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•Original article•

Bazi Bushen alleviates reproductive aging in aged male mice

MA Kun[1Δ], LI Mengnan[2, 3Δ], SONG Yahui[1, 2], MAO Xinjing[4, 5], ZHANG Shaolan[1, 6], ZHANG Yaping[1, 6], JIANG Yuning[4, 5], JI Chuanyuan[4, 5], NIU Kunxu[3, 5], LI Hongrong[2, 5], HOU Yunlong[2, 5*], WEI Cong[1, 2*]

[1] *Graduate School, Hebei University of Chinese Medicine, Shijiazhuang 050091, China;*
[2] *State Key Laboratory for Innovation and Transformation of Luobing Theory, Shijiazhuang 050035, China;*
[3] *Key Laboratory of State Administration of TCM (Cardio-Cerebral Vessel Collateral Disease), Shijiazhuang 050035, China;*
[4] *College of Traditional Chinese Medicine·College of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing 210023, China;*
[5] *Hebei Provincial Key Laboratory of Luobing, Shijiazhuang 050035, China;*
[6] *Key Disciplines of State Administration of TCM for Luobing, Shijiazhuang 050035, China*

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[ABSTRACT] Bazi Bushen (BZBS), a traditional Chinese medicine (TCM), has demonstrated therapeutic efficacy in testicular dysfunction within D-galactose and $NaNO_2$ mouse models. This study aimed to ascertain if BZBS could also mitigate the decline in testicular function associated with natural aging. Therefore, male aged mice were employed to evaluate the preventive effects of BZBS on male reproductive aging. This was achieved by assessing sex hormone production, testicular histomorphology, and spermatogenesis. Relative to the untreated aged control group, BZBS administration elevated the levels of sex hormones and spermatocyte populations and preserved normal testicular structure in aged mice. Notably, spermatogenesis was maintained. Further analyses, including malondialdehyde (MDA) assays and real-time PCR, indicated that BZBS diminished testicular oxidative stress and the inflammatory burden. Corroborating these findings, mice treated with BZBS exhibited reductions in the populations of senescent and apoptotic cells within the seminiferous tubules, suggesting alleviated cellular damage. In contrast, we observed that rapamycin, a drug known for its longevity benefits, induced excessive testicular apoptosis and did not decrease lipid peroxidation. Collectively, our results highlight BZBS's promising clinical potential in counteracting male reproductive aging, underlining its mechanisms of action.

[KEY WORDS] Traditional Chinese medicine; Male reproductive aging; Spermatogenic cells; Cell senescence; Apoptosis
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Introduction

The global demographic is currently grappling with a profound aging crisis. Population aging, propelled by falling fertility rates and enhanced longevity, is a significant worldwide demographic shift, impacting societies on a global scale. The ramifications of reproductive aging extend beyond individual health, bearing considerable social and economic consequences. As the global population ages, the repercussions of reproductive aging are set to intensify, marking it as an imperative public health concern globally.

Reproductive aging is a multifaceted process influenced by both genetic and environmental factors, affecting both genders as they age. In women, this process is predominantly characterized by a marked decline in ovarian hormone secretion, diminished follicular response to hormonal stimuli, and an eventual cessation of reproductive capability [1]. In men, aging may result in diminished sperm quality and quantity, alongside erectile dysfunction, primarily due to reduced testosterone levels and alterations in the testes and prostate [2, 3]. Furthermore, the hypothalamic-pituitary-gonadal (HPG) axis undergoes dysregulation with age, disrupting the secretion of

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[*]**Corresponding author]** E-mails: houyunlonghrb@hotmail.com (HOU Yunlong); weitcm@163.com (WEI Cong)
[Δ]These authors contributed equally to this work.
These authors have no conflict of interest to declare.

several hormones, including hypothalamic gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). These hormonal imbalances contribute to spermatogenesis defects and increased infertility rates in older men. The gradual decline in testicular spermatogenesis, alterations in testicular structure, and a notable reduction in sperm quantity and quality are accompanied by a rise in reproductive defects and male infertility rates [4]. Additionally, reproductive aging is linked to other age-related ailments. Thus, addressing reproductive aging is crucial for ameliorating the broader issue of population aging.

Although the mechanisms of reproductive aging are still not fully understood, many different factors have been proven to be involved in the complex process [5], including alterations in hormone levels, mitochondrial function, and cellular senescence [6]. Cellular senescence, a state in which cells cease to divide and lose functionality, has emerged as a potential key player in reproductive aging. As individuals age, senescent cells accumulate in various tissues, potentially driving the aging process. These senescent cells can release pro-inflammatory factors and other molecules, fostering inflammation and tissue damage, which are implicated in the progression of reproductive aging. A notable study highlighted the significant accumulation of senescent cells in the testes of older humans, particularly under conditions of excessive stress, leading to a localized inflammatory microenvironment [7]. Gaining a deeper understanding of cellular senescence' s impact on reproductive aging is crucial for the development of novel therapeutic strategies aimed at enhancing reproductive and overall health in the aging population.

Bazi Bushen (BZBS) is composed of 14 herbal components, including Renshen, Gouqizi, Tusizi, Shechuangzi, Jiucaizi, Wuweizi, Fupenzi, Jinyingzi, Chuanlianzi, Yinyanghuo, Bajitian, Roucongrong, Shengdihuang, and Chuanniuxi, among others. Many of these herbs have been revered as longevity agents throughout thousands of years of medical practice [8]. Clinically, BZBS has been utilized to enhance vitality, and subsequent studies have unveiled its expansive medicinal benefits. Earlier research demonstrated BZBS's capacity to safeguard testicular function through the modulation of the Sirt6/P53 and Sirt6/NF-κB signaling pathways in mouse models predisposed to disease by D-galactose and $NaNO_2$ exposure [9]. Additionally, our recent investigations have shown that BZBS extends the health span of mice undergoing natural aging [10]. These findings prompt an intriguing inquiry into BZBS's potential impact on male reproductive aging within the natural aging continuum.

