

Salvia miltiorrhiza activates Nrf2/HO-1 signaling and restores steroidogenesis in Leydig TM3 cells and an aging rat model

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ABSTRACT

Male aging is often accompanied by a gradual decline in testosterone production, referred to as andropause, which is associated with fatigue, reduced libido, hormonal imbalances, and metabolic disturbances. Testosterone replacement therapy poses risks such as prostate and cardiovascular complications, prompting interest in natural alternatives. This study explored the therapeutic effects of *Salvia miltiorrhiza* extract (SME) in an *in vitro* H₂O₂-induced Leydig TM3 cell model and an *in vivo* aged rat model. HPLC/MS analysis confirmed the presence of tanshinone IIA (10.629 mg/g) in SME. SME (1–2 µg/mL) attenuated oxidative stress, restored antioxidant gene and protein expression (Nrf2, HO-1, SOD, CAT, and GPx), and enhanced the steroidogenic pathway by upregulating STAR, CYP11A1, CYP17A1, 3β-HSD, and 17β-HSD while downregulating 5α-reductase. In aged rats, oral administration of SME (particularly 50 mg/kg) restored testosterone, LH, FSH, and progesterone levels, while reducing SHBG and DHT levels. SME also improved liver function markers (ALT and AST) and lipid profiles (TG, TC, LDL, and HDL), and reduced MDA and serum PSA levels. Also, HPLC/MS analysis detected the presence of tanshinone IIA in the serum of SME-administered animals. Furthermore, fecal microbiome analysis revealed an abundance of the propionate-producing microbe *Succinispira mobilis*, indicating a possible role of SME in improving gut health and hormone levels. These findings suggest that SME may serve as a promising natural intervention against andropause by regulating oxidative stress, steroidogenesis, and gut dysbiosis.

1. Introduction

The term andropause is derived from the Greek words “Andras,” which means human male, and “pause,” which means cessation. It is defined as a syndrome linked to decreased sexual satisfaction or feeling of general well-being owing to lower testosterone levels in older men [1]. Testosterone maintains the overall health of a male by preserving

muscle mass, regulating fat metabolism, and maintaining reproductive development and functioning [2]. A decline in testosterone levels may lead to impaired puberty, impotence, gynecomastia, infertility, or decreased spermatogenesis [3]. The general symptoms include reduced libido, diminished sexual function, erectile dysfunction, fatigue, muscle loss, depression, and increased risk of metabolic diseases [4,5].

Luteinizing hormone (LH) regulates the production of testosterone

Abbreviations: SME, *Salvia miltiorrhiza* extract; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; AST, aspartate transaminase; ALT, alanine transaminase; TG, triglyceride; TC, total cholesterol; MDA, malondialdehyde; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PSA, prostate-specific antigen; SHBG, sex hormone-binding globulin; OS, oxidative stress; DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; OS, oxidative stress.; DAD, diode array detector; H&E, hematoxylin and eosin; NGS, next generation sequencing; ASV, amplicon sequence variant; UV, ultraviolet.

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from Leydig cells, which is released into the blood [6]. Testosterone production by Leydig cells is stimulated when LH binds to its receptor, activates adenyl cyclase, and increases cAMP and cAMP-dependent protein kinase levels. This signal activates the steroidogenic pathway, as the steroidogenic acute regulatory (STAR) protein and translocator protein in the inner membrane of the mitochondria are activated. This is considered the most crucial step in the initiation of steroidogenesis in the testes. The cytochrome P450 cholesterol side-chain cleavage enzyme (P450_{scc} or CYP11A1) metabolizes the substrate cholesterol into pregnenolone, which is further metabolized by a series of enzymes, such as 3 β -hydroxysteroid dehydrogenase (3 β -HSD), cytochrome P450 17 α -hydroxylase/C17–20 lyase (P450_{c17} or CYP17A1), and 17 β -hydroxysteroid dehydrogenase (17 β -HSD), ultimately leading to the production of testosterone [7]. In andropause, testosterone production from Leydig cells decreases because of an impaired steroidogenic pathway, decreased LH-stimulated cAMP production, downregulation of STAR and CYP11A1 in the mitochondria, and CYP17A1 in the smooth endoplasmic reticulum [7–9].

Oxidative stress (OS) is closely associated with increased cellular senescence and age-related degenerative diseases, including hypogonadism [10]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor and primary regulator of the cellular redox response [11]. It regulates various downstream antioxidants, such as heme oxygenase (HO)-1, catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [12]. However, this system is compromised in patients with hypogonadism. Recent studies conducted in animals have shown that the depletion of the antioxidant glutathione (GSH) is linked to reduced testosterone synthesis [13]. The disrupted mitochondrial function, membrane potential, poor ATP synthesis, and deregulated calcium concentrations in Leydig cells owing to OS reduce steroidogenesis through interference with STAR expression levels, cAMP production, and cytochrome P450 activity [10]. Moreover, hydrogen peroxide (H₂O₂) generated in testicular macrophages also affects 3 β -HSD, SOD, CAT, and glutathione-S-transferase activities, thereby increasing lipid peroxidation and apoptosis and impairing steroidogenesis [14,15]. Additionally, the complex community of microorganisms residing in the gastrointestinal tract influences systemic inflammation and metabolic function, and interacts with the endocrine system [16]. Gut microbiota-derived metabolites, such as short-chain fatty acids (SCFAs), lipopolysaccharides (LPS), and secondary bile acids, have been shown to impact testosterone synthesis, hypothalamic-pituitary-gonadal (HPG) axis regulation, and OS levels [17].

