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Bazi Bushen mitigates age-related muscular atrophy by alleviating cellular senescence of skeletal muscle

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ABSTRACT

Background and aim: Muscular atrophy is one of the most common age-related conditions characterized by the deterioration of skeletal muscle structures and impaired functions. It is associated with cellular senescence and chronic inflammation, which impair the function of muscle stem cells. Bazi Bushen (BZBS) is a patent compound Chinese medicine that has been shown to have anti-aging effects in various animal models. In this study, we investigated the effects and mechanisms of BZBS on muscular atrophy in naturally aged mice.

Experimental procedure: A muscular atrophy model of naturally aged mice (18 months) was employed with administration of BZBS (2 g/kg/d, 1 g/kg/d) and nicotinamide mononucleotide (NMN, 200 mg/kg/d). After six months of drug administration, muscle weight loss, muscle function and muscle histopathology were measured to evaluate the therapeutic effect of BZBS. The expression of cellular senescence, inflammatory and satellite cell-related factors were used to assess the effects of BZBS in inhibiting cellular senescence, reducing inflammation and improving muscle atrophy.

Results and conclusion: Compared with age matched natural aging mice, we found that BZBS improved muscle strength, mass, and morphology by reducing senescent cells, inflammatory cytokines, and intermyofiber fibrosis in aged muscle tissues. We also found that BZBS prevented the reduction of Pax7 positive stem cells and stimulated the activation and differentiation into myocytes. Our results suggest that BZBS might be a promising intervention in senile muscular atrophy.

1. Introduction

Due to the decline in fertility rates and the increase in life expectancy, the global population is aging rapidly, especially in countries like China, Japan, and Germany.[1] Physiologically, aging is a natural and unavoidable process that affects various aspects of the body. One of the most significant effects is the loss of skeletal muscle mass and function, which is also known as muscular atrophy.[2] Muscular atrophy is a complex phenomenon characterized by reductions in the size, number, and performance of skeletal muscle cells, leading to decreased force production and a smaller cross-sectional area of muscle fibers.[3] Due to the lack of effective pharmacological treatments,[4–6] muscular atrophy remains an unmet clinical need and poses a serious public health challenge worldwide.

Although the exact mechanisms underlying muscular atrophy are still unclear, there are several risk factors for this condition, including reduced protein synthesis, declined hormonal levels, chronic

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List of abbreviations

NMN	nicotinamide mononucleotide
BZBS	Bazi Bushen
SCs	satellite cells
NF-κB	nuclear factor kappa-B
HE	Hematoxylin and eosin
Pax7	Paired-box seven
MyoD	Myogenic differentiation antigen
MyoG	myogenin
SASP	senescence-associated secretory phenotype
CD45	cluster of differentiation 45
CD11b	cluster of differentiation 11b
CD206	cluster of differentiation 206
IL-4	interleukin-4
TNF-α	tumor necrosis factor-α
IL-10	interleukin-10
IL-1β	interleukin-1β
IL-13	interleukin-13

inflammation, and aging.[7] Recently, the relationship between chronic inflammation and aging, as well as their influence on the process of muscle atrophy, has garnered growing attention.[8] Inflammaging is the term used to describe the chronic, low-grade inflammation that occurs in older individuals.[9] Senescent cells, a distinctive cell state accumulated throughout the aging process, have the ability to release pro-inflammatory substances such as cytokines, chemokines, and growth factors, collectively referred to as the senescence-associated secretory phenotype (SASP).[10] The SASP may contribute to inflammaging through various pathways, including the activation of NF-κB signaling in neighboring cells or the alteration of the immune system.[9] Conversely, inflammaging can promote cell senescence by inducing oxidative stress and DNA damage.[11] Therefore, inflammaging and cell senescence establish a vicious cycle that accelerates aging and increases the risk of age-related diseases.[12,13] Targeting cellular senescence could potentially serve as an effective therapeutic strategy for treating muscular atrophy. Animal studies have demonstrated that senolytics, which selectively eliminate senescent cells, and senomorphics, which modulate the SASP profile, can reduce inflammation, enhance muscle regeneration, and improve physical function.[14]

Bazi Bushen (BZBS), as a patent compound Chinese medicine, is an extract composed of eight plant seeds and several famous longevity herbs such as *Radix Ginseng* and *Herba Cistanches*. The formulation of BZBS is based on the essence, qi, and spirit theory derived from the Qiluo Doctrine.[15] Accumulating evidence from animal studies has revealed the therapeutic properties of BZBS in natural aging and age-related diseases. For instance, BZBS has been found to extend the health span of the naturally aging mice.[16] Additionally, BZBS has demonstrated physiological improvements in the cognitive function and synaptic plasticity, the bone micro-structure and composition, the muscle strength and endurance of aged mice, and the reproductive function and hormone levels in several aging mice models.[17–20] Mechanistically, BZBS exerts its therapeutic effects through various pathways, including maintaining telomere length and telomerase activity in cells, regulating the expression of longevity and aging-related proteins, and reducing senescent cells.[19,20]

In this study, we aim to thoroughly explore the therapeutic effects and underlying mechanisms of BZBS on the improvement of motor ability in naturally aging mice. We demonstrated BZBS mitigated muscular atrophy of naturally aged mice by inhibiting cellular senescence, reducing macrophage mediated inflammation, and potentially promoting skeletal muscle regeneration through the activation and differentiation of satellite cells (SCs). Thus, we hope to find a new strategy to treating senile muscular atrophy.

2. Materials and methods

2.1. Preparation of BZBS

The components and mass ratio amounts in application of BZBS were shown in Table 1[19] The production of BZBS accorded with the standard procedure of China FDA (No. B20020585). Ultra performance liquid chromatography/mass spectrometry (UPLC/MS) had been used for chemical analysis of BZBS in previous studies.[21]

2.2. Animal maintenance and drug administration

Sixty female BALB/c mice of specific pathogen-free grade, aged 18 months, were procured from Beijing Huafukang Biotech Co., Ltd., China (production license No.SCXK2019-0008). The mice were housed in laboratory conditions with a temperature of 25 ± 2 °C, relative humidity of 60%–70 %, and a 12-h dark-light cycle. They were provided with free access to food and water.

After acclimating for one week, the mice were randomly divided into four groups (15 mice per group), including the naturally aged (Aged), NMN, high-dose BZBS (BZ-high), and low-dose BZBS (BZ-low) groups. The aged group received only ordinary feed, others received drug-containing feed daily. The following is the dosage of the administration group：NMN group (200 mg/kg/d), BZ-high group (2 g/kg/d), and BZ-low group (1 g/kg/d). The dose of NMN (Lot: BT05N120A005, Bontac Bio-engineering Co. Ltd., China) and BZBS (Lot: SYB2112001, Yiling Pharmaceutical Co., Ltd., China) were used according to our previous study.[17] After six months of drug administration, BALB/c mice at the age of 23 months underwent biomolecular testing and pathological examination. The experimental animal ethics committee of Hebei Medical Research Institute of Integrated Traditional Chinese and Western Medicine (No. N2022-158) approved this experiment.