In this study, we explored the male reproductive characteristics of naturally aged mice subjected to prolonged BZBS treatment. Our results indicate that BZBS successfully maintained sex hormone levels, enhanced testicular structure, preserved the count of spermatogenic cells in the testes, minimized cellular apoptosis and senescence, and mitigated inflammatory responses. This study provides experimental validation of BZBS's effectiveness in mitigating reproductive aging, highlighting the benefits of traditional Chinese medicine (TCM) in clinical applications.

Materials and Methods

Animal maintenance and drug administration

This study was conducted using male C57BL/6J mice at the age of 52 weeks, sourced from Beijing Weitong Lihua Experimental Animal Technology. The animals were housed under a 12-hour light/dark cycle at a controlled temperature of 20–22 °C at the Hebei Yiling Chinese Medicine Research Institute, where they were provided with a standard mouse diet. The mice were randomly divided into four groups. The aged control group received daily saline injections, while the treatment groups were administered a BZBS suspension orally each day (at dosages of 2 $g \cdot kg \cdot d^{-1}$ for the high-dose group, BZ-High, and 1 $g \cdot kg \cdot d^{-1}$ for the low-dose group, BZ-Low, both dissolved in 0.5% sodium carboxymethyl cellulose). The BZBS used (Lot: XB2103001) was supplied by Shijiazhuang Yiling Pharmaceutical Co., Ltd., Shijiazhuang, China. Additionally, a rapamycin solution was added to the drinking water of a separate group (RAPA group), with the water being refreshed every 48 hours. The rapamycin (Lot: S115842) was provided by Shanghai Aladdin Biochemical Technology Co., Ltd.. After a three-month period of treatment, the mice, now at the age of 63 weeks, were subjected to biomolecular assays and pathological evaluations. The research protocols received approval from the Ethics Commission of Animal Care at the Hebei Yiling Chinese Medicine Research Institute and were carried out in accordance with the institute's approved guidelines (approval number: N2020107). The chemical composition of BZBS was analyzed using ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) [11].

Overall appearance score of mice

Quantitative scoring of the overall appearance of mice based on the degree of Alopecia, Fur Color Loss, Coat Condition, and Loss of whiskers [12-14]. Alopecia: Gently restrain the animal and inspect it for signs of fur loss including signs of barbering, 0 = normal fur density, 0.5 = < 25% fur loss, 1 = > 25% fur loss; Fur Color Loss: Note any change in fur color from black to grey or brown, 0 = normal color, 0.5 = patchy, focal grey, brown or white changes, 1 = grey, brown or white fur on > 25% of body; Coat Condition: Inspect the animal for signs of poor grooming. Alopecia and loss of fur color can contribute to poor coat condition, 0 = smooth, sleek, shiny coat, 0.5 = coat is slightly ruffled, 1 = unkempt and un-groomed, matted appearance; Loss of Whiskers: Inspect the animal for signs of a reduction in the number of whiskers, 0 = no loss, 0.5 = reduced number of whiskers, 1 = absence of whiskers. The appearance of each group of mice was scored according to the above, and the mean value and the proportion of mice in each group with different score segments were calculated.

Measurement of serum hormones

The serum was separated through centrifugation of blood at 3000 rpm for 15 min. FSH levels were determined using a

Luminex Assay (UNIV, Shanghai, China, Catalog No. 3760310), adhering to the manufacturer's guidelines. Testosterone concentrations were measured *via* radioimmunoassay conducted by Beijing North Institute of Biotechnology Co., Ltd. (Beijing, China, Catalog No. 1220).

Histopathological analysis

Testicular tissue sections (4 μm thick) were stained using hematoxylin and eosin (H&E). The process involved initially deparaffinizing the sections with xylene, followed by rehydration through a series of alcohol gradients and distilled water. Subsequently, the sections were stained with hematoxylin for 10 min. After a differentiation step and rinsing, eosin was applied to counterstain the nucleus for 1 min. Morphological alterations were examined using a Nano Zoomer-SQ digital slide scanner (Hamamatsu, Japan).

Sperm collection

Sperm were extracted from the caudal (tail) epididymis of mice. The epididymis was placed in 1 mL of saline and incised using ophthalmic scissors. Following this, the samples were incubated in a constant temperature water bath at 37 °C for 30 min and then filtered through a 200-mesh sieve into a 1.5 mL centrifuge tube. This process allowed for the separation of the sperm suspension from tissue fragments. The isolated sperm suspension was subsequently diluted with 2 mL of normal saline for further dilution and observation.

Malondialdehyde (MDA) Assay

Testes were homogenized in normal saline and subsequently subjected to centrifugation at 4 °C with a speed of 3000 rpm for 10 min. The supernatant obtained from this process was then utilized for MDA assessment, employing a lipid peroxidation (MDA) content assay kit (Abbkine, Shanghai, China, Catalog No. KTB1050).

RNA isolation and quantitative real-time PCR

Total RNA was extracted using the TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China, Catalog No. ER501-01) and subsequently reverse-transcribed using the GoScript™ Reverse Transcription System (Promega, Madison, USA, Catalog No. A5001). The resultant cDNA products served as templates for quantitative PCR (qPCR), conducted with the MonAmp™ ChemoHS qPCR Mix kit (Monad, Suzhou, China, Catalog No. MQ00401S). The primer sequences utilized are detailed in Table 1. All procedures were conducted strictly in accordance with the manufacturer's protocols.

Table 1 Primer sequences used for quantitative real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
TNF-α	GTGCCTATGTCTCAGCCTCT	TGGTTTGTGAGTGTGAGGGT
NF-κB	GGAGGCATGTTCGGTAGTGG	CCCTGCGTTGGATTTCGTG
GAPDH	ACTGGCATGGCCTTCCG	CAGGCGGCACGTCAGATC

Aging-associated β-galactosidase staining

Frozen testicular tissue sections (10 μm) were fixed and rinsed at room temperature, followed by incubation at 37 °C overnight in dark conditions with suitable volumes of premixed staining solution. After staining, the slides were dehydrated and sealed, then examined using a light microscope. Quantitative analysis of the observed results was conducted utilizing ImageJ software.