Salvia miltiorrhiza has been widely used in East Asian traditional medicine for centuries, particularly in treating cardiovascular and cerebrovascular disorders [18]. Its therapeutic efficacy is primarily attributed to its antioxidant, anti-inflammatory, antifibrotic, and anti-apoptotic properties, which are relevant to various conditions, including atherosclerosis, myocardial infarction, cardiac hypertrophy, and hepatic and neurological injuries [18–20]. Previous studies have reported that *S. miltiorrhiza* extracts exhibit DPPH radical scavenging activity [21], enhance estrogen levels in immature/ovariectomized mice [22], and have anti-osteoporotic effects in both ovariectomized and naturally menopausal animal models [23]. Phytochemical investigations have identified over 200 bioactive compounds in *S. miltiorrhiza*, including tanshinones, salvianolic acids, and polysaccharides, which are considered responsible for its diverse pharmacological effects [24–26]. Among them, tanshinone IIA, a major lipophilic diterpenoid quinone, has potent antioxidant, anti-apoptotic, and endocrine-modulating properties [26, 27]. Recent reports have positioned *S. miltiorrhiza* as a promising candidate in managing age-related conditions, particularly those involving OS and endocrine dysregulation. However, despite these promising insights, the role of *S. miltiorrhiza* in male aging, particularly in H₂O₂-induced *in vitro* and *in vivo* models that mimic early-stage andropause, remains underexplored. Recent studies focus either on acute pharmacological effects or disease-specific models, leaving a gap in our understanding of how it influences the gradual physiological

changes associated with aging. The present study aims to address this gap by investigating the effects of *S. miltiorrhiza* extract (SME) in H₂O₂-induced Leydig TM3 cells and in 36-week-old male rats, which exhibit early markers of age-related decline but are not yet fully senescent. By focusing on its antioxidant, endocrine, and metabolic actions, this study provides novel insights into the preventive potential of *S. miltiorrhiza* in age-related male health decline.

2. Materials and methods

2.1. Materials

The mouse Leydig TM3 cells (ATCC® CRL-1714™) used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, USA). The primary antibodies anti-CYP11a1 (ab175408), anti-CYP17A1 (ab134910), anti-STAR (ab203193), and anti- β -actin (ab6276) were purchased from Abcam (Cambridge, UK). Santa Cruz Biotechnology (Dallas, TX, USA) provided the humanized mouse monoclonal primary antibodies for Nrf2 (sc-365949), HO-1 (sc-136960), SOD (sc-101523), GPx (sc-133160), and 3 β -HSD (sc-515120). The anti-17 β -HSD (NBP1–56295) antibody was bought from Novus Biologicals (Toronto, Canada), and the anti-5 α -reductase (LS-C749770) antibody was from LSBio (Washington, United States). The primary antibody for CAT was purchased from OriGene (Rockville, MD, USA). The testosterone assay kit (KGE010) was obtained from R&D Systems (Minneapolis, Minnesota, United States). Rat testosterone (MBS282195), dihydrotestosterone (DHT, MBS265478), progesterone (MBS703208), 3 β -HSD (MBS2604899), 17 β -HSD (MBS9323846), 5 α -reductase (MBS9711737), follicle-stimulating hormone (FSH, MBS2021901), luteinizing hormone (LH, MBS764675), and SHBG (MBS2502327) were provided by MyBioSource (San Diego, USA). An ELISA kit for prostate-specific antigen PSA (E-EL-R0796) was obtained from Elabscience (Wuhan, China). SME was obtained from FromBio (Yongin-si, Gyeonggi-do, Korea).

2.2. HPLC/MS detection of tanshinone IIA in the serum of experimental animals

To quantify tanshinone IIA in both the *S. miltiorrhiza* extract and serum of experimental animals, HPLC-MS analysis was conducted. The extract was initially dissolved in methanol at a concentration of 1 mg/mL, then diluted to 1000 ng/mL using acetonitrile. Subsequently, 3 μ L of the diluted extract was spiked into 27 μ L of the biological matrix.

For analysis, 30 μ L of either standard solution, extract solution, or serum sample was transferred into microcentrifuge tubes, followed by the addition of 150 μ L of the internal standard (IS) working solution containing Warfarin at 1 ng/mL. The mixture was vortexed at 2500 rpm for 3 min and centrifuged at 12,000 rpm for 5 min. A volume of 150 μ L of the resulting supernatant was then transferred to vials for analysis. Quantitative analysis of tanshinone IIA was conducted using a QTRAP 6500 + mass spectrometer coupled with an Exion LC high-performance liquid chromatography (HPLC) system (AB SCIEX). Data acquisition and processing were performed using Analyst software (version 1.7.2). Chromatographic separation was achieved using a Kinetex C18 column (50 $\times$ 2.1 mm, 2.6 μ m, Phenomenex) maintained at 40°C. The mobile phase comprised 0.1 % formic acid in distilled water (A) and 0.1 % formic acid in acetonitrile (B). The gradient elution program was as follows: B was held at 50 % from 0 to 0.5 min, increased to 95 % by 2.0 min, maintained at 95 % until 3.0 min, then returned to 50 % at 3.1 min and held until 4.0 min. The flow rate was set at 0.45 mL/min, the injection volume was 1 μ L, and the autosampler temperature was maintained at 15°C. Mass spectrometric detection was carried out in positive ion mode using electrospray ionization (ESI). Quantification was conducted in multiple reaction monitoring (MRM) mode. The MRM transitions were *m/z* 195.1 $\rightarrow$ 189.1 for tanshinone IIA and *m/z* 308.9 $\rightarrow$ 163.0 for the internal standard, Warfarin. The MS/MS parameters were

Table 1
Forward and reverse primer sequences used to amplify target mouse genes.

Target	Forward primer sequence	Reverse primer sequence
β-Actin	5'- CTGTCCTGTATGCCTCTG-3'	5'- ATGTCACGCACGATTTC-3'
CYP11A1	5'- ACA TGGCCAAGATGGTACAGTTG-3'	5'- ACGAAGCACCAGGTCATTAC-3'
CYP17A1	5'- CTCCAGCCTGACAGACATTCTG-3'	5'- TCTCCACCGTGACAAGGAT-3'
STAR	5'- GTGCTTCATCCACTGGCTGGAA-3'	5'- GTCTGCGATAGGACCTGGTTGA-3'
3β-HSD	5'- AGAACTGCAGGAGGTGACAGCT-3'	5'- GGCATCCAGAATGTCTCTCTCC-3'
17β-HSD	5'- CTGTGACACCGTACAACGGA-3'	5'- GCTCGGGTGATCCGATTCT-3'
5α-Reductase	5'- CGTCCTGCTGGCTATGTTTC-3'	5'- GAAGGCCAAGACAAAGGTGA-3'
Nrf2	5'- CACATCCAGACAGACACCAGT-3'	5'- CTACAAATGGGAATGTCTCTGC-3'
HO-1	5'- ACAGGGTGACAGAAGAGGCTAA-3'	5'- CTGTGAGGGACTCTGGTCTTTG-3'
CAT	5'-GAGGCAGTGTACTGCAAGTTC-3'	5'-GGGACAGTTCACAGGTATCTGC-3'
SOD	5'-GGTGAACCAAGTGTGTGTCAGG-3'	5'-ATGAGGTCTGCACCTGGTACAG-3'
GPx	5'-AGTTCGGACATCAGGAGAATGGCA-3'	5'-TCACCAATTCACCTGCACCTTCTCA-3'

