O-Glycan biosynthesis	1.49x10 ⁻²	0.133333333
hsa00830-Retinol metabolism	1.65x10 ⁻²	0.09375
hsa05010-Alzheimer's disease	2.01x10 ⁻²	0.065088757
hsa04115-p53 signaling pathway	2.32x10 ⁻²	0.086956522
hsa05410-Hypertrophic cardiomyopathy	2.42x10 ⁻²	0.078651685
hsa05414-Dilated cardiomyopathy	2.84x10 ⁻²	0.076086957
hsa00190-Oxidative phosphorylation	2.97x10 ⁻²	0.066666667
hsa05412-Arrhythmogenic right ventricular cardiomyopathy	3.52x10 ⁻²	0.078947368
hsa00970-Aminoacyl-tRNA biosynthesis	4.20x10 ⁻²	0.097560976

Pathways whose corresponding upper-tailed P-value of the hypergeometric cumulative distribution was <0.05 were deemed to be significantly enriched by candidate target genes. Those pathways were ranked according to the order of the increasing P-values. The coverage for each pathway was referred to as the fraction of candidate target genes among all the pathway member genes. KEGG, Kyoto Encyclopedia of Genes and Genomes; BZ, Bu Zhong Yi Qi decoction.

these 451 candidate targets, including calcium signaling pathway and tricarboxylic acid cycle (TCA cycle; Table II). The calcium signaling pathway is well established in muscle dystrophy, which is activated by ROS to produce Ca²⁺ entry, resulting in a significant effect of muscle damage (30). TCA cycle flux and adenosine triphosphate (ATP) synthesis rate, TCA cycle and oxidative phosphorylation pathway genes were downregulated in muscle atrophy (31-34).

In addition, GO biological process enrichment analysis was conducted and this indicated that candidate targets were significantly enriched in biological processes including biosynthetic process, cellular nitrogen compound metabolic process, lipid metabolic process, cellular amino acid metabolic process and response to stress (Table III). Protein and amino acid metabolism is well known to be disrupted during muscle atrophy (35). In addition, fatty acid metabolism and lipid oxidation has been identified to be impaired in the majority of muscle disorders (36-38). The significantly enriched biological pathways and processes are potential target pathways and proteins of BZ, providing important evidence for elucidating the molecular mechanism of BZ.

To further determine the key candidate targets of BZ, the topological property of these candidate targets was assessed in the protein interaction network. The degree of a protein was referred to as its number of interaction partners in the protein interaction network, which could reflect the biological importance of a protein. The higher the degree of a protein was, the higher its biological importance. The degree was used to rank the candidate targets, proteins with relatively high degrees included splicing factor 3a subunit 1 (SF3A1), COP9

signalosome subunit 5 (COPS5) and other targets (Table IV). SF3A1, necessary for the *in vitro* conversion of 15S U2 snRNP into an active 17S particle that performs pre-mRNA splicing (39), has been demonstrated to have stability in relative quantity in skeletal muscle (40). Genome wide association studies indicated that COPS5 interacted with key regulators of beef cattle carcass intermuscular fat (41). These proteins may serve key roles in muscle physiology and BZ-induced protection. Among the targets predicted, NCoR1 was identified to have a high degree and betweenness centrality in the protein interaction network (Table IV), and is a well-known gene closely associated with muscle growth and oxidative function.

BZ reduces NCoR1 expression levels and promotes myogenesis under simulated weightlessness-induced muscle atrophy. To establish whether NCoR1 is a target for BZ to counteract muscle atrophy, the transcription levels of NCoR1 were detected in hindlimb-suspended and BZ-fed mice in GAS by RT-qPCR analysis. As presented in the results, NCoR1 expression was increased in the muscles of HS mice, and was markedly downregulated upon BZ administration (Fig. 2A), confirming that BZ may inhibit the upregulation of NCoR1 expression in muscles under conditions of simulated microgravity.

As reported previously, the expression of Mef2c and Mef2d negatively correlated with NCoR1 expression. These are the key transcription factors involved in the expression of muscle remodeling genes (42-44). The mRNA levels were reduced in HS mice compared with that of the Ctrl mice. Following BZ treatment, the expression levels of Mef2c were markedly increased. In line with this, mRNA levels of two MEF2 targets,

Table III. Significantly enriched GO biological process term among top 0.1% candidate target genes of BZ composite compounds.