Immunohistochemistry

Following deparaffinization, hydration, and antigen retrieval, sections were blocked using an SP kit (ZSGB, Beijing, China). The sections were then incubated with primary antibodies (rabbit anti-p16^{INK4a} polyclonal antibody, 1∶200 dilution, Abcam, Cambridge, UK) at 4 °C overnight. The next day, after washing with phosphate-buffered saline, the sections were treated with biotin-labeled goat anti-rabbit IgG polymer, followed by incubation with HRP-labeled streptavidin for 15 min. Subsequent to the final washes, the sections were exposed to diaminobenzidine at room temperature for 3–5 min. Observation and image capture of the sections were performed using a Nano Zoomer-SQ digital slide scanner (Hamamatsu, Japan).

Immunofluorescence

Sections were dewaxed, rehydrated, and subjected to antigen retrieval in a microwave using a citrate-EDTA solution. Subsequently, 5% bovine serum albumin (BSA) was applied to block non-specific binding for 15 min. Primary antibodies against p16^{INK4a} (1:500 dilution, Abcam, Cambridge, UK, ab189034), c-Kit (1∶1000 dilution, Abcam, Cambridge, UK, ab256345), and transition nuclear protein 1 (TNP1, 1∶200 dilution, Proteintech, USA, 17178-1-AP) were added to the sections, which were then incubated overnight at 4 °C. Following incubation, the sections were washed three times with PBS and incubated with goat anti-rabbit IgG (1:500 dilution, Proteintech, USA, SA00013-2) at 37 °C for 1 h. A ZEISS laser scanning confocal microscope (Zena, Dogern, Germany) was utilized for section analysis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assays were conducted using the CoraLite® 594 TUNEL Apoptosis Detection Kit (Proteintech, USA, Catalog No. PF00009). The sections underwent dewaxing and hydration, followed by treatment with Proteinase K working solution and incubation at 37 °C for 25 min. A permeabilization solution was then applied to the sections, which were incubated for 20 min at room temperature. The TDT enzyme, DUTP, and buffer from the TUNEL kit were combined in a 1∶5∶50 ratio. After blocking with 3% BSA, the sections were incubated overnight at 4 °C with c-Kit rabbit monoclonal antibody (1∶1000 dilution, Abcam, Cambridge, UK, Catalog

No. ab256345) and SCP3 rabbit polyclonal antibody (1 : 200 dilution, Proteintech, USA, Catalog No. 23024-1-AP). Following rinsing, goat anti-rabbit IgG (1 : 500 dilution, Proteintech, USA, Catalog No. SA00013-2) was applied to the sections, which were then incubated at room temperature for 50 min. The sections were sealed with an anti-fluorescence quencher containing DAPI, and images were captured using a ZEISS laser scanning confocal microscope (Zena, Dogern, Germany).

Western blotting (WB) assay

Proteins were extracted from testicular tissues using a high-performance RIPA tissue/cell lysate and PMSF (Solarbio, Beijing, China). The protein concentrations were determined using the BCA protein quantification kit (Beyotime Biotechnology, Shanghai, China). Forty micrograms of protein from each sample were loaded into each well of a precast 4%–20% polyacrylamide gel (GenScript Biotech, Nanjing, China) and separated at 120 V. Following electrophoresis, proteins were transferred from the gel to polyvinylidene fluoride (PVDF) membranes, with careful alignment to ensure the accurate location of target proteins.

The membranes were blocked for 1 h at 37 °C using a blocking solution provided by Beyotime Biotechnology, Shanghai, China, and then incubated overnight at 4 °C with monoclonal or polyclonal antibodies. Primary antibodies against P53 and SCP3 were acquired from Abcam (dilutions of 1 : 800 and 1 : 2000, respectively, Cambridge, UK, Catalog Nos. ab131442 and 23024-1-AP). β-Actin and GAPDH antibodies, used as internal reference proteins, were also obtained from Abcam (dilutions of 1 : 2000 and 1 : 5000, respectively, Cambridge, UK, Catalog Nos. ab8227 and ab181602). Following this, goat anti-rabbit IgG antibody (1 : 1000 dilution, Abcam, Cambridge, UK, Catalog No. ab216773) was applied to the membranes, which were then incubated for 1 h at 37 °C away from light. The quantification of target proteins was performed by analyzing grayscale values using an Odyssey imaging system and image analysis software (LI-COR Biosciences, Lincoln, NE).

Statistical analysis

Statistical analyses were conducted using IBM SPSS 22. Prior to selecting the suitable statistical test, data were subjected to the Shapiro–Wilk test to assess normal distribution and the Levene's test to evaluate the homogeneity of variances. For data following a normal distribution, a one-way analysis of variance (ANOVA) was employed to determine significant differences. For data not normally distributed, the Kruskal–Wallis test was applied. *P*-values less than 0.05 were deemed statistically significant for all analyses, with $^{*}P < 0.05$ and $^{**}P < 0.01$ indicating different levels of significance. All results are reported as mean ± standard deviation (SD).

Results

BZBS ameliorates the hair appearance of naturally aged mice

Hair loss and whitening are typical signs of aging in both humans and C57BL/6 mice. As illustrated in Fig. 1A, the fur of mice in the aged control group (63 weeks) exhibited significant graying and extensive hair loss. In contrast, mice treated with BZBS maintained both the density and the black color of their fur. Mice treated with rapamycin displayed a less severe degree of hair loss compared to the aged control group (Fig. 1A). A quantitative assessment of hair condition, evaluating the extent of alopecia, fur color loss, coat quality, and whisker loss, indicated that BZBS treatments could mitigate the visual signs of aging in naturally aged mice, suggesting a potential anti-aging pharmacological benefit (Figs. 1B–1F).