CYP11A1, cytochrome P450 cholesterol side-chain cleavage enzyme; CYP17A1, cytochrome P450 17α-hydroxylase/C17–20 lyase; STAR, steroidogenic acute regulatory; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme-oxygenase-1; CAT, catalase; SOD, superoxide dismutase; and GPx, glutathione peroxidase.

as follows: for tanshinone IIA, DP 176 V, EP 10 V, CE 83 V, and CXP 18 V; for warfarin, DP 46 V, EP 10 V, CE 21 V, and CXP 10 V. The ion source temperature was set at 600°C, and the ionspray voltage was 5500 V.

2.3. Cell culture

Cells were maintained in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12; Sigma-Aldrich, St. Louis, USA) supplemented with 5 % horse serum (A&E Scientific PAA, Austria), 2.5 % fetal bovine serum (FBS; Merck, Berlin, Germany), 2.5 mM/L- glutamine (Sigma-Aldrich, St. Louis, USA), and 1 % penicillin/streptomycin (Invitrogen, CA, USA) in a humidified incubator with 5 % CO₂ at 37°C.

2.4. Cell viability assay

Mouse Leydig TM3 cells (1 × 10⁴ cells/well) were seeded and allowed to adhere for 24 h in 96-well plates. The cells were exposed to various concentrations of H₂O₂ (12.5, 25, 100, 200, 400, and 800 μM) or SME (1, 2, 3, 4, 5, 6, 7 and 8 μg/mL) for 24 h or treated with 200 μM H₂O₂ in the presence and absence of 1, 2, 3 and 4 μg/mL of SME for 24 h at 37 °C under 5 % CO₂ atmosphere. Cell viability was determined using the Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA). SME prepared in DMSO was used as the control. The H₂O₂ concentration was selected based on the previously published reports [28–30], while SME was tested at a wide range of concentrations, including 0–500 μg/mL (data not shown), to select the 0–8 μg/mL concentration range.

2.5. Laboratory animals and diet

Sprague-Dawley (SD) male rats aged 8 and 36 weeks were procured and subjected to a one-week acclimation period. Subsequently, the rats were randomly assigned to groups of eight animals each, and the samples were administered orally for 6 weeks (n = 48). The experimental design comprised six distinct groups: YC (young control, normal control, 8 weeks old, vehicle administration), OC (old control, aging control, 36 weeks old, vehicle administration), PC (positive control, testofen 300 mg/kg administration), SEL (low-dose SME, 50 mg/kg administration), SEM (medium-dose SME, 75 mg/kg administration), and SEH (high-dose SME, 100 mg/kg administration). All experimental animals were housed in an animal facility with a consistent 12-h light/dark cycle, a temperature of 23 ± 2°C, and humidity levels maintained at 50 ± 10 %. The animals had ad libitum access to food and water and were fed standard rat feed (Purina, Seoul, Korea). The Testofen dose was decided based on the previously published report on andropause [31], and SME doses were selected following the criteria for human and rat

application. If the human body (60 kg) requires 480, 720, and 920 mg per day, it's the same as 50, 75, and 100 mg per day for a rat [32].

2.6. Male hormones and biochemical indicators

To analyze the testosterone levels *in vitro*, mouse Leydig TM3 cells (8 × 10⁵ cells/well) were seeded and allowed to adhere for 24 h in 6-well plates. The cells were exposed to 200 μM H₂O₂ in the presence and absence of 1, 2, 3, and 4 μg/mL of SME for 12 h at 37 °C under a 5 % CO₂ atmosphere. The culture medium was collected, centrifuged (300x g, 4 °C), and stored at –20°C until hormone measurement. According to the manufacturer's instructions, the testosterone concentration in the samples was measured using an ELISA kit. The absorbance was measured at 450 nm using a microplate reader.

To analyze biochemical parameters, blood was collected from anesthetized rats by cardiac puncture after completion of the experiments, and sera were separated using SST tubes. Serum concentrations of testosterone, DHT, progesterone, 3β-HSD, 17β-HSD, 5α-reductase, FSH, LH, SHBG, and PSA were quantified using specific ELISA kits. Additionally, liver function and lipid metabolism indicators, including triglycerides (TG, AM157S-K), total cholesterol (TC, AM202-K), aspartate transaminase (AST, AM103-K), alanine transaminase (ALT, AM102-K), and high-density lipoprotein (HDL) were analyzed using a biochemical analysis kit from Asan Pharmaceutical (Seoul, Korea). LDL was calculated as follows: LDL = TC-(TG/5 + HDL).

2.7. RNA preparation and real-time PCR

For cellular mRNA expression analysis, TM3 cells (8 × 10⁵ cells/well) were seeded and allowed to adhere for 24 h, in 6-well plates. The cells were exposed to 200 μM H₂O₂ in the presence and absence of 1, 2, 3, and 4 μg/mL of SME for 6 h. For animal tissue analysis, rat testicular tissue was analyzed. Total RNA was extracted using the RNA extraction kit (iNTRON Biotechnology). RNA (50 ng) was reverse-transcribed into complementary DNA by PCR (TaKaRa Bio, Japan). RT-PCR was performed using TB Green (TaKaRa Bio, Kusatsu, Shiga, Japan) in an ABI QuantStudio 3 PCR system (Applied Biosystems). Each experiment was performed in triplicate. Specific primer sets for the target genes are listed in Table 1. The β-actin gene was used as an endogenous control to normalize gene expression.