2.3. Grip test

The grip test reflects the burst force of the muscle and is an indicator to evaluate the muscle strength of the mice.[17] Eight mice per group were tested for grip. Each mouse was positioned on a detection net, in the same position as in the grip-measuring test (Chatillon, Largo, FL). Once their limbs securely grasped the net, their tail were pulled and maintained parallel to the detection net. A digital force gauge measured the grip strength and displayed the maximum value. This procedure was repeated thrice for each animal examined, and the resulting average value was subjected to statistical analysis.

Table 1
The components and ratio amounts in application of BZBS.

Latin name	Chinese name	Amount in application (Ratio)
Cuscuta	菟丝子Tù sī zǐ	250
Fructus lycii	枸杞子Gǒu qǐ zǐ	138
Fructus schisandrae	五味子Wǔ wèi zǐ	46
Fructus cnidii	蛇床子Shé chuáng zǐ	35
Fructus Rosae	金樱子Jīn yīng zǐ	35
Raspberry	覆盆子Fù pén zǐ	35
Semen Allii Tuberosi	韭菜子Jiǔ cài zǐ	35
Morinda officinalis	巴戟天Bā jǐ tiān	35
Herba cistanches	肉苁蓉Ròu cóng róng	35
Medicinal cyathula root	川牛膝Chuān niú xī	35
Herba epimedii	淫羊藿Yín yáng huò	70
Rehmannia glutinosa	生地黄Shēng dì huáng	46
Hippocampus Kelloggi	海马Hǎi mǎ	21
Pilose antler	鹿茸Lù róng	16
Ginseng	人参Rén shēn	25
Toosendan fructus	川楝子Chuān liàn zǐ	23

2.4. Rotarod test

Muscle endurance is one of the evaluation indicators of muscle function. The purpose of the rotarod test is to measure muscle endurance of mice.[16] Eight mice per group were tested for Rotarod. Each mouse was positioned on the rods using a rotarod test (IITC Inc. Life Sciences, Woodland Hills, CA) with following specific parameters: Run limit 200, Start rpm 5, Top rpm 23, Ramp time 30. Subsequently, each mouse underwent three rotarod trials, and the duration of time that the animals remained on the rods was recorded. The total time and distance were determined by calculating the mean value.

2.5. Mouse frailty assessment

The frailty index (FI) is defined as among all possible health losses in the individual, which represents the overall health status of the mice.[22] FI was determined by measuring multiple aging indicators, including hair loss severity, visual acuity, hearing, whisker, kyphosis of the spine, and abdominal distension, etc.[23,24] Mice were scored according to the severity grade of each indicator (0 = normal, 0.5 = light, 1 = severe) to create a quantitative frailty assessment.

2.6. Gastrocnemius-sample collection

At the end of the experiment, the gastrocnemius tissues on right hind legs were harvested, weighed, and photographed, then stored at −80 °C. The left gastrocnemius muscles were immersed in a 10 % solution of neutral buffered formalin for a duration of 24 h at ambient temperature. Subsequently, the muscles were embedded in paraffin and sliced into sections with a thickness of 4 μm.

2.7. Hematoxylin and eosin (HE) and masson staining

HE and masson are commonly used staining methods. In this study, the morphology of the muscle fibers was observed by HE staining, and the cross-sectional area of the muscle fibers was calculated to evaluate the muscular atrophy. In addition, intermuscular fiber deposition is one of the pathological manifestations of muscle atrophy. The collagen fibers between muscles were stained with masson to further reflect the degree of muscle atrophy. Collected gastrocnemius samples were processed as previously described. The gastrocnemius specimens underwent dewaxing using varying concentrations of ethanol and xylene, followed by staining with HE and masson. Subsequently, the sections were affixed with neutral resin. The resulting stained sections were examined for conventional morphology and intermyoidal fiber deposition using a fully automated inverted microscope (Hamamatsu, Japan).

2.8. Senescence-associated β-galactosidase (SA-β-gal) staining

Senescence-associated β -galactosidase activity level was upregulated during cellular senescence, and the purpose of SA-β -gal staining is to observe cellular senescence.[25] Gastrocnemius tissues were embedded in Sakura Tissue-Tek OCT Compound (Sakura, USA, 4583) and sectioned into slices of 8 μm thickness. Then the frozen sections were fixed and washed at room temperature and then were incubated at 37 °C overnight under light-proof conditions with appropriate amounts of preconfigured staining working solution (Solarbio, China, G1580). Slides were sealed by dehydration and observed under a light microscope (DMi8 Thunder, Leica, Germany).

2.9. Immunofluorescence

Anti-Pax-7 (Pax7) antibody is used to dye the satellite cells in the muscle. Anti-laminin α-2 (laminin α2) AF488 antibody Labels of laminin. 4′,6-diamidino-2-phenylindole (DAPI)was labeled the cell nuclei. Frozen sections (8 μm) of muscle tissue were incubated with 3 % hydrogen peroxide solution at 37 °C in the dark for 10 min. After that, the sections were washed with PBS and incubated with 5 % BSA at room temperature for 30 min. The primary antibodies used in the study were mouse anti-Pax-7 (Pax7) antibody (1:50 dilution, Santa Cruz, USA, sc-81648), and rat anti-laminin α-2 (laminin α) AF488 antibody (1:50 dilution, Santa Cruz, USA, sc-59854). Following incubation with the primary antibody overnight at 4 °C, sections were washed with PBS and incubated with fluorescein isothiocyanate-labeled goat anti-mouse antibody (1:500 dilution, Abcam, UK, ab150113) and fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:500 dilution, Abcam, UK, ab150081) for 2h at room temperature in the dark. The sections were rinsed and stained with DAPI, and mounted with glycerol. Sections were observed using a fluorescence microscope and the images were taken on fixed scaling (DMi8 Thunder, Leica, Germany).

Anti-CD80 antibody and anti-CD206 antibody were used to label M1 and M2 macrophages, respectively. After conventional dewaxing to water, the gastrocnemius sections underwent microwave heat repair with EDTA sodium citrate antigen repair solution. Then the sections were incubated with 3 % hydrogen peroxide solution at 37 °C in the dark for 10 min. After that, the sections were washed with PBS and incubated with 5 % BSA at room temperature for 30 min. The primary antibodies used in the study were rabbit anti-CD80 antibody (1:500 dilution, Abcam, UK, ab254579), and rabbit anti-CD206 antibody (1:500 dilution, Abcam, UK, ab64693). Following incubation with the primary antibody overnight at 4 °C, sections were washed with PBS and incubated with fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:500 dilution, Abcam, UK, ab150081) for 2h at room temperature in the dark. The sections were rinsed and stained with DAPI, and mounted with glycerol. Sections were observed using a fluorescence microscope and the images were taken on fixed scaling (DMi8 Thunder, Leica, Germany).

2.10. Immunohistochemistry

After dewaxing to water and antigen retrieval with sodium citrate antigen retrieval solution (1:50 dilution, Solarbio, China, C1032), gastrocnemius sections were blocked using an SP kit (ZSGB, Beijing, China). Then the gastrocnemius were incubated overnight at 4 °C with P16INK4A (1:200 dilution, Santa Cruz, USA, sc-1661) primary antibodies. HRP-labeled sheep anti-mouse IgG was employed as a secondary antibody and subjected to incubation with the samples. Following a 5-min visualization with diaminobenzidine (DAB), the slices were subsequently restained with hematoxylin. All images were captured utilizing a fully automated inverted microscope. Quantitative analysis was conducted employing the Image J software (Bethesda, USA).