BZBS improves sex hormone production in naturally aged mice

The stability of the HPG axis is critical for preserving normal reproductive functions. FSH and testosterone, two principal sex hormones, play pivotal roles in regulating spermatogenesis and are known to diminish with age [15-17]. To assess the impact of BZBS on hormone secretion, we utilized a Luminex assay for FSH measurement—a bead-based immunoassay renowned for its precision in detecting minute quantities of analytes—and a radioimmunoassay for testosterone determination. As depicted in Fig. 2A, BZBS treatment significantly elevated serum FSH and testosterone levels in a dose-responsive manner when compared to the untreated aged group. Notably, the increase in testosterone production induced by the high dose of BZBS was strikingly potent, being approximately tenfold greater than that observed in the aged control mice (Fig. 2B). Intriguingly, rapamycin treatment also notably raised serum FSH levels and modestly enhanced testosterone secretion (Fig. 2).

BZBS preserves testicular morphology and improves spermatogenesis in naturally aged mice

The enhancement of sex hormone production holds promise for improving testicular morphology and function. Nevertheless, both low and high doses of BZBS treatments resulted in testicular indices similar to those observed in aged control mice (Fig. 3A). Remarkably, rapamycin treatment led to a significant decrease in the testicular index when compared to the other three groups (Fig. 3A). Further investigation of testicular morphology using H&E staining revealed significant degeneration in the testes of aged mice, characterized by noticeably enlarged seminiferous tubules, a reduced interstitial area, and a scarcity of spermatocytes (Figs. 3B–3E). Despite the increase in sex hormone levels, rapamycin treatment exacerbated testicular damage, even resulting in a completely disrupted blood-testis barrier (Fig. 3B). Importantly, however, treatment with both doses of BZBS preserved the normal testicular structure (Figs. 3B–3E) and maintained a significant number of mature sperm within the seminiferous tubules (Fig. 3B).

BZBS promotes germ cell meiosis in naturally aged mice

Synaptonemal Complex Protein 3 (SCP3) is an integral part of the synaptonemal complex that forms between homologous chromosomes during the prophase of meiosis [1],

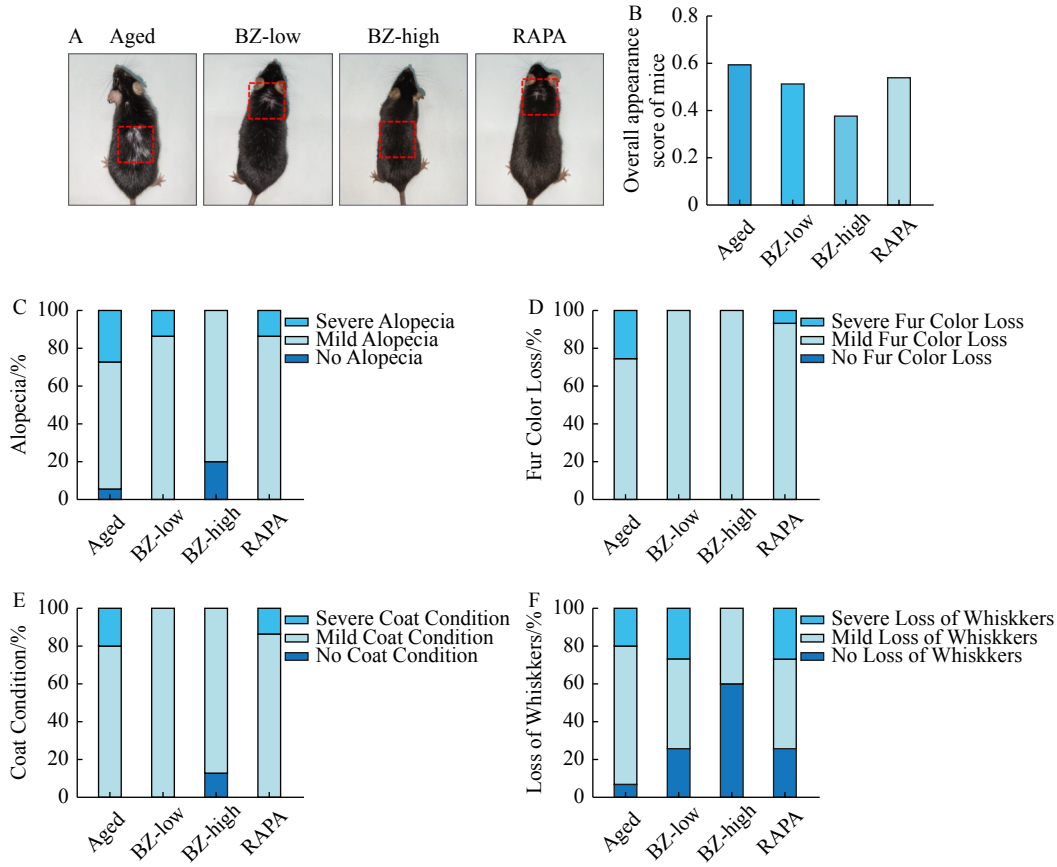

Fig. 1 Impact of BZBS treatment on the hair appearance of naturally aged mice. (A) Representative images of the hair appearance of the mice. (B) The overall rating of the hair condition (n = 15). (C–F) The quantitative score of alopecia, fur color loss, coat condition, and loss of whiskers.