2.8. Western blotting

For cellular protein expression analysis, mouse Leydig TM3 cells (8 × 10⁵ cells/well) were seeded and allowed to adhere for 24 h in 6-well plates. The cells were exposed to 200 μM H₂O₂ in the presence and absence of 1, 2, 3, and 4 μg/mL of SME for 12 h. To determine the protein expression levels, total proteins from different treatment groups

were isolated using a lysis buffer (iNtRON Biotechnology) with protease and phosphatase inhibitors. RIPA buffer supplemented with protease and phosphatase inhibitors was used for protein extraction from the rat testicular tissue. A total of 40 µg of protein was separated via SDS-PAGE and subsequently transferred onto a PVDF membrane. The membrane was blocked with 5 % skim milk for 1 h and then incubated overnight at 4°C with primary antibodies anti-β-actin (1:1000), anti-CYP11A1 (1:500), anti-CYP17A1 (1:1000), anti-STAR (1:500), anti-3β-HSD (1:500), anti-17β-HSD (1:500), anti-5α-reductase (1:500), anti-Nrf2 (1:500), anti-HO-1 (1:1000), anti-CAT (1:500), anti-SOD (1:500), and anti-GPx (1:500). The membrane was then incubated with secondary antibody at room temperature for 1 h. An ECL solution (Miracle-Star™, iNtRON Biotechnology, Korea) was then applied to visualize the proteins using an ImageQuant LAS500 (GE Healthcare Life Sciences, USA) device, and quantitative analysis was conducted using Amersham Imager 680 software (GE Healthcare Life Sciences). Some membrane blots were stripped by submerging them in the stripping buffer (iNtRON Biotechnology, Seongnam, Korea), covering them with foil paper, and incubating them at 37 °C for 25 min. Following this, the membrane blots were washed using 1 × TBST three times at 10-min intervals, blocked for 1 h, and re-probed by incubating the membrane blots with another primary antibody for 2 h at room temperature. After incubation with the primary antibody, the membranes were washed and incubated with the corresponding secondary antibodies for 1 h at room temperature, followed by quantitative analysis.

2.9. Histological analysis

Testicular tissues were fixed in 10 % neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 µm. Hematoxylin and eosin (H&E) staining was performed to examine the overall histological architecture, including the morphology of seminiferous tubules and the organization of germ cells. Apoptotic cells were detected using a TUNEL assay kit (ab206386, HRP-DAB method), according to the manufacturer's protocol. All stained sections were observed under a light microscope (BX51; Olympus, Japan).

2.10. Microbiome analysis

Metagenomic amplicon sequencing of the 16S rRNA region was performed using next-generation sequencing (NGS) on an Illumina platform. The samples were prepared according to the NGS library preparation procedure. The sequencing library was constructed using Herculase II fusion DNA polymerase and the Nextera XT index V2 Kit (Agilent Technologies, Santa Clara, CA, USA). Paired-end sequence reads were generated using the sequencing-by-synthesis approach employed in the Illumina sequencing system.

2.11. Gut microbiome sequence analysis

The Quantitative Insights into Microbial Ecology (QIIME) version 2 pipeline was used to analyze paired-end reads derived from amplicon sequences of the 16S rRNA region [33]. Divisive amplicon denoising algorithm 2 (DADA2) was used for quality control analysis, including denoising, trimming, and filtering of low-quality reads, and chimeras were generated after manual visualization using QIIME modules [34]. Unique error free-sequences termed as Amplicon sequence variants (ASVs) obtained after DADA2 analysis were subjected to multiple sequence alignments for phylogenetic studies [35]. Phylogenetic trees were constructed from this alignment using FastTree [36].

2.12. Taxonomic annotation

Greengenes 13.8 99 % OTU-based taxonomy classifier [37] was used to annotate ASVs unique sequences using the QIIME classifier module, which uses the naïve Bayes algorithm for taxonomic annotations. Bar

plots were used to graphically depict the taxonomic annotations of different groups [38].

2.13. Alpha and beta diversity analyses

Alpha diversity was assessed using Faith's phylogenetic diversity and Pielou's evenness in the QIIME workflow. Beta diversity was studied through community dissimilarity using both qualitative and quantitative measures, specifically the Jaccard and Bray-Curtis distance measures.

2.14. Abundance of taxa in the groups

The linear discriminant analysis effect size (LEfSe) method was employed to identify differentially abundant species among the various groups [39–42]. A standalone Docker-based LEfSe analysis was used to study differentially abundant taxa. The ASV table, metadata information, and 7th-level (species-level) collapsed taxonomy results were used for the differential abundance analysis. Bar graphs and cladograms were constructed to visualize the differentially abundant species among different groups [39].

2.15. Statistical analysis

The sample size, $n = 3$ for *in vitro* and $n = 6$ for *in vivo* experiments, was used, and the results are presented as the mean ± standard deviation. To assess the significance of differences between experimental groups, one-way ANOVA was conducted using GraphPad Prism 10.3.1 (GraphPad Software, San Diego, CA, USA), followed by Dunnett's test for group comparisons. Statistical significance was set at a p -value of less than 0.05.