2.11. Flow cytometry

To detect the proportion of M2 macrophages in muscle tissue, we used the flow cytometry for detection. The single cell suspension of muscle tissues were prepared by enzyme treatment. Skeletal muscles were collected, minced and then gently digested with 0.5 % II collagenase (Worthington, USA, LS004176) at 37 °C shaker for 30 min. Then 10 % FBS was added to terminate digestion. After that, debris removal solution (Miltenyi Biotec, Germany, no.130-109-398) was used to remove cell debris. Then the total cells isolated from muscle homogenate were resuspended in fluorescence-activated cell sorting buffer (phosphate buffer solution, 0.5 % bovine serum albumin, 2 mM EDTA) to obtain a single-cell suspension.

The single cell suspension incubated with the mouse flow cytometry specific antibodies anti-CD45-AF700 (Biolegend, USA, 147715), anti-CD11b-PerCP/Cy5.5 (Biolegend, USA, 101227), anti-F4/80-PE (Biolegend, USA, 123110) for 20 min for staining in dark. Then, cells were fixed with fixed membrane breaking reagent (Biolegend, USA, 554714) and incubated at 4 °C for 20min. CD206 antibody (anti-CD206-APC, Biolegend, USA, 141707) was added to the treated cells and incubated

for 20 min in dark. Then, cells were collected and resuspended with a staining buffer, and the proportions of CD45+CD11b + F4/80+CD206+ macrophage cells were determined using flow cytometry by Attune NXT Cell Analyzer (Attune NXT, Invitrogen, Singapore).

2.12. RNA isolation and quantitative real-time PCR

Total RNA was isolated utilizing the RNA Kit (TransGen Biotech, China, ER501-01) and subjected to reverse transcription using the reverse transcription system (Promega, USA, A5001). The resulting cDNA products were employed as templates for quantitative polymerase chain reaction (Monad, China, MQ00401S). The primer sequences utilized are designed by Shenggong Bioengineering (Shanghai) Co., LTD. All experimental procedures adhered to the guidelines specified by the manufacturer. The primer sequences are provided in Appendix Table 1.

2.13. Statistical analysis

Statistical analysis was conducted using SPSS 22.0 software (IBM, USA). Prior to selecting an appropriate statistical test, the data underwent assessment for normal distribution and homogeneity of variances using the Shapiro-Wilk test and Levene's test for equality of variances. Comparisons between groups were performed with one way analysis of variance and post-hoc Bonferroni test. The data are presented as mean ± standard deviation (SD). Statistical significance was defined as $P < 0.05$. Graphs were prepared using GraphPad Prism 6.0 (CA, USA).

3. Results

3.1. Bazi Bushen improved muscular performance in naturally aged mice

As it was shown in Fig. 1A, the aged group exhibited severe fur depilation, an indicative signs of aging. Besides, the FI is a common feature observed in both elderly individuals and aged mice. As it was shown in Fig. 1B, the aged group exhibited higher FI Score, an indicative signs of aging, while the BZBS and NMN treated mice had significantly lower FI score ($P < 0.05$). To confirm the effect of BZBS on older BALB/c mice, we conducted grip and rotarod tests on each group. The aged group exhibited the lowest levels of strength, endurance, and motor coordination (Fig. 1C–E, $P < 0.01$). Conversely, the BZ-low group demonstrated significantly higher strength, while the BZ-high group exhibited greater muscular performance (Fig. 1C–E, $P < 0.01$). Importantly, the BZ-high group demonstrated similar improvements in appearance and behavior tests to that of the NMN group (Fig. 1A–E), indicating a comparable efficacy between BZ-high and NMN in anti-aging potency.

3.2. Bazi Bushen improved muscle atrophy in naturally aged mice through restoring the activities of satellite cells

At the end of drug administration, the degree of muscle atrophy was determined by weighing. As shown in Fig. 2A and B, the volume and weight of the gastrocnemius muscle were higher in the BZ-high group compared to the aged group ($P < 0.01$). HE and masson staining was conducted to assess the histopathological changes in each group. We observed considerable deterioration in the structure of the gastrocnemius tissues in the aged group. This includes the presence of disordered and broken muscle fibers, a decrease in the cross-sectional area of myofibers, and an increase in intermyoidal fiber deposition (Fig. 2C–F). However, these histopathological changes were mitigated by both BZBS treatment and NMN treatment (Fig. 2C–F, $P < 0.01$). Therefore, these findings suggest that BZBS has the potential to be a promising drug for improving muscle atrophy.

Paired-box seven (Pax7) represents the number and myogenic function of satellite cells, which can serve as a marker for activated satellite cells initiating the myogenic program.[26] Immunofluorescence and real-time PCR analysis indicated a significant elevation in Pax7 expression in the BZ-high group compared to the aged group (Fig. 3A and B, $P < 0.01$). Additionally, myogenic differentiation antigen (MyoD) and myogenin (MyoG) are important regulators of the myogenic program. MyoD can promote the proliferation and differentiation of SCs.[27,28] While MyoG enhances the myogenic differentiation of SCs to form new muscle fibers.[29] Real-time PCR results indicated that, similar to Pax7, the expressions of MyoD and MyoG were increased in BZ-high group compared to the aged group (Fig. 3C–D, $P < 0.01$). Notably, the expression of MyoD in the NMN group was lower than that of BZ-high

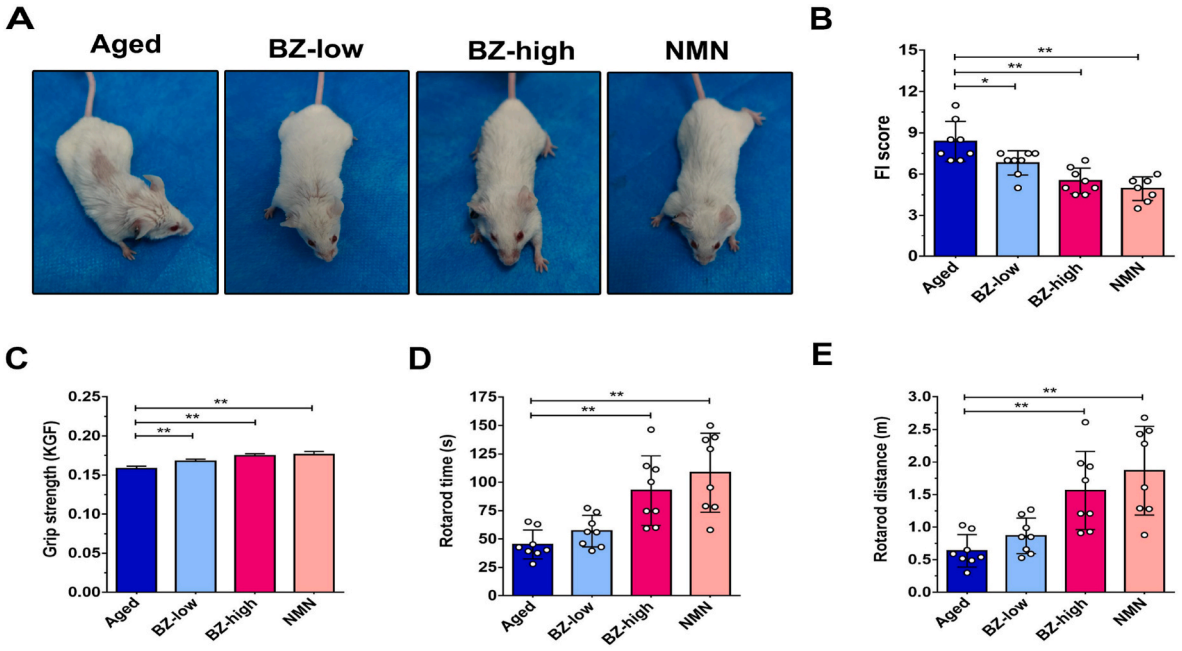

Fig. 1. BZBS improves muscular performance of naturally aged mice. (A) The representative images of mice in each group. (B) The FI score in each group (n = 8). (C–E) The results of grip and rotarod test (n = 8). **P < 0.01. Data are shown as means ± SD.