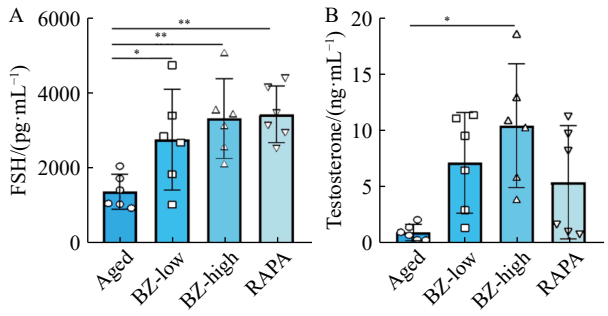

Fig. 2 BZBS improved sex hormone production in naturally aged mice. (A) Serum FSH hormone levels. (B) Serum testosterone levels. Data are expressed as means ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars indicate SD.

serving as a critical marker for germ cells undergoing meiosis in mouse testes. Immunofluorescence analysis revealed that SCP3 expression was significantly higher in the BZBS-treated groups compared to both the aged and rapamycin-treated groups, as shown in (Fig. 4A). WB analysis corroborated these findings, indicating a pronounced increase in SCP3 levels in the BZBS high-dose group (Fig. 4B). Conversely, TNP1, which is pivotal in histone-to-protamine replacement in elongating spermatids and serves as a marker for mature sperm cells [18], also showed increased expression in response to BZBS treatments, aligning with SCP3 staining results, as evidenced in (Fig. 4C). Sperm count analyses further demonstrated a significantly higher number of sperm in the BZBS treatment groups compared to both the aged control and rapamycin groups (Fig. 4D). These findings, in conjunction with hormone level measurements and histomorphological assessments, affirm the positive impact of BZBS on counteracting the natural testicular aging process.

BZBS alleviates testicular senescence in naturally aged mice

Throughout the aging process, cells experiencing extensive stress may either enter senescence or undergo apoptosis, triggered by various signaling pathways. Cellular senescence, primarily induced by the P53/P21$^{WAF1/CIP1}$ and p16$^{INK4A/RB}$ pathways, is marked by changes such as a flattened/enlarged cellular morphology, a permanent halt in cell proliferation, and the release of pro-inflammatory cytokines [19]. Additionally, senescence-associated β-galactosidase activity is known to increase during cellular senescence [20]. *β*-Galactosidase staining revealed that the positive area was significantly larger in the aged control group compared to the BZ-low, BZ-high, and rapamycin-treated groups, highlighting BZBS's therapeutic effects (Fig. 5A). In alignment with *β*-galactosidase staining, p16^{INK4a} expression was also reduced in the three treatment groups relative to the aged control group (Fig. 5B). Co-immunostaining of c-Kit (a marker for spermatogo-

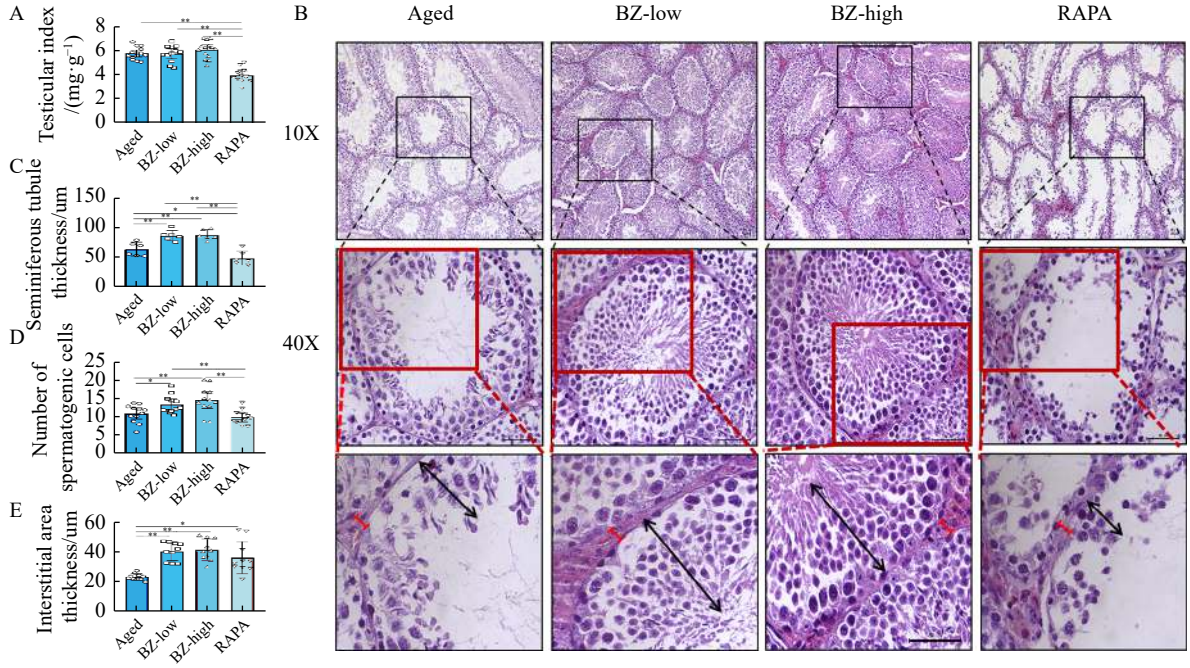

Fig. 3 Effect of BZBS treatment on maintaining testicular structure. (A) Testicular indexes of each group. (B) HE staining of mouse testes. (C–E) Quantification of seminiferous tubule thickness, number of spermatogenic cells, and interstitial area thickness of the mouse testes. Data are expressed as means ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars indicate SD.

nia and Leydig cells) with $p16^{INK4a}$ indicated that cellular senescence in the natural aging process predominantly affects spermatogonia and Leydig cells (Fig. 5C). P53 acts as a sensor of cellular stress and a critical regulator of the P53/P21 senescence signaling pathway [21]. WB analysis for P53 expression further verified the heightened senescence in aged testes. Interestingly, BZBS treatment significantly curtailed cellular senescence, as evidenced by the markedly reduced $p16^{INK4a}$ throughout the testes, particularly in the seminiferous tubules (Fig. 5C). Although rapamycin treatment also led to decreases in p16INK4a and P53 expressions, these reductions were comparatively modest (Figs. 5B–5D).

BZBS reduces cell apoptosis in aged testicular tissue

Previous research has demonstrated that testicular aging is associated with an increase in cell apoptosis [22]. TUNEL staining, as illustrated in Figs. 6A and 6B, revealed a significant presence of apoptotic cells at the periphery of spermatogenic tubules in the aged control mice. In comparison to the aged group, both the BZ-low and BZ-high groups exhibited a markedly reduced number of apoptotic cells, indicating the anti-apoptotic properties of the formula. Notably, the rapamycin-treated group displayed a significantly higher level of apoptosis signal than the other three groups, as shown in (Figs. 6A and 6B). Given rapamycin's known effects on hormone regulation and the clearance of senescent cells, these accumulating apoptotic cells might explain the observed disruption in the testicular structure mentioned earlier (Fig. 3).