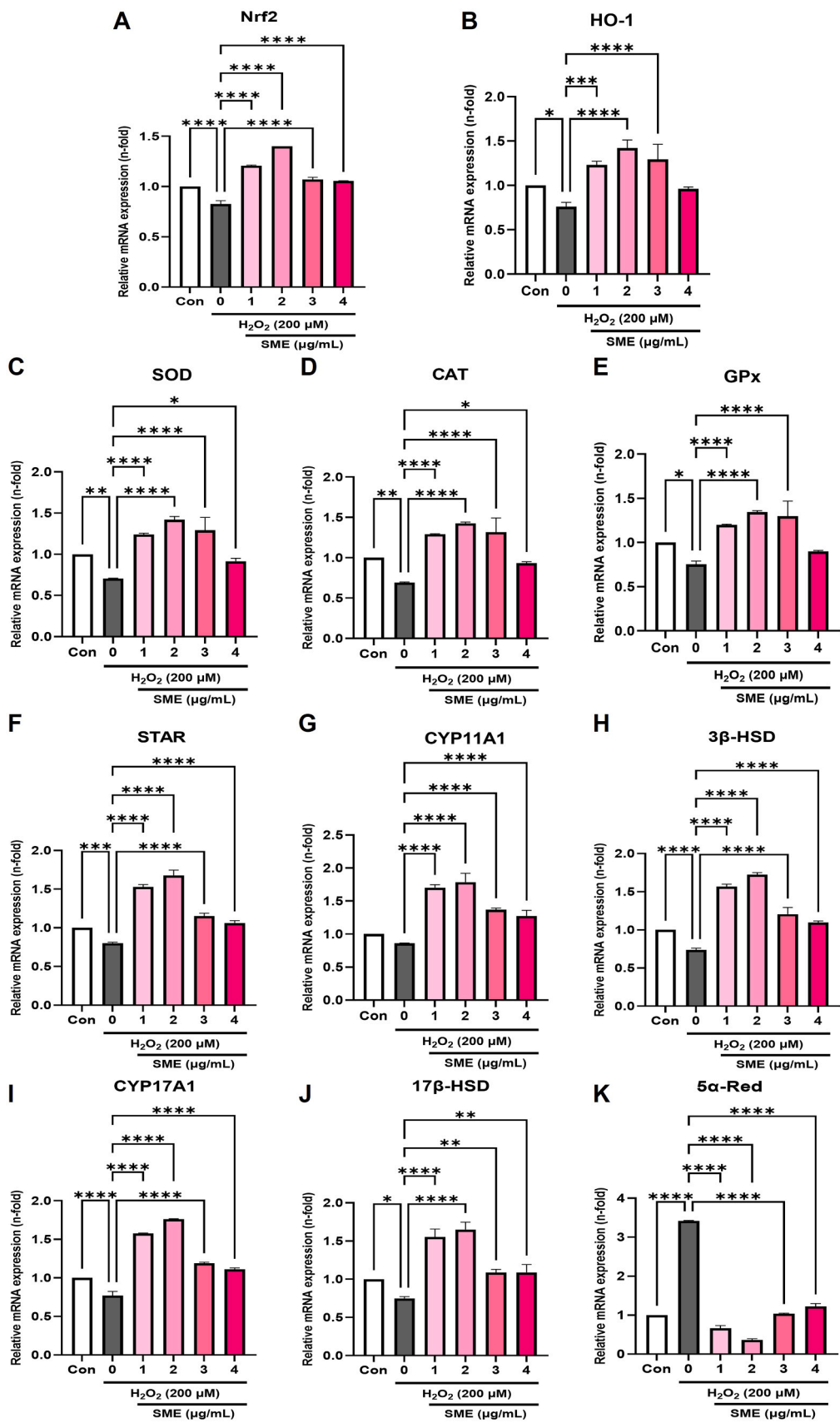
3. Results

3.1. Quantitative analysis of tanshinone IIA in SME and serum of experimental animals

The quantification of tanshinone IIA content in the SME was conducted using HPLC/MS analysis, with warfarin (1 ng/mL) serving as an internal standard to enhance the accuracy and precision of the measurement. A matrix-matched method was utilized to assess the tanshinone IIA content in the extract, which was determined to be 10.629 mg/g to simulate an *in vivo* environment. Following oral administration of the extract, serum levels of tanshinone IIA were analyzed. No tanshinone IIA was detected in the YC and OC groups. Conversely, all SME-treated groups exhibited detectable levels of tanshinone IIA in the serum, with average concentrations of 1.146 ng/mL in SEL, 0.775 ng/mL in SEM, and 0.708 ng/mL in SEH groups (Supplementary Table S1). These findings suggest that tanshinone IIA from the extract was absorbed or distributed systemically following oral administration. The peaks showing the presence of tanshinone IIA across different groups are shown in Supplementary Fig. S1.

3.2. SME protects TM3 cells from H₂O₂-induced cell death

The concentration of H₂O₂ was screened to observe its toxic effects by treating Leydig TM3 cells with 12.5, 25, 50, 100, 200, 400, and 800 µM. H₂O₂ was not toxic up to 50 µM concentration and only significantly reduced the cell viability at concentrations of 100 µM and above (Supplementary Fig. S2A). The 200 µM concentration was selected for further experiments. Cells were treated with 1, 2, 3, 4, 5, 6, 7, and 8 µg/mL of SME. The SME at 1–4 µg/mL did not show any cytotoxic effects and was used for further experiments. To observe the cytoprotective effects of SME on H₂O₂-induced cell death in TM3 cells, the cells were exposed to 200 µM of H₂O₂ in the presence and absence of various concentrations (1, 2, 3, and 4 µg/mL) of SME. The results



(caption on next page)

Fig. 1. SME modulates the transcriptional expression level of antioxidants and steroidogenic pathway-associated markers *in vitro*. Mouse Leydig TM3 cells were exposed to 200 μM H_2O_2 in the presence and absence of SME (0, 1, 2, 3, and 4 $\mu\text{g}/\text{mL}$) for 6 h. The mRNA expression levels of antioxidants, Nrf2, HO-1, SOD, CAT, and GPx (A-E), and steroidogenic pathway-associated markers STAR, CYP11A1, 3β -HSD, CYP17A1, 17β -HSD, and 5α -reductase (F-K) were observed through RT-PCR ($n = 3$). SME, *Salvia miltiorrhiza* extract; H_2O_2 , hydrogen peroxide; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme-oxygenase-1; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; STAR, steroidogenic acute regulatory; CYP11A1, cytochrome P450 cholesterol side-chain cleavage enzyme; CYP17A1, cytochrome P450 17α -hydroxylase/C17–20 lyase; 3β -HSD, 3β -hydroxysteroid dehydrogenase; 17β -HSD, 17β -hydroxysteroid dehydrogenase. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

showed that SME had cytoprotective effects, as it attenuated the H_2O_2 -induced reduction in cell viability (Supplementary Fig. S2B and S2C).