GO term ID	GO term name	P-value
GO:0009058	Biosynthetic process	2.27x10 ⁻⁹
GO:0034641	Cellular nitrogen compound metabolic process	5.20x10 ⁻⁶
GO:0044281	Small molecule metabolic process	2.22x10 ⁻⁵
GO:0006629	Lipid metabolic process	5.33x10 ⁻⁵
GO:0019748	Secondary metabolic process	1.45x10 ⁻⁴
GO:0003013	Circulatory system process	2.89x10 ⁻³
GO:0051276	Chromosome organization	1.08x10 ⁻²
GO:0006520	Cellular amino acid metabolic process	1.14x10 ⁻²
GO:0051186	Cofactor metabolic process	1.46x10 ⁻²
GO:0006399	tRNA metabolic process	2.28x10 ⁻²
GO:0006950	Response to stress	4.69x10 ⁻²
GO:0042592	Homeostatic process	4.87x10 ⁻²

The top 12 candidate target genes are significantly enriched with genes annotated with a GO term when its corresponding upper-tailed P-value of hypergeometric cumulative distribution was <0.05. These GO terms are ranked according to the order of the increasing P-values. GO, gene ontology; BZ, Bu Zhong Yi Qi decoction.

Mb and Mck, which were two markers of myogenesis, were upregulated in GAS subsequent to administration with BZ (Fig. 2B). These results further confirmed that could stimulate myogenesis, and NCoR1 mediated the effect of BZ on muscle atrophy.

Muscle differentiation has been previously reported to be impaired during hindlimb suspension-induced muscle atrophy (45). Pax 7, myogenin and myosin heavy chain (MyHC) are markers of muscle proliferation and differentiation. The expression levels of Pax 7 and myogenin were reduced in HS mice. By contrast, BZ-fed mice displayed a marked increase in the mRNA expression of Pax 7, myogenin and MyHC. Pax 7 and myogenin expression increased 3-fold and MyHC increased by >44 fold compared with that of HS mice (Fig. 2C), indicating that BZ-fed mice were resistant to muscle atrophy by enhancing muscle differentiation and growth.

BZ reduces NCoR1 levels and promotes myogenesis and oxidative function in vitro. To further confirm the regulation of BZ on myogenesis and its mechanism, the effect of serum from BZ-fed rats on myoblasts was investigated. Subsequent to incubation of the C2C12 myoblasts in medium with serum containing BZ, the expression of NCoR1 was significantly reduced in comparison with the cells incubated with the serum from rats that did not receive BZ (Fig. 3A). Subsequently, the effect of BZ on the proliferation and differentiation of C2C12 cells *in vitro* was investigated. Following treatment of C2C12 cells with the medium containing serum from the rats fed with BZ, the cell numbers were markedly higher than those without

Table IV. Drug target prediction and analysis.

Potential drug-target	Degree in the protein interaction network	Interaction partners in the protein interaction network (gene ID: gene symbol)
10291:SF3A1	126	9939:RBM8A
10987:COPS5	113	6095:RORA
7520:XRCC5	111	6418:SET
9611:NCoR1	95	7704:ZBTB16
2091:FBL	85	3190:HNRNPK
6601:SMARCC2	82	10262:SF3B4
408:ARRB1	79	1855:DVL1
6633:SNRPD2	79	10250:SRRM1
6605:SMARCE1	76	7030:TFE3
6602:SMARCD1	74	1024:CDK8
86:ACTL6A	73	5469:MED1
7421:VDR	72	8204:NRIP1
50813:COPS7A	70	22919:MAPRE1
5705:PSMC5	70	2099:ESR1

The proteins with the highest degree/betweenness centrality are referred to as those with the top 5% degree/betweenness centrality in the protein interaction network. SF3A1, splicing factor 3a subunit 1; COPS5, COP9 signalosome subunit 5; XRCC5, X-ray repair cross complementing 5; NCoR1, nuclear receptor corepressor 1; FBL, fibrillarlin; SMARCC2, SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily c member 2; ARRB1, arrestin β 1; SNRPD2, small nuclear ribonucleoprotein D2 polypeptide; SMARCE1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1; SMARCD1, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1; ACTL6A, actin-like 6A; VDR, vitamin D receptor; COPS7A, COP9 signalosome subunit 7A; PSMC5, proteasome 26S subunit, ATPase 5.