BZBS mitigates oxidative stress and inflammation in aged mouse testes

Oxidative stress is a primary factor contributing to cellular dysfunction and plays a significant role in testicular aging. An excess of reactive oxygen species can trigger a series of damaging effects, starting with lipid peroxidation of cellular membranes and leading to overall cellular dysfunction [23]. Particularly vulnerable are testicular cells, including Leydig cells, which are rich in lipid-filled vesicles [24]. To evaluate lipid peroxidation within the testes of each group, we conducted an MDA assay. This assay detects the formation of an MDA-TBA adduct, which exhibits strong absorption at 532 nm. Remarkably, both groups treated with BZBS displayed significant reductions in MDA-TBA2 levels, indicating reduced lipid peroxidation, whereas the rapamycin treatment did not diminish lipid peroxidation in aged testes, as shown in (Fig. 7A). Inflammation also presents a significant risk to genome stability and the fate of testicular cells [25]. Tumor necrosis factor-alpha (TNF-α), primarily produced by round spermatids, pachytene spermatocytes, and testicular macrophages, plays a crucial role in testicular inflammation. Through real-time PCR analysis, we observed that both TNF-α and its downstream effector, NF-κB, were downregulated in the BZBS-treated groups compared to the aged control group, as illustrated in (Figs. 7B–7C).

Discussion

Aging is a multifaceted process influenced by various external and internal factors promoting aging. Reproductive aging may represent one of the earliest indicators of the broader aging phenomenon, with diminished sex hormone levels playing a pivotal role in this aspect of aging [26, 27]. In men, sex hormones begin to decline from around 30 years of age, leading to reduced activity of testicular cells and con-

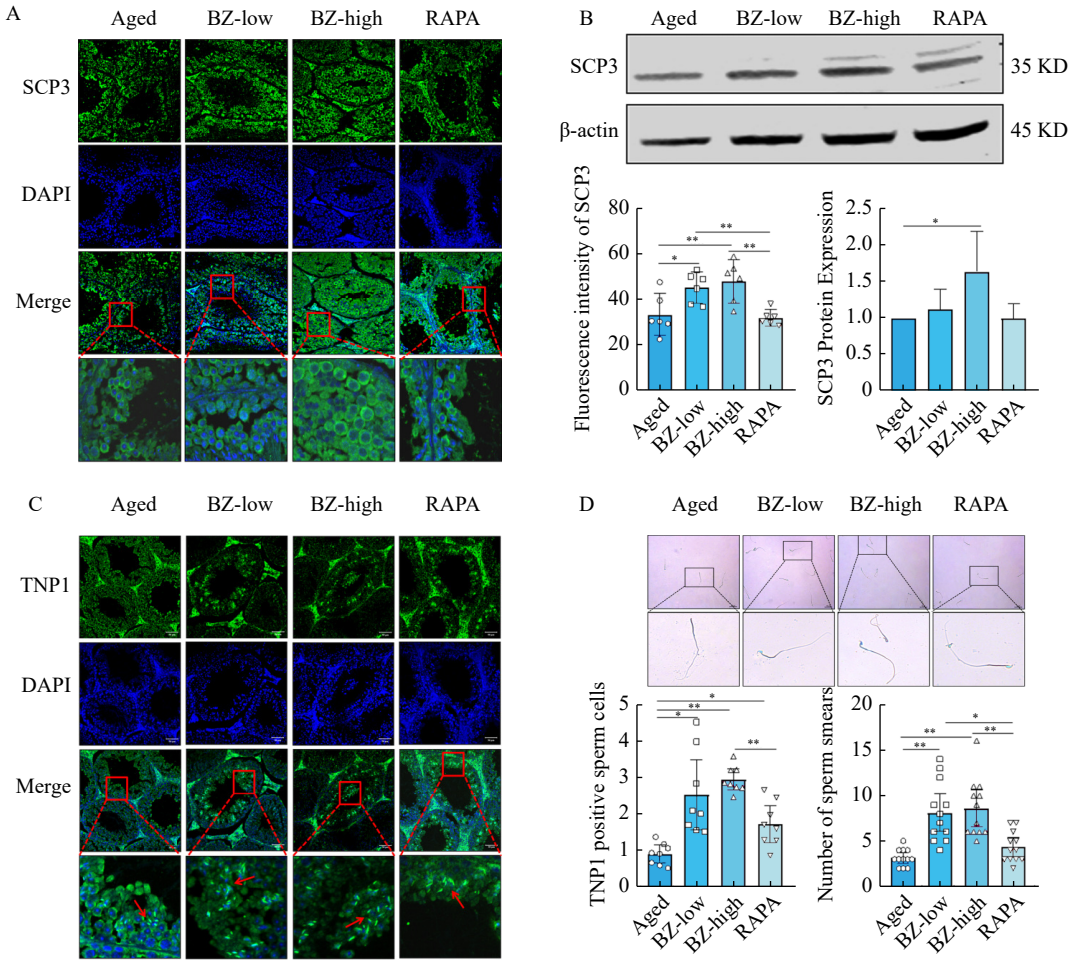

Fig. 4 BZBS promotes germ cell meiosis and increases the sperm cell population. (A) The expression of SCP3 analyzed by immunofluorescence. (B) WB analysis of SCP3 and β-actin in testicular tissue. (C) Immunofluorescence of TNP1. (D) Quantification of sperm number from diluted epididymal sperm suspension smears. Data are expressed as means ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars indicate SD.