3.3. SME modulates the transcriptional expression level of antioxidants and steroidogenic pathway-associated markers *in vitro*

The mRNA expression levels of the transcription factor Nrf2 and antioxidants such as HO-1, SOD, CAT, and GPx were significantly downregulated in H_2O_2 -induced TM3 cells; however, treatment with SME reversed the H_2O_2 -induced downregulation of these antioxidants (Fig. 1A–1E). Additionally, exposure to H_2O_2 in TM3 cells reduced the mRNA expression levels of STAR, CYP11A1, 3β -HSD, CYP17A1, and 17β -HSD, while enhancing the mRNA expression levels of 5α -reductase, indicating impaired steroidogenic signaling. However, the treatment with SME at all concentrations significantly attenuated the H_2O_2 -induced reduction in STAR, CYP11A1, CYP17A1, 3β -HSD, and 17β -HSD, and reversed the increase in 5α -reductase (Fig. 1F–1K).

3.4. SME modulates the translational expression level of antioxidants and steroidogenic pathway-associated markers *in vitro*

The translational expression levels followed the same trend as the transcriptional levels, showing decreased protein expression of Nrf2, HO-1, SOD, CAT, and GPx upon H_2O_2 -exposure. However, treatment increased the protein expression levels of Nrf2, HO-1, SOD, CAT, and GPx. Similarly, in the case of steroidogenic pathway-associated marker expression, H_2O_2 downregulated the protein expression levels of STAR, CYP11A1, 3β -HSD, CYP17A1, and 17β -HSD and upregulated the protein expression levels of 5α -reductase (Fig. 2A–2E). SME treatment ameliorated H_2O_2 -induced effects, as SME at all the concentrations significantly upregulated the protein expression of STAR, CYP11A1, 3β -HSD, CYP17A1, and 17β -HSD and downregulated the protein expression of 5α -reductase (Fig. 2F–2K).

3.5. SME modulates the transcriptional levels of antioxidants *in vivo* and improves testicular histology and apoptosis

The *in vitro* results were complemented by the *in vivo* findings as the positive control (PC, testofen), and different doses of SME (SEL, SEM, and SEH) also enhanced the mRNA expression levels of these antioxidants compared to the old age control (OC) group (Fig. 3A–3E). TUNEL assays revealed increased apoptosis accompanied by substantial tissue damage in the testicular tissue of the OC group. In addition, H&E staining revealed disrupted seminiferous tubule architecture, reduced germ cell layers, and degenerated Leydig cells in the OC group. All these histological alterations were attenuated in testes treated with orally administered SME and PC (Fig. 3F). These findings suggest that SME possesses antioxidant properties and confers protective effects on testicular tissues [19,43].

3.6. SME regulates steroidogenic pathway-associated markers

Similarly, in the *in vivo* experiments, the serum levels of 3β -HSD and 17β -HSD, along with the protein expression levels of enzymes CYP11A1, CYP17A1, and STAR, were reduced in the OC group, indicating an impaired steroidogenic pathway. However, oral administration with testofen and all SEM doses (SEL, SEM, and SEH) attenuated the old age-induced effects on the steroidogenic pathway, as they significantly

upregulated the serum levels of 3β -HSD and 17β -HSD and the protein expression levels of CYP11A1, CYP17A1, and STAR (Fig. 4A–4E). The OC group showed increased serum levels of 5α -reductase compared to the young age control (YC), and oral administration with PC and three doses of SME reduced serum levels of 5α -reductase compared to OC (Fig. 4F). These findings suggest that SME effectively enhanced the expression of steroidogenic pathway-associated markers.

3.7. SME regulates hormone levels both *in vitro* and *in vivo*

The H_2O_2 -exposed TM3 cells showed reduced (non-significant) testosterone levels; however, SME, at 1 and 2 $\mu\text{g}/\text{mL}$ of concentration only, increased testosterone levels (Fig. 5A). FSH and LH secreted by the hypothalamic-pituitary axis are crucial for the maintenance of male reproductive function. LH stimulates Leydig cells in the testes to produce testosterone, thereby regulating spermatogenesis initiation and maintenance. In conjunction with LH, FSH stimulates Sertoli cells, facilitating sperm maturation and the overall reproductive function. In the present study, the serum FSH and LH levels were significantly lower in the OC group than in the YC group. However, the FSH and LH levels were higher in the PC- and SME-treated groups (SEL, SEM, and SEH) than in the OC group. Note that all orally administered PC- and SME-treated groups showed a significant increase in FSH, but only SEL significantly increased serum LH levels (Figs. 5B and 5C). Progesterone levels were substantially reduced in the OC group compared to the YC group; however, the orally administered PC- and SME-treated groups showed increased progesterone levels, indicating positive steroidogenic downstream signaling (Fig. 5D). In addition, the serum SHBG and DHT levels were elevated in the OC group compared to the YC group, whereas the PC- and SME-treated groups (SEL, SEM, and SEH) demonstrated significantly reduced SHBG and DHT levels (Figs. 5E and 5F). This indicates that SME may inhibit SHBG and DHT, as it directly and indirectly influences testosterone levels. Finally, the age-associated testosterone decline observed in the OC group was ameliorated by orally administered PC and all three selected doses of SME (Fig. 5G). The effects of different doses of SME were compared and interpreted (Supplementary Table S2). Also, a table summarizing the p-values for all the markers studied in *in vitro* and *in vivo* experiments is shown in Supplementary Table S3.

3.8. SME regulates liver injury-associated markers and PSA

To observe the effects of SME on liver injury, liver injury-associated markers such as ALT and AST were measured in the different groups. Serum AST levels were significantly increased, with no change in ALT levels in the OC group compared to the YC group (Figs. 6A, 6B). However, SME treatment led to a decrease in serum AST levels compared to those in the OC group. Lipid biomarkers such as TG and TC were found to be higher in the OC group than in the YC group, indicating age-associated liver diseases; however, treatment with PC and SME (SEL, SEM, and SEH) reversed the age-associated increase in TG and TC levels (Figs. 6C and 6D). These data support the anti-hyperlipidemic properties of SME in older rats. In addition, an imbalance between LDL and HDL levels indicates age-associated impaired liver function or a risk of heart disease and stroke. The oral administration of PC and all three doses of SME reduced the age-associated increase in LDL levels and significantly increased the age-associated decrease in HDL levels (Figs. 6E and 6F). Additionally, significantly enhanced levels of malondialdehyde (MDA)