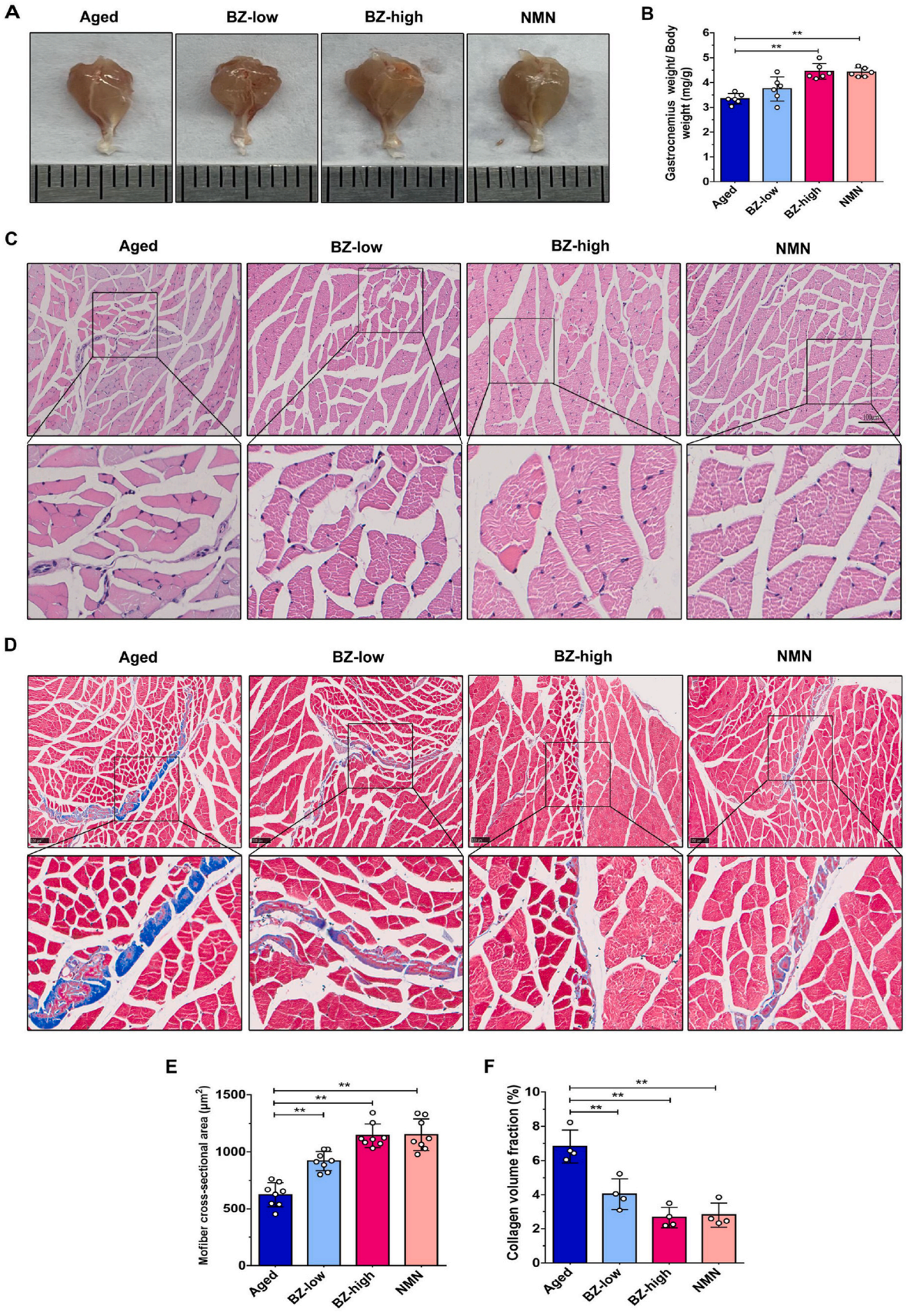

Fig. 2. BZBS ameliorates muscle atrophy in naturally aged mice. (A, B) Representative images and the ratio of total gastrocnemius weight/body weight (n = 6). (C) HE staining of myofibers 7 (200 × , n = 4). (D) Masson staining of muscles (200 × , n = 4). (E, F) Statistical analysis of myofiber cross-sectional area (n = 4, two random fields of view per slice) and collagen volume fraction (n = 4). **P < 0.01. Data are shown as means ± SD.