BZ treatment (Fig. 3B). The expression levels of muscle differentiation marker genes were also upregulated following incubation in BZ serum (Fig. 3C). These results demonstrated that BZ promoted the proliferation of C2C12 in addition to enhancing the expression of muscle differentiation marker genes such as myogenin and MyHC.

BZ treatment was indicated to enhance muscle peak force of BZ-fed mice (Fig. 1C), which is consistent with the results of bioinformatic analysis (Tables II and III). The enriched potential KEGG biological pathways included calcium signaling pathway, TCA cycle, cardiac muscle contraction and arachidonic acid metabolism. TCA cycle is the energy generating reaction through the oxidation of carbohydrates, fats and proteins in the form of ATP. Calcium levels in the mitochondrial matrix are necessary for the activation of isocitrate dehydrogenase, one of the key regulatory enzymes of the TCA cycle (46). The significantly enriched GO biological processes, which indicated the functional distribution of those candidate targets, included several metabolic processes, including cellular nitrogen compound, small molecule and lipid and secondary metabolic process. These results indicated that the mechanism of BZ may be closely associated with the energy

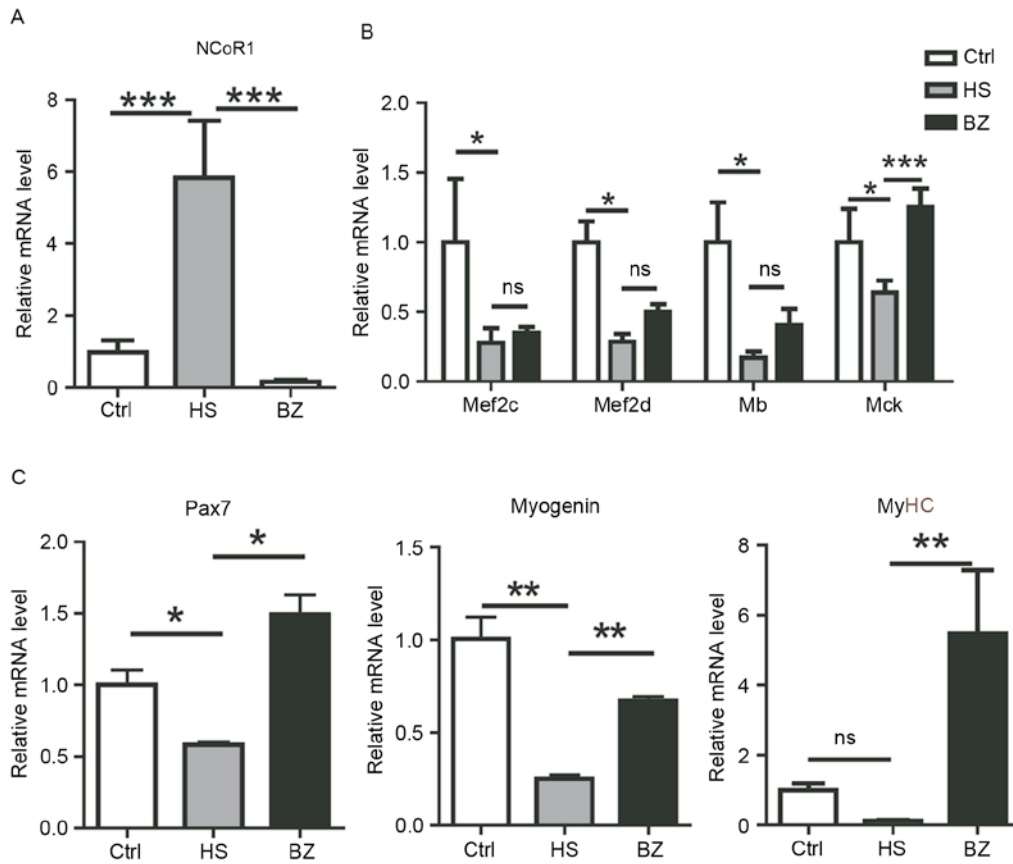


Figure 2. BZ reduces NCoR1 expression levels and promotes myogenesis in mice. (A) The expression of NCoR1 in GAS in the mice of Ctrl, tail suspended and BZ fed mice was detected by RT-qPCR. The values are normalized to Ctrl. (B) The expression of target genes of NCoR1 in GAS in Ctrl, tail suspended and BZ fed mice were detected by RT-qPCR. The values are normalized to the Ctrl. (C) The expression of Pax7, Myogenin and MyHC of GAS in Ctrl, tail suspended and BZ fed mice, detected by RT-qPCR. The values are normalized to the Ctrl. Data are presented as the mean \pm standard deviation. *P<0.05; **P<0.01; n=8 for each group. BZ, Bu Zhong Yi Qi decoction; NCoR1, nuclear receptor corepressor 1; GAS, gastrocnemius; Ctrl, control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HS, hindlimb suspended; ns, non-significant.