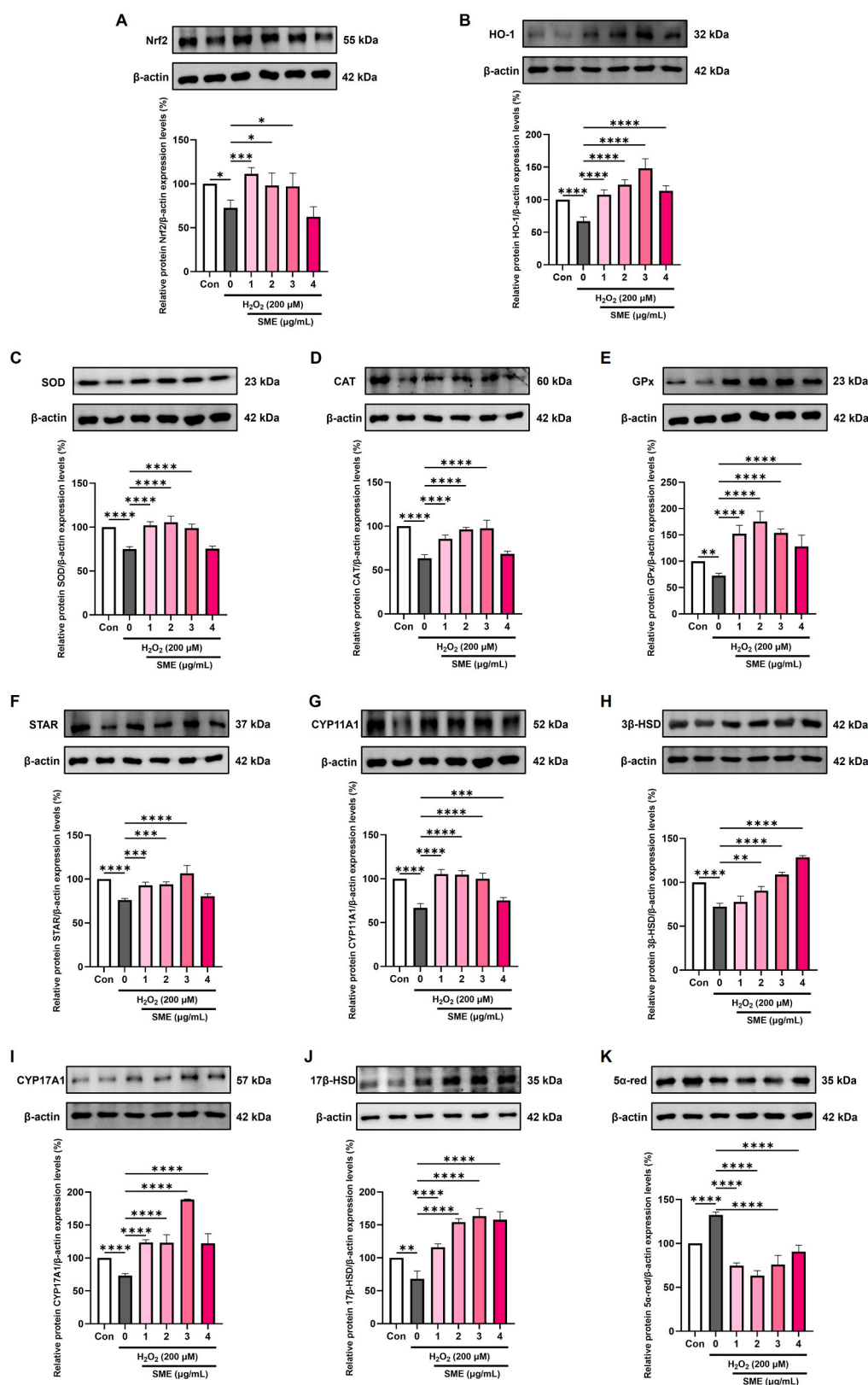


Fig. 2. SME modulates the translational expression level of antioxidants and steroidogenic pathway-associated markers *in vitro*. Mouse Leydig TM3 cells were exposed to 200 μ M H₂O₂ in the presence and absence of SME (0, 1, 2, 3, and 4 μ g/mL) for 12 h. Protein expression levels of antioxidants, Nrf2, HO-1, SOD, CAT, and GPx (A-E) and steroidogenic pathway-associated markers STAR, CYP11A1, 3 β -HSD, CYP17A1, 17 β -HSD, and 5 α -reductase (F-K) were observed through western blotting. SME, *Salvia miltiorrhiza* extract; H₂O₂, hydrogen peroxide; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme-oxygenase-1; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; STAR, steroidogenic acute regulatory; CYP11A1, cytochrome P450 cholesterol side-chain cleavage enzyme; CYP17A1, cytochrome P450 17 α -hydroxylase/C17–20 lyase; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase. * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