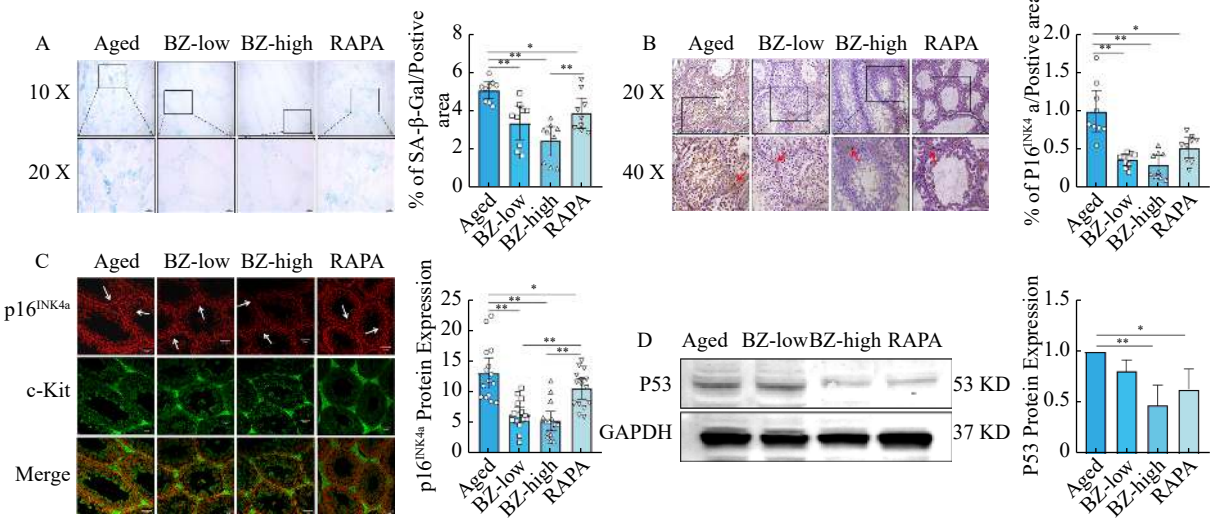

Fig. 5 BZBS reduces testicular senescence. (A) SA-β-Gal staining images. (B) IHC-stained image of p16^{INK4a} protein. (C) IF stained image of p16^{INK4a} protein. (D) WB analysis of P53 and GAPDH in testicular tissue. Data are expressed as means ± SD (n = 3). $^{**}P < 0.01$. Error bars indicate SD.

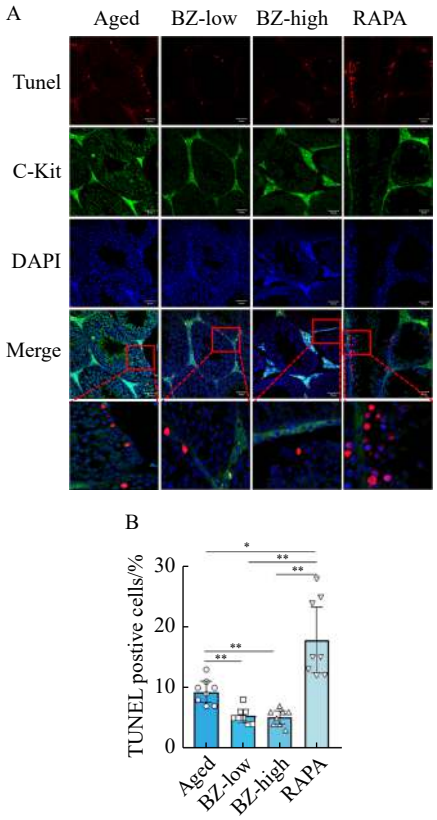

Fig. 6 BZBS inhibits testicular apoptosis. (A) TUNEL staining apoptosis image. (B) Quantification of TUNEL staining for apoptosis. Data are expressed as means ± SD (n = 6). $^{**}P$ < 0.01. Error bars indicate SD.

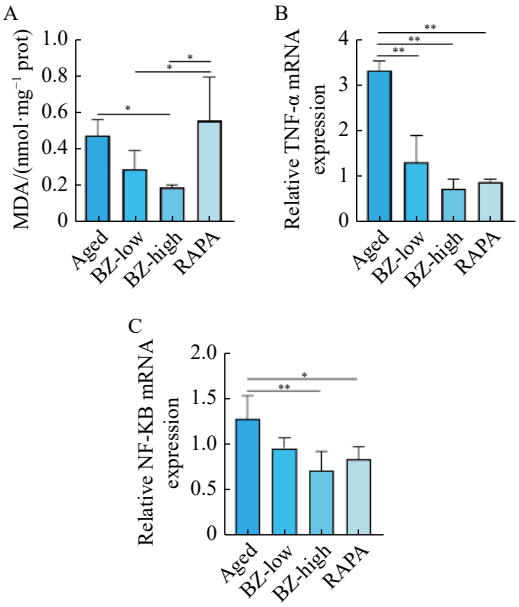

Fig. 7 BZBS mitigates testicular oxidative stress and inflammation. (A) MDA content in testicular tissue. (B) The mRNA expression of TNF-α. (C) The mRNA expression of NF-κB. Data are expressed as means ± SD (n = 3). $^{**}P$ < 0.01. Error bars indicate SD.