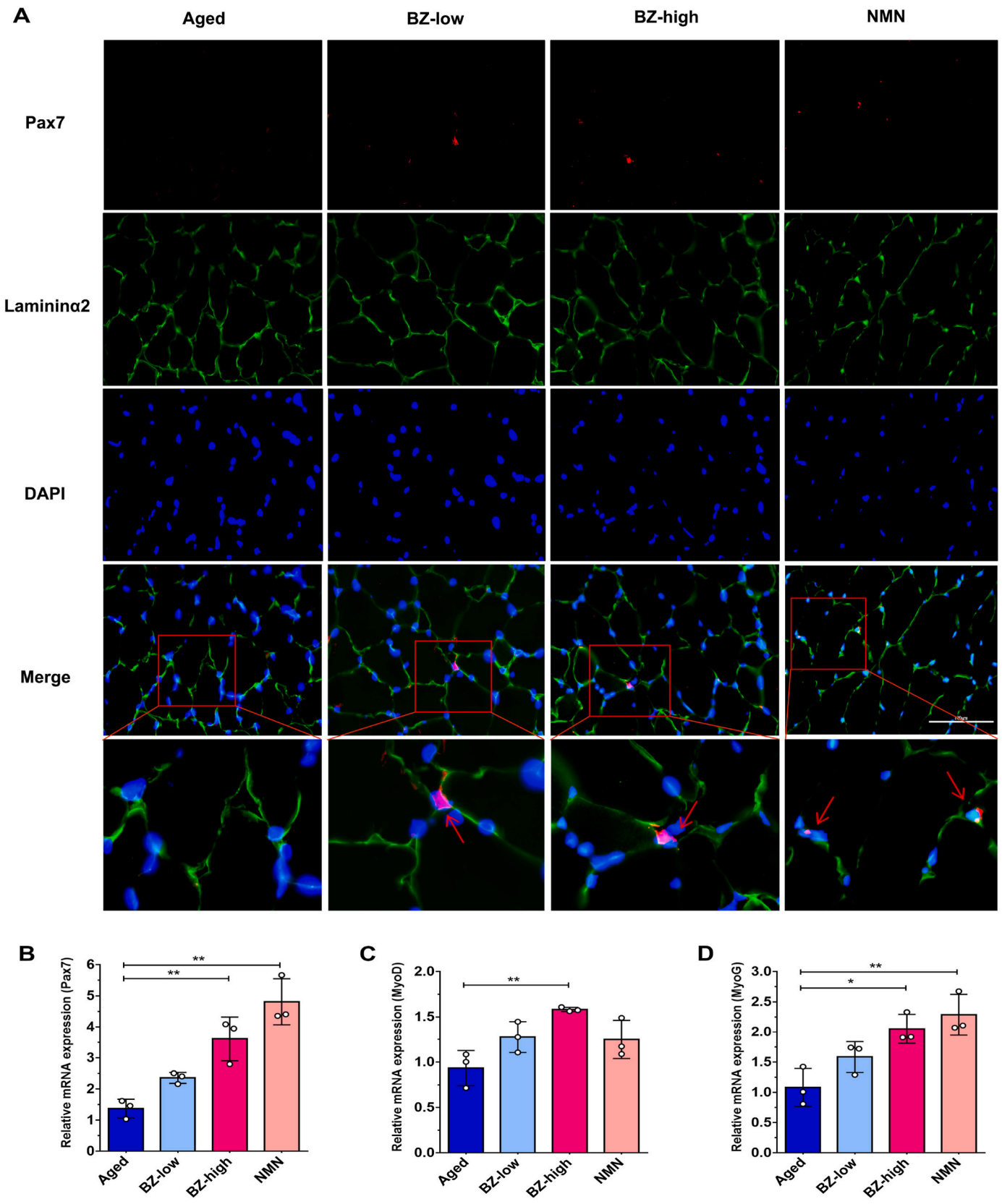

Fig. 3. BZBS restores the activities of satellite cells of SCs in naturally aged mice. (A) Immunofluorescent staining of Pax7 in mice gastrocnemius muscles (400 × , n = 4). (B–D) The mRNA expression of Pax7, MyoD, and MyoG in mice gastrocnemius muscles (n = 3). *P < 0.05, **P < 0.01. Data are shown as means ± SD.

group and showed no statistical differences compare to the aged group (Fig. 3C). These results demonstrate the promising potential of BZBS in attenuating age-related muscular atrophy by restoring the normal activities of satellite cells.

3.3. Bazi Bushen alleviated cellular senescence of skeletal muscle in naturally aged mice

Cells undergo senescence in response to the accumulating cellular stress during the aging process. Senescence associated β-galactosidase activity upregulates during cell senescence.[25] SA-β-gal staining showed that the positive area in the aged group was larger than that in the BZ-low, BZ-high and NMN-treated groups (Fig. 4A). Besides, the activation of p53/p21 and p16 signaling pathways initiates cellular senescence, leading to the irreversible cessation of cell proliferation and the release of proinflammatory cytokines.[30] Immunohistochemistry demonstrated significantly lower protein expressions of p16 in the BZBS treated groups compared to the aged group (Fig. 4B–C, $P < 0.01$ or 0.05), and real-time PCR revealed comparable findings on the mRNA level (Fig. 4D, $P < 0.01$). SA-β-gal staining and nuclear expression of p16 illustrate the senescence of skeletal muscle cells. In addition, p53 and p21 function as cellular stress sensors and serve as the principal regulators of the p53/p21 senescence signaling pathway.[31] Real-time PCR analysis of p53 and p21 gene expression provided additional confirmation of cell senescence in the aged skeletal muscles (Fig. 4E and F). Upon BZBS treatment, cellular senescence was largely suppressed (Fig. 4C–F, $P < 0.01$ or 0.05). Notably, there were slight differences between NMN and BZ-high groups, with higher suppression rate of p16 and p53 and lower suppression rate of p21 in the NMN treated group (Fig. 4A–F). Collectively, our observations confirmed the occurrence of cellular senescence in the muscle tissues and revealed the potential serotherapeutic effects of BZBS in muscular aging.

3.4. Bazi Bushen inhibited inflammatory in skeletal muscle of naturally aged mice

Senescent cells release a secretory phenotype known as SASP. SASP includes pro-inflammatory cytokines, such as TNF-α and IL-1β, which exacerbates inflammation.[10] This inflammation affects the stem cell niche and the proliferation and differentiation of satellite cells, ultimately leading to muscle atrophy.[32,33] Real-time PCR analysis indicated that BZBS and NMN significantly decreased the gene expressions of SASP (TNF-α, IL-1β) and NF-kB (an activation of the SASP regulator) in the muscle tissues compared with the aged group (Fig. 5A–C, $P < 0.01$ or 0.05). Besides, the anti-inflammatory factor IL-4 increased in the BZBS and NMN group (Fig. 5D, $P < 0.01$), futher demonstrating the anti-inflammatory effect of BZBS and NMN. Macrophages are polarized like the M1 type as aging, which leading to an imbalance of M1 and M2 macrophages.[34] The imbalance leads to a pro-inflammatory state which also impairs the activation of satellite cells.[35] After drug administration, we found a decrease in M1 macrophages and an increase in M2 macrophages (Fig. 6A–F, $P < 0.01$). Considering the pro-inflammatory effects of M1 macrophages and the anti-inflammatory effects of M2 macrophages, we speculated that the anti-inflammatory effect of BZBS and NMN might be involved.

4. Discussion

Previous research has demonstrated that BZBS can enhance grip strength and motor ability in 13-month-old C57BL/6 mice.[16] However, it is important to note that 13-month-old mice are still considered to be in the early stages of the aging process. Consequently, there is a dearth of