metabolism. The expression levels of genes associated with the oxidative capacity of muscle cells in mitochondria were measured. The mitochondrial DNA marker, cyclooxygenase 2, normalized by genomic DNA marker, uncoupling protein 2, was significantly higher in BZ-incubated C2C12, indicative of increased mitochondrial content (Fig. 3D). NCoR1 targeting genes citrate synthase, long-chain acyl-CoA dehydrogenase, vascular endothelial growth factor isoform a-121 and -b were analyzed by RT-qPCR (Fig. 3E). These genes are involved in the regulation of mitochondrial function. Their mRNA expression levels in cells incubated with BZ containing rat serum were significantly increased in comparison with that of rat Ctrl serum-incubated cells. These results suggest that the aerobic capacity of myocytes was promoted by downregulation of NCoR1 following BZ treatment.

Discussion

In the present study, it was demonstrated that BZ could protect from simulated weightlessness-induced muscle atrophy. The muscle mass and isometric twitch force was observed to be ameliorated. Using the drugCIPHER-CS method, the potential targets of the compounds in BZ were predicted, and the interaction between these targets and potential pathways were analyzed. The results indicated that

calcium signaling pathways, TCA cycle and lipid metabolism process serve an important role in the network. NCoR1 is one of the important targets that is involved in muscle differentiation and metabolism (47). The experiments of the present study further verified their associations with muscle remodeling.

BZ was originally prescribed by Li Dongyuan in his book *Treatise on the Spleen and Stomach* (18). BZ is believed in TCM to ‘tonify qi and replenish the spleen’, leading to an improvement to muscle health (18).

In the present study, BZ was observed to regulate calcium signaling, TCA cycle, lipid metabolism process and NCoR1 protein expression by drugCIPHER-CS. Calcium signaling pathway and TCA cycle were identified to be involved in muscle metabolism and remodeling. Slow twitch oxidative muscle fibers maintain relatively high levels of Ca²⁺ concentrations, while fast twitch fibers require high-amplitude Ca²⁺ transients and lower ambient Ca²⁺ levels. The coupling of ATP production through TCA cycle, which skeletal muscle function is largely reliant on, to the requirement of a contracting muscle is ensured by Ca²⁺ on aerobic metabolism. In addition, Calcineurin has been demonstrated to be required for muscle regeneration and hypertrophy (48).

Muscle wasting caused by spaceflight or unloading, is associated with a decreased number of quiescent and proliferating