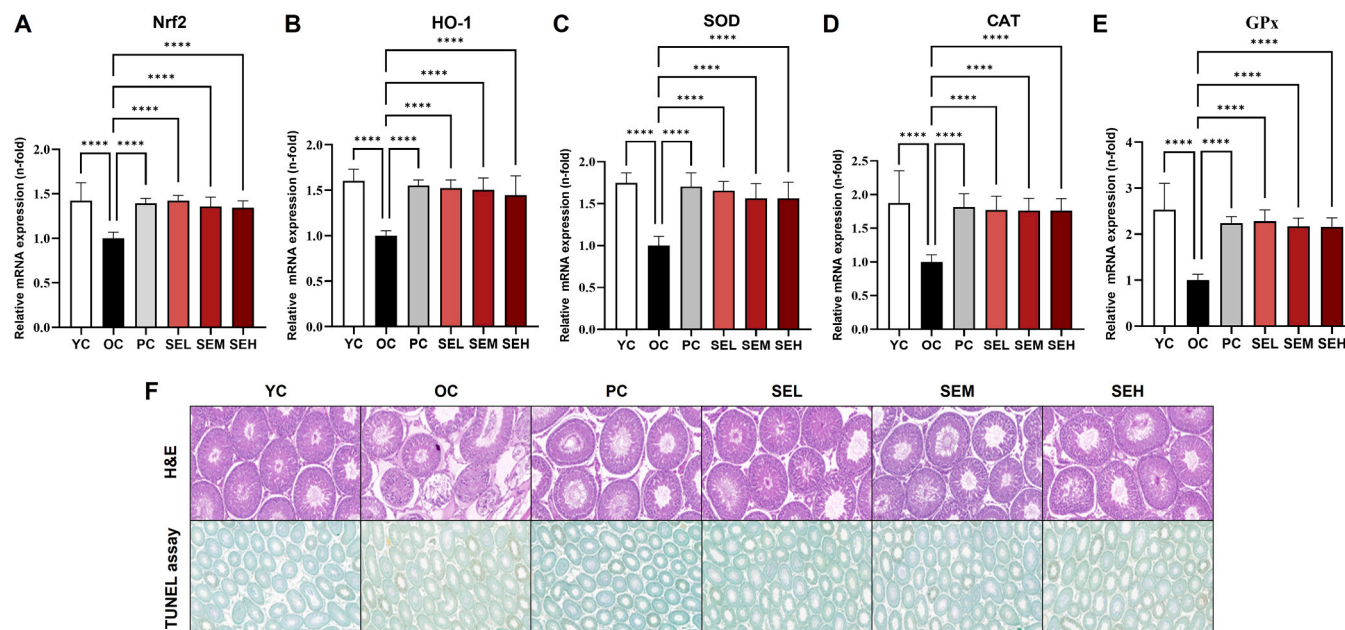


Fig. 3. SME modulates the expression level of antioxidants *in vivo* and improves testicular histology and apoptosis in testicular tissues. SD rats were divided into six groups and given oral administration for 6 weeks as follows: YC (young control, 8 weeks old, vehicle administration), OC (old control, aging control, 36 weeks old, vehicle administration), PC (positive control, 36 weeks old, Testofen 300 mg/kg administration), SEL (SME low-dose, 36 weeks old, 50 mg/kg administration), SEM (SME medium-dose, 36 weeks old, 75 mg/kg administration), and SEH (SME high-dose, 36 weeks old, 100 mg/kg administration). mRNA expression levels of Nrf2 (A), HO-1 (B), SOD (C), CAT (D), and GPx (E) were measured by RT-PCR ($n = 6$). (F) H&E and TUNEL staining were performed to observe histological alterations in testicular tissue. SME, *Salvia miltiorrhiza* extract; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme-oxygenase-1; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase. Data are expressed as mean $\pm$ SD. **** $p < 0.0001$.

in the liver tissues of the OC group indicated age-associated severe liver damage, which was significantly reduced in the PC- and SME-treated groups (Fig. 6G). These findings reflected the hepatoprotective and cardioprotective properties of SME.

Furthermore, PSA, a prostate health-associated marker, was also higher in the OC group than in the YC group, indicating age-associated poor prostate health. However, the PC- and SME-treated groups (SEL, SEM, and SEH) showed significantly reduced serum PSA levels, revealing the protective effects of SME against age-associated prostate health impairment (Fig. 6H).

3.9. Microbiome and diversity analyses

A total of 869,355 paired-end reads from the four groups were used for analysis. After quality control analysis (preprocessing), 11,645 unique features were identified in all samples. The lowest alpha diversity was observed in the high-dose treatment group compared to the low- and medium-dose treatment groups, but these differences were not significant. Similarly, Pielou's evenness was also weakest in the high-dose treatment group compared to the low- and medium-dose treatment groups, but the differences were not significant (Supplementary Fig. S3A, S3B). In the beta diversity analysis, slight differences were observed between the groups, which were only significant when compared to the young controls (Supplementary Fig. S3C, S3D).

3.10. Taxonomy and differential abundance of taxa between the groups

In box plot analysis, Firmicutes was the most abundant phylum in all samples. Bacteroidetes was the second most abundant phylum in most samples (Supplementary Fig. S4). Differential abundance analysis revealed the abundance of *Clostridium disporicum* and *Bifidobacterium breve* in the YC group, and *Ruminococcus callidus* and *Desulfovibrio oxamicus* were more abundant in the OC group. *Clostridium islandicum* and *Succinispira mobilis* were more abundant in the PC and SEL treatment groups, respectively (Fig. 7).

4. Discussion

Testosterone, the pivotal hormone in male physiology, decreases by approximately 1–2 % per year after age 30 [44]. The decrease in testosterone levels with age is related to various age-associated physiological changes, and the symptoms may include reduced libido, decreased sexual activity, impotence, fertility issues, changes in body composition, reduced body and facial hair, and osteoporosis [45,46]. Although testosterone-associated illness or andropause has long been recognized, it has not gained attention because various conflicting theories question its authenticity as a medical issue. Additionally, men's general hesitation in seeking medical assistance hinders medical advancement. Although andropause is associated with impaired puberty, impotence, gynecomastia, infertility, and a decrease in spermatogenesis, several studies have explained the relationship between low testosterone levels and an increased risk of cardiovascular disease, bone fractures, metabolic syndrome, and type 2 diabetes. Additionally, with age, the levels of SHBG increase, reducing the bioavailable form of testosterone from the blood, leading to decreased sex drive, erectile dysfunction, fatigue, changes in mood and cognition, and reduced muscle mass and density [47,48]. Testosterone replacement therapy is commercially available to increase testosterone levels. However, its prolonged use has been reported to have potential side effects, including an increased risk of prostate cancer and cardiovascular illness [49]. Natural compounds or plant products could be alternative therapeutic options in this case because they are less toxic and more cost-effective. *S. miltiorrhiza*, which exhibits various bioactivities, has not yet been studied in the context of andropause. We explored the beneficial effects of *S. miltiorrhiza* on age-associated OS, steroidogenic pathway impairment, and gut dysbiosis.

Leydig TM3 mouse cells were used to design an OS-induced *in vitro* andropause-like pathology. H_2O_2 induces cell cycle arrest and cell death and induces OS in TM3 cells [28]. In the present study, it was used at various concentrations to induce cell death in TM3 cells. H_2O_2 was cytotoxic to cells at concentrations of 100 μ M or higher. Thus, a 200 μ M