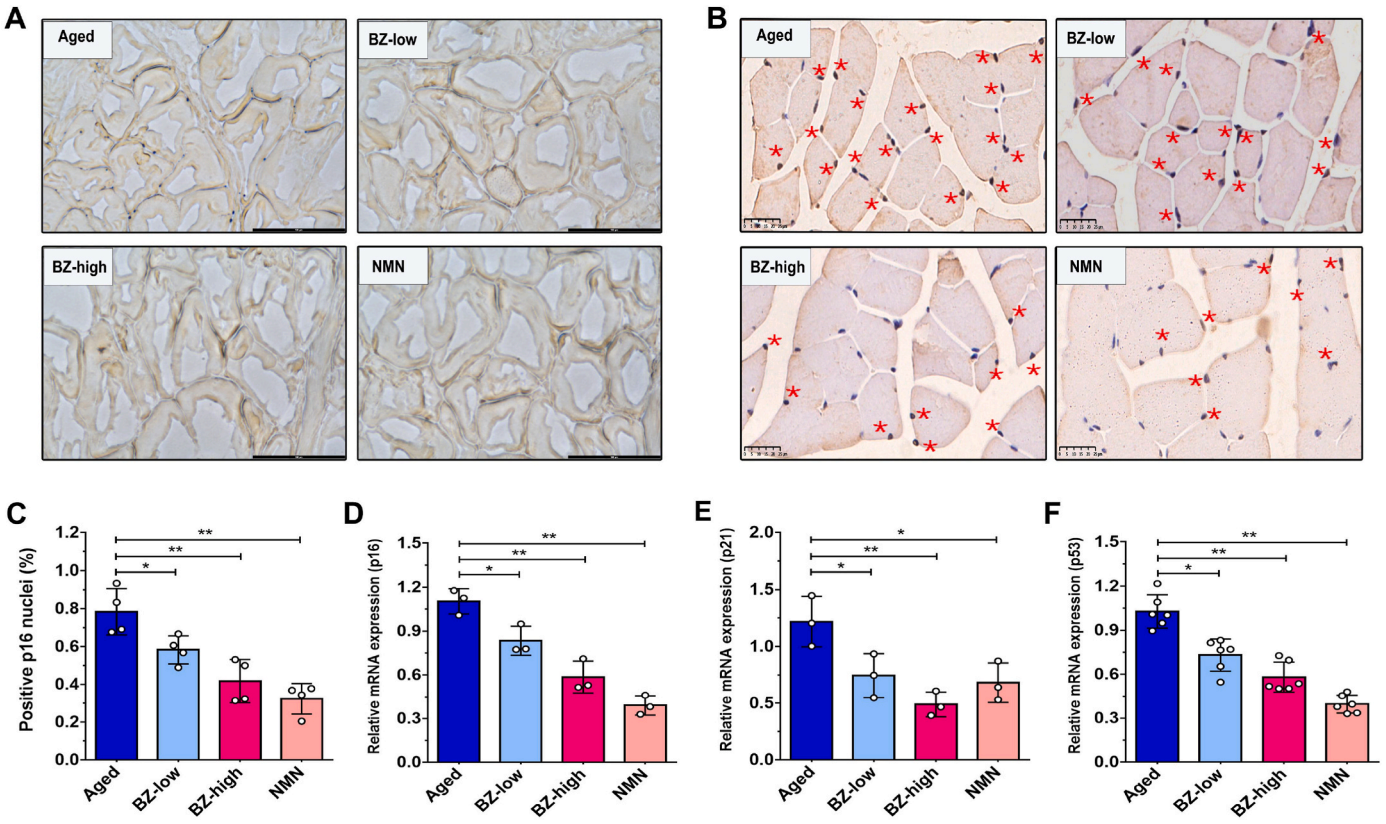

Fig. 4. BZBS alleviates cellular senescence in skeletal muscle of naturally aged mice. (A) SA-β-Gal staining images (n = 3). (B, C) Immunohistochemistry of P16 (400 ×, marked in red stars) and percentage of P16 positive cell in each group (n = 4). (D–F) The mRNA expression of p16, p53, p21 in mice gastrocnemius muscles (n = 3). *P < 0.05, **P < 0.01. Data are shown as means ± SD.

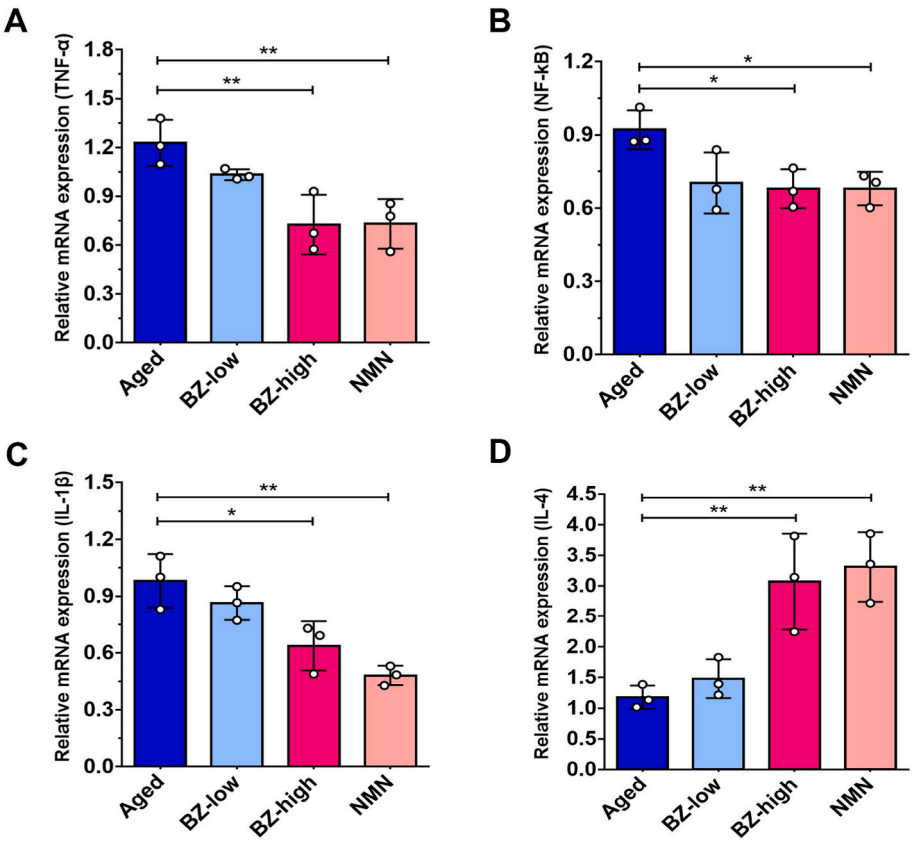

Fig. 5. BZBS inhibits inflammation of skeletal muscle in naturally aged mice. (A–D) The mRNA expression of TNF-α, NF-kB, IL-1β and IL-4 in mice gastrocnemius muscles (n = 3). *P < 0.05, **P < 0.01. Data are shown as means ± SD.

supportive evidence regarding the effectiveness of BZBS in improving motor system function during the later stages of aging in various mouse strains. Moreover, comprehensive mechanistic research on this topic is lacking. Therefore, the purpose of this study was to investigate the impact and potential mechanisms of long-term BZBS administration in safeguarding skeletal muscle in aged mice using naturally aging 18-month-old BALB/c animals. Our findings indicate that treatment with BZBS significantly ameliorated muscle atrophy. We also found that BZBS reduced the accumulation of senescent cells and the secretion of SASP in skeletal tissues with aging. Furthermore, we analyzed the macrophage polarization in aged muscle tissues and found that BZBS reversed the increase in M1 macrophage proportion and the decrease in M2 macrophage proportion, indicating its anti-inflammatory and pro-proliferative effects. By fluorescence staining and real-time PCR, we verified that BZBS stimulated the activation and differentiation of skeletal muscle satellite cells.