sequently impairing spermatogenesis [28]. Translating mouse age to human years, 52 weeks in male mice approximately equates to 44 human years, aligning with the onset of declining fertility in men [29]. A decrease in LH impairs the normal functioning of Leydig cells [30], while the diminished testosterone output from these cells intensifies the negative feedback on the HPG axis, further inhibiting overall hormone secretion [31]. Concurrently, FSH interacts with Sertoli cells within the seminiferous tubules and, together with testosterone, supports the survival of germinative cells and fosters spermatogenesis [32, 33]. Apart from HPG axis dysregulation, aging also leads to structural deterioration of the testes, such as reduced testicular size and increased thickness and rigidity of the seminiferous tubules [34, 35]. Testicular cells, including Leydig and Sertoli cells, experience functional decline and alterations in cell fate due to cumulative cellular stress [35]. These changes, coupled with hormonal fluctuations, contribute to defects in spermatogenesis. Our study demonstrated that BZBS significantly raised serum FSH and testosterone levels in aged mice (Figs. 2A–2B). The herbal ingredients in BZBS are known for their hormone-enhancing or hormone-like properties [8]. For instance, polysaccharides from Lycium barbarum have been shown to augment spermatogenesis in oligospermic rats by promoting Leydig cell-mediated testosterone release [36]. Similarly, raspberries have been used clinically to adjust serum sex hormone levels for treating male reproductive dysfunctions, with their active compounds directly affecting the HPG axis [37]. The specific mechanisms through which these components facilitate BZBS-induced hormone elevation warrant further exploration. Additionally, BZBS was found to safeguard the structure of seminiferous tubules and interstitial areas, maintaining the count of spermatocytes in aged mice (Figs. 3B–3E). Given the existence of the blood-testis barrier, the potential direct protective mechanisms of BZBS on seminiferous tubule cells are intriguing and merit further investigation.

The prevailing view on biological aging posits that it culminates either in programmed cell death (apoptosis) or in a state of permanent cell cycle arrest (cellular senescence) [38]. Our observations in aging mouse testes revealed a concurrent occurrence of cellular senescence and apoptosis, displaying a region-specific distribution. Cellular senescence was predominantly observed in both the seminiferous tubules and the interstitial area, as illustrated in (Figs. 5A–5C). In contrast, apoptotic cells were relatively infrequent and localized specifically to the margins of seminiferous tubules, as shown in (Figs. 6A–6B). Nonetheless, cellular senescence and apoptosis may act in concert to amplify organ damage. For example, senescent cells can secrete the senescence-associated secretory phenotype (SASP), deteriorating the cellular microenvironment and impacting the function of adjacent normal cells. This can either initiate apoptosis or further promote cellular senescence, depending on the severity of cellular damage [39, 40]. Remarkably, in the context of senotherapeutic effects on aged testes, BZBS demonstrated superior efficacy over rapamycin. Further analysis showed that BZBS significantly reduced the levels of MDA-TBA2 (Fig. 7A) and curtailed the activation of the TNF-α/NF-κB signaling pathway

in testicular tissues (Figs. 7B–7C), whereas rapamycin did not effectively suppress testicular lipid peroxidation. Previous studies have highlighted BZBS's antioxidative and anti-inflammatory properties in a disease-prone mouse model [41], and our findings further validate its effectiveness in mitigating the natural testicular aging process, as evidenced in Fig. 8 [41].

Rapamycin is a potential longevity medicine that can extend the lifespan of mice. In general, the aging of organs is believed to have a clear intrinsic correlation with overall aging. Therefore, drugs with anti-aging effects may also have therapeutic effects on age-related diseases and delay organ aging. However, in regard to the testes, rapamycin accelerates the age-related degeneration process with an incompletely explored mechanism. Studies in rats and mice have shown that rapamycin can increase testicular degeneration by inhibiting the proliferation and differentiation of spermatogonia, leading to the loss of all stages of spermatogenesis [42-45]. Our findings reveal that while rapamycin may improve certain age-related appearances in mice, it adversely impacts the testicular index and triggers apoptosis in seminiferous tubule cells, potentially leading to a complete loss of spermatogonia (Fig. 3A and 3B). Conversely, BZBS treatment preserved testicular weight and mitigated morphological alterations in the testes of aged mice by reducing cellular senescence and apoptosis and by bolstering the counts of spermatocytes and spermatozoa. This study underscores the advantage of TCM for sustained anti-aging interventions. Notably, some literature suggests that prolonging male lifespan may come at the cost of fertility [42, 45]. However, we argue that even if male hormones pose risks to healthspan or lifespan, interventions like BZBS can simultaneously support overall aging mitigation and reproductive aging delay. BZBS's role in diminishing chronic inflammation and harmonizing the internal environment may contribute broadly to slowing the aging of other organs as well.

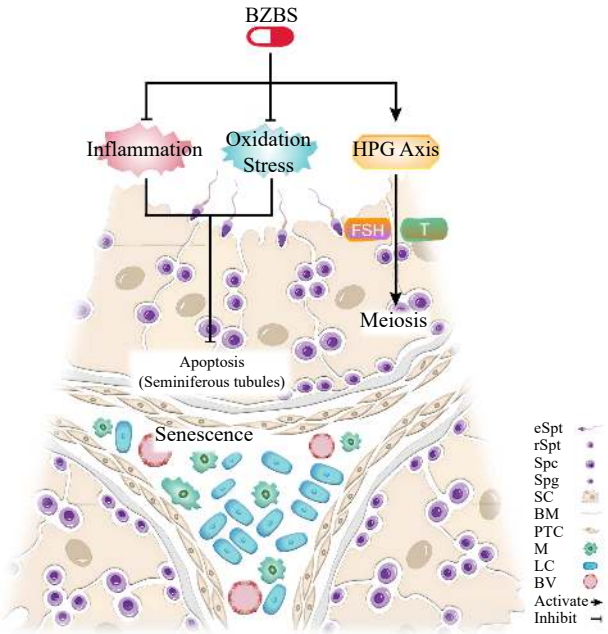

Fig. 8 Summary figure of BZBS acting on the natural testicular aging process. Sertoli cells (SC); spermatogonia (Spg); spermatocytes (Spc); round spermatids (rSpt); elongating spermatids (eSpt); basement membrane (BM); peritubular myoid cells (PTC); Leydig cells (LC); vascular network (BV); macrophages (M).

In conclusion, this research establishes BZBS's efficacy against reproductive aging, highlighting its comparative advantage over rapamycin in preserving testicular function and structure in naturally aged male mice. The benefits of BZBS extend beyond hormone regulation, encompassing the preservation of spermatogenic cell numbers and testicular integrity. These findings advocate for TCM's potential to offer holistic patient care through its multi-component, multi-pathway, and multi-target approach, enhancing synergy and minimizing toxicity.

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