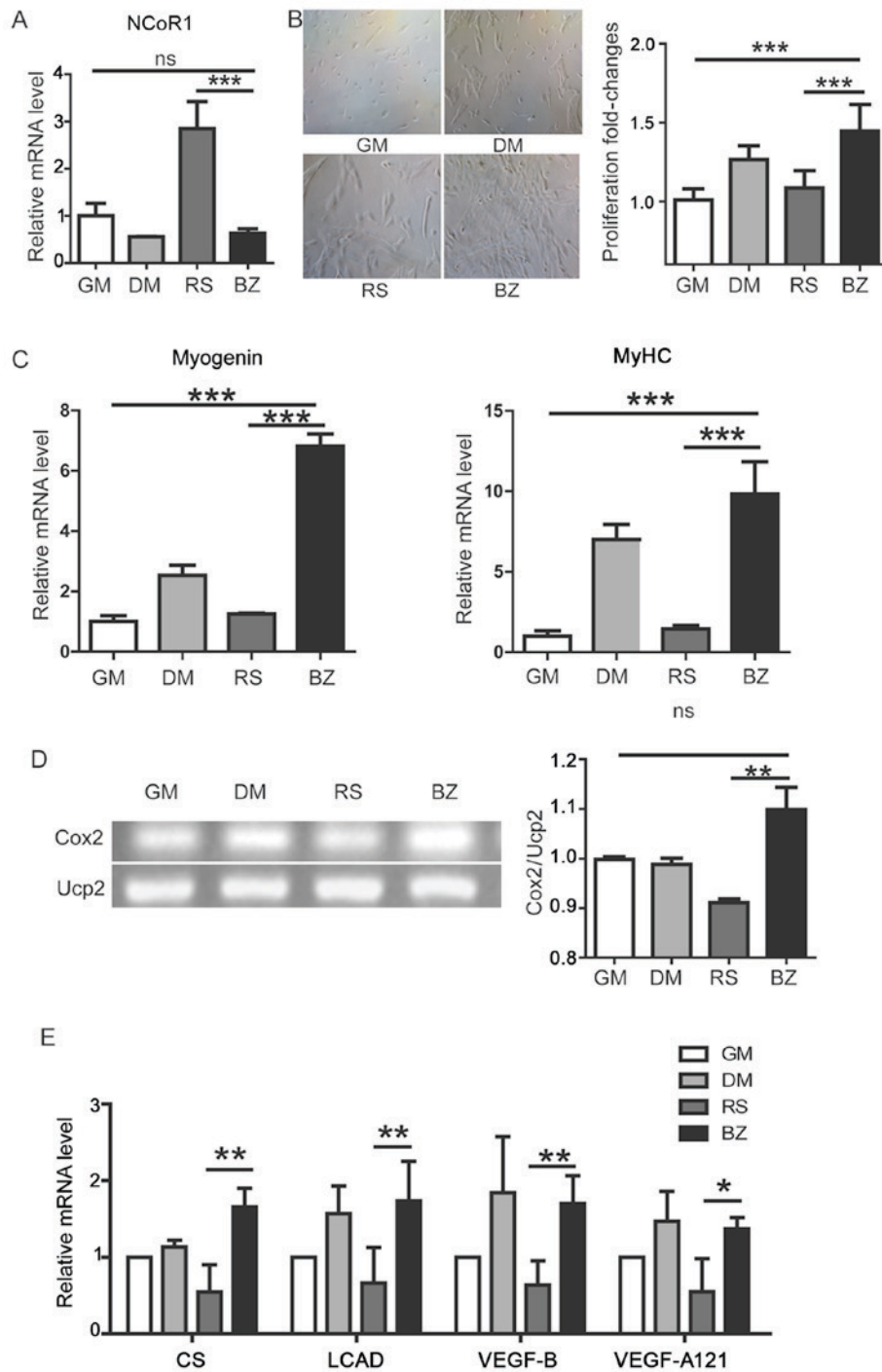


Figure 3. BZ could reduce NCoR1 level and promote myogenesis and oxidative function. (A) The expression of NCoR1 in C2C12 cells cultured in GM, DM, high glucose DMEM with 4% RS or high glucose DMEM with 4% rat BZ serum for 3 days, was detected by RT-qPCR. The values are normalized to Ctrl. Data are presented as the mean \pm standard deviation. * $P < 0.05$; *** $P < 0.005$; $n = 8$ for each sample. (B) Light microscopic images and MTS assay for the proliferation of C2C12 cells cultured in GM, DM, RS and BZ for 3 days. (Cellular experiments were repeated in triplicate). (C) Expression of myogenin and MyHC in C2C12 cells cultured in GM, DM, RS and BZ for 3 days, was detected by RT-qPCR. The values are normalized to the Ctrl. Data are presented as the mean \pm standard deviation. * $P < 0.05$; *** $P < 0.005$; GM/DM, $n = 3$; RS/BZ, $n = 4$. (D) Relative mitochondrial DNA content (Cox2) in C2C12 cells cultured in GM, DM, RS and BZ for 3 days was measured and normalized by genomic DNA content (Ucp2). $n = 3$. (E) The expression of target genes of NCoR1 in different groups of C2C12 cells cultured in GM, DM, RS and BZ for 3 days, were detected by RT-qPCR. The values are normalized to CtrlC2C12 cells. Data are presented as the mean \pm standard deviation. * $P < 0.05$; *** $P < 0.005$; GM/DM, $n = 3$; RS/BZ, $n = 4$. BZ, Bu Zhong Yi Qi decoction; NCoR1, nuclear receptor corepressor 1; GM, growth medium; DM, differentiation medium; DMEM, Dulbecco's modified Eagle's medium; RS, rat serum; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Ctrl, control; Cox2, cyclooxygenase 2; ns, non-significant.

satellite cells, which begin the repairing process by differentiating and fusing with each other. In addition, satellite cell transplantation appears to be a clinical application for multiple muscle atrophies (49,50).

The impairment of oxidative metabolism and signs of mitochondrial dysfunction serves a major role in weightless muscle atrophy (51). Previous studies indicated downregulation of metabolic enzymes and decreasing mitochondrial

oxidative capacity appeared in the later phase of atrophic muscles (52,53). In addition, it has been demonstrated that the activation of mitochondrial fission is sufficient to lead to muscle wasting (54,55).