Muscle strength and muscle mass start to decline in humans after the age of 40.[36] According to the age conversion between mice and humans, mice that are 18 months old are equivalent to humans that are 67 years old.[37] This is the age when muscle strength and mass have already decreased significantly. The underlying pathology of muscular atrophy involves the atrophy of muscle cells and the increased deposition of intermyofibers.[38] Muscle cell atrophy leads to a reduction in muscle mass, while intermyofiber fibrosis increases skeletal muscle stiffness, thereby limiting its extension and contraction. These changes in muscle cells and intermyofibers result in muscle dysfunction. In our study, we observed the deterioration of muscle strength, muscle mass, myofiber cross-sectional area, and intermyofiber deposition in aged mice, which were consistent with human muscular atrophy. Moreover, BZBS treatment significantly improved these symptoms (Figs. 1 and 2). Considering that the main reason for the decline of skeletal muscle repair ability with age may be due to the reduced proliferation and differentiation capacity of stem cells in the tissue,[39,40] we explored whether BZBS protects against age-related muscular atrophy through muscle regeneration with NMN as a positive control, an anti-aging agent against age-related muscle atrophy and weakness.[41]

Generally, muscle stem cells that are essential for skeletal muscle repair and regeneration. They are in the satellite cell niche, which is a specialized microenvironment that regulates their quiescence, activation, proliferation, and differentiation. In aged muscles, the satellite cell niche is disrupted by various factors, among them, inflammation plays a pivotal role in inhibiting the proliferation and differentiation of stem cells.[32,33] Inflammation can induce cellular senescence, which can reduce the number and quality of muscle stem cells.[11] Senescent cells can also secrete SASP, which can further aggravate inflammation and muscle damage.[10] Moreover, inflammatory niche microenvironment can alter the macrophage polarization in the muscle tissue,[34] which can affect the interaction between macrophages and Pax7 positive stem cells.[42–44] Thus, cellular senescence and inflammation influence each other,[45,46] together create a vicious cycle and synergistically exacerbate muscle damage. When myocytes atrophy, M1 macrophages remove the muscle debris and secrete large number of cytokines to induce activation and proliferation of stem cells. After the activation of the stem cells, the M1 macrophages (pro-inflammatory) switch to the M2 macrophages (anti-inflammatory).[47] M2 macrophages produce anti-inflammatory cytokines to repress the local inflammatory response,[48,49] and promoting the differentiation of stem cells and muscle regeneration.[43,50] As aging, macrophages are polarized like the M1 type.[34] In naturally aged mouse muscles, we found the accumulation of senescent cells, the increased gene expression of SASP, and the polarization of M1 macrophages. We found that BZBS had similar effects to NMN in inhibiting the levels of cellular senescence (SA-β-gal, p16, p53, p21) and reducing the SASP releasing (IL-1β, TNF-α) , and regulating the polarization of macrophages (Figs. 4, Fig. 5, Fig. 6). Furthermore, the inflammation

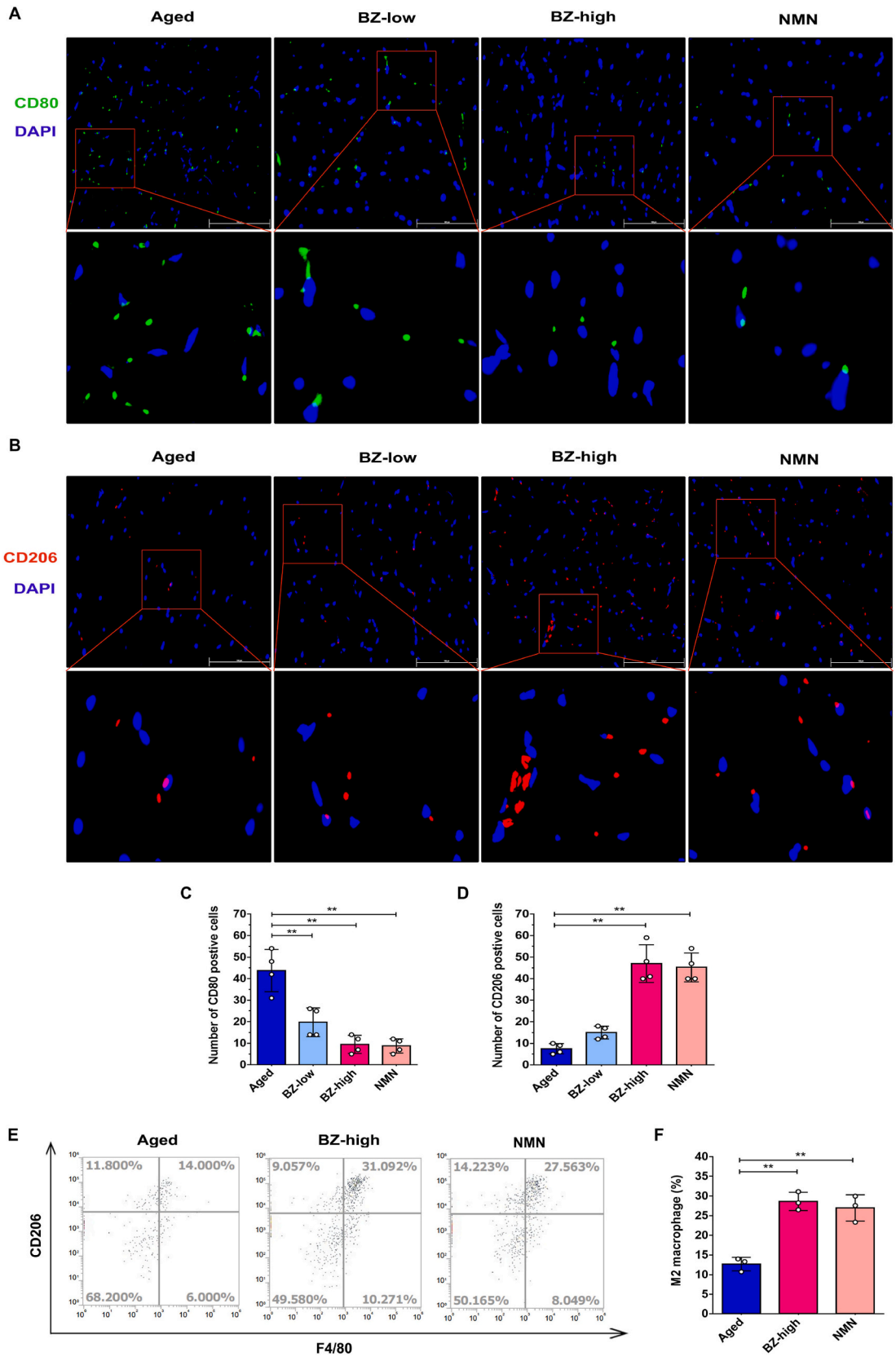

Fig. 6. BZBS regulates macrophages polarization in skeletal muscle of naturally aged mice. (A, B) Immunofluorescent staining of M1 macrophage ($CD80^+$) and M2 macrophage (CD206+) in mice gastrocnemius muscles (400 × , n = 4). (C, D) Statistical analysis of $CD80^+$ and CD206+ macrophage proportions (n = 4). (E, F) Flow cytometry analysis of M2 macrophages in mice gastrocnemius muscles (n = 3). **P < 0.01. Data are shown as means ± SD.

caused by aging leads to abnormally increased fibrosis. The abnormal fibrosis limits the extension and contraction of skeletal muscle, increases the stiffness degree of skeletal muscle, and reduces the muscle strength and exercise ability.[51] In naturally aged mouse muscles, we found the accumulation of deposition of intermyofibers, and BZBS decreased the deposition of intermyofibers (Fig. 2D and F). All these results suggested that BZBS might have a great potential in promoting the activation and differentiation of stem cells to delay muscle atrophy and restore muscle strength by inhibiting inflammatory response.

When skeletal muscle atrophy, SCs will activate and give rise to cohorts of committed SCs and thus progenitors. Myogenic progenitors proliferate, and eventually differentiate through fusion with each other

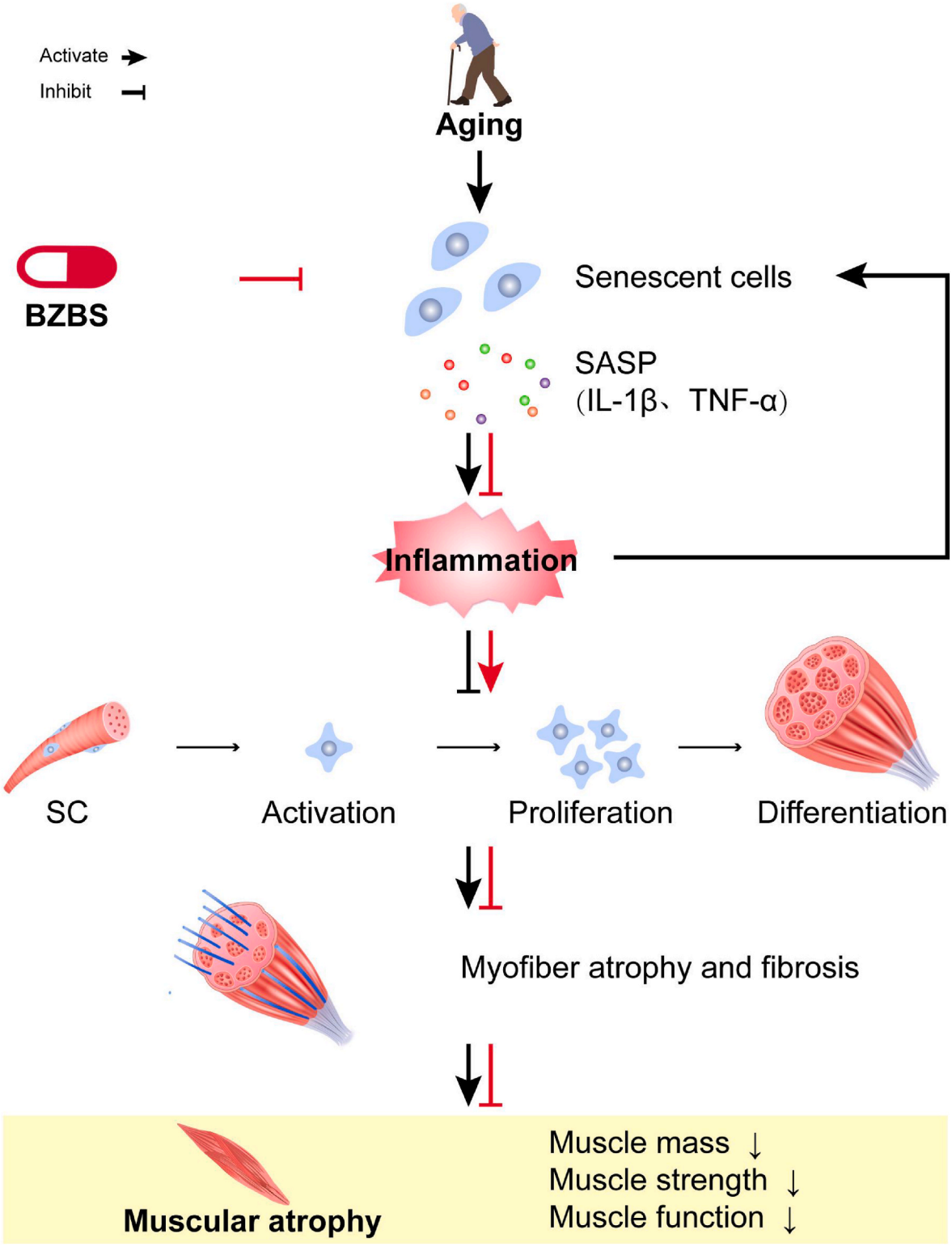

Fig. 7. Summary figure illustrating the hypothesis of BZBS ameliorating age-related motor system dysfunction. BZBS mitigates muscular atrophy by targeting cellular senescence, inflammation, and muscle stem cells.

to reconstitute fiber integrity and function.[52] Pax7 is the SCs marker, which represents the survival of SCs and the myogenic function of myogenic stem cells.[26] The activation of SCs enables skeletal muscle to initiate the myogenic program. MyoD is another major regulator of the skeletal myogenic program. Following the activation of SCs, MyoD is activated to promote the proliferation and differentiation of myogenic stem cells,[27,28] which means new muscle tubes will form. When muscle tubes fuse to form new muscle fibers, MyoG will increase and promote the formation of new muscle fibers by promoting the myogenic differentiation of SCs.[29] Briefly, in quiescent condition, SCs express Pax7 but rarely express MyoD. After activation, the SCs express Pax7, MyoD and begin further proliferation. Then at the late differentiation, MyoD expression decreases and MyoG promotes terminal differentiation.[53] In this study, BZBS increased the level of Pax7 (Fig. 3A and B), which indicated that BZBS promoted the activation of SCs, enabling skeletal muscle to initiate the myogenic program. Furthermore, BZBS improved the gene expression level of MyoD and MyoG (Fig. 3C and D), indicating BZBS could promote Pax7 positive cells to complete terminal differentiation and the formation of muscle fibers.

In summary, we demonstrated that BZBS treatment could effectively ameliorate the signs of muscular atrophy in aged mice. We found that BZBS reduced the accumulation of senescent cells and the secretion of SASP in skeletal tissues and modulated the macrophage polarization in aged muscle tissues. Moreover, we verified that BZBS stimulated the activation and differentiation of Pax7 positive stem cells, which are essential for muscle repair (Fig. 7). Our findings suggest that BZBS might be a promising intervention for preventing and treating muscular atrophy by inhibiting cellular senescence, targeting the inflammatory niche and muscle stem cells.

Author contributions

Kunxu Niu: Data curation, Conceptualization, Validation, Writing – original draft, Visualization. Liping Chang, Runtao Zhang, Yuning Jiang, Xiaogang Shen, Xuan Lu, Shixiong Zhang: Validation, Formal analysis. Kun Ma, Zhiqin Zhao: Formal analysis. Mengnan Li, Yunlong Hou and Yiling Wu: Methodology, Writing – review & editing, Supervision. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix

Table 1
Primer sequences used for real-time PCR reaction

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Pax7	GGAAAACCAGTGTGCCATCT	CCTTGTCTTTGGCACCATTT
MyoD	GACAGGGAGGAGGGGTAGAG	TGCTGTCTCAAAGGAGCAGA
MyoG	CCTACAGACGCCCACAATCT	CAGGGCTGTTTTCTGGACAT
P16	GCGAGGACCCCACTACCTTCTC	CACAAAGACCACCCAGCGGAAC
P21	ACAGGAGCAAAGTGTGCCGTTG	GCTCAGACACCAGAGTGCAAGAC
P53	ACCGCCGACCTATCCTTACCATC	ACTCCTCTGTAGCATGGGCATCC
IL-1β	TGCCACCTTTTGACAGTGATG	TGTGCTGCTGCGAGATTTGA
NF-κB	GGAGGCATGTTCGGTAGTGG	CCCTGCGTTGGATTTCGTG
IL-4	ATGGATGTGCCAAACGTCCT	GGCATCGAAAAGCCCGAAAG
TNF-α	GTGCCTATGTCTCAGCCTCT	TGGTTTGTGAGTGTGAGGGT
GAPDH	ACTGGCATGGCCTTCCG	CAGGCGGCACGTCAGATC

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