It has been previously observed that NCoR1 is a 'master modulator' of the mitochondrial metabolism in muscle, which can reprogram muscle metabolism from a glycolytic to a more oxidative mitochondrial state, in addition, muscle-specific NCoR1 knockout mice exhibit increased muscle quantity and oxidative profile (47). NCoR1 docks histone deacetylases, such as HDAC3 (56), negatively correlated with the expression of MEF2, a transcription factor controlling muscle differentiation and development (57). Chinese herbs have been identified to be closely associated with the regulation of muscle physiology. Astragali radix has been identified to exhibit protective and therapeutic effects on tail-suspension induced SOL muscle atrophy. These effects are associated with the control of increased mATPase activity (58). A previous study indicated that ginsenoside Rb1 stimulated adiponectin signaling through upregulation of AdipoR1 and AdipoR2 proteins in C2C12 muscle cells, in addition, glucose transporter 4, a crucial membrane protein transporting glucose into myocytes, was promoted to translocate to the cell surface (59). Adiponectin signaling has been well established in the modulation of glucose levels and stimulation of fatty acid oxidation in myocytes (60). In addition, mice fed with Angelica exhibited prolonged physical exercise duration and higher blood glucose and muscle glycogen level, indicating that Angelica (61) improved energy utilization and exercise performance in mice. Angelica polysaccharides additionally promoted skeletal muscle satellite cell proliferation and stem cell factor receptor, c-kit, expression (62). These herbs are also included in the formula of BZ.

The therapeutic application of BZ provides a novel means to counteract unloading-induced skeletal muscle atrophy. In the present study, through *in vitro* and *in vivo* experiments, it was established that BZ could protect from weightlessness-induced muscle atrophy and promote the proliferation and differentiation of myoblast cells. Its effect on muscle remodeling is by activating signaling pathways to reprogram gene expression to sustain muscle performance. This new understanding of the molecular mechanism of BZ makes the design of therapeutic compounds with improved pharmacological properties for the treatment of weightlessness-induced muscle atrophy possible.

Acknowledgements

The authors would like to thank Professor Jingping Song, from the China Astronaut Research and Training Center, for the help with cell culture. In addition, they would like to thank Mr. Guanghan Kan and Ms. Hongqing Song, from the China Astronaut Research and Training Center, for the help with animal experimental preparation. The present study was financially supported by the National Natural Scientific Foundation of China (project nos. 31170811, 31271225, 31325012, 30500663 and 11475035) and 1226 Major Project (project no. AWS16J018).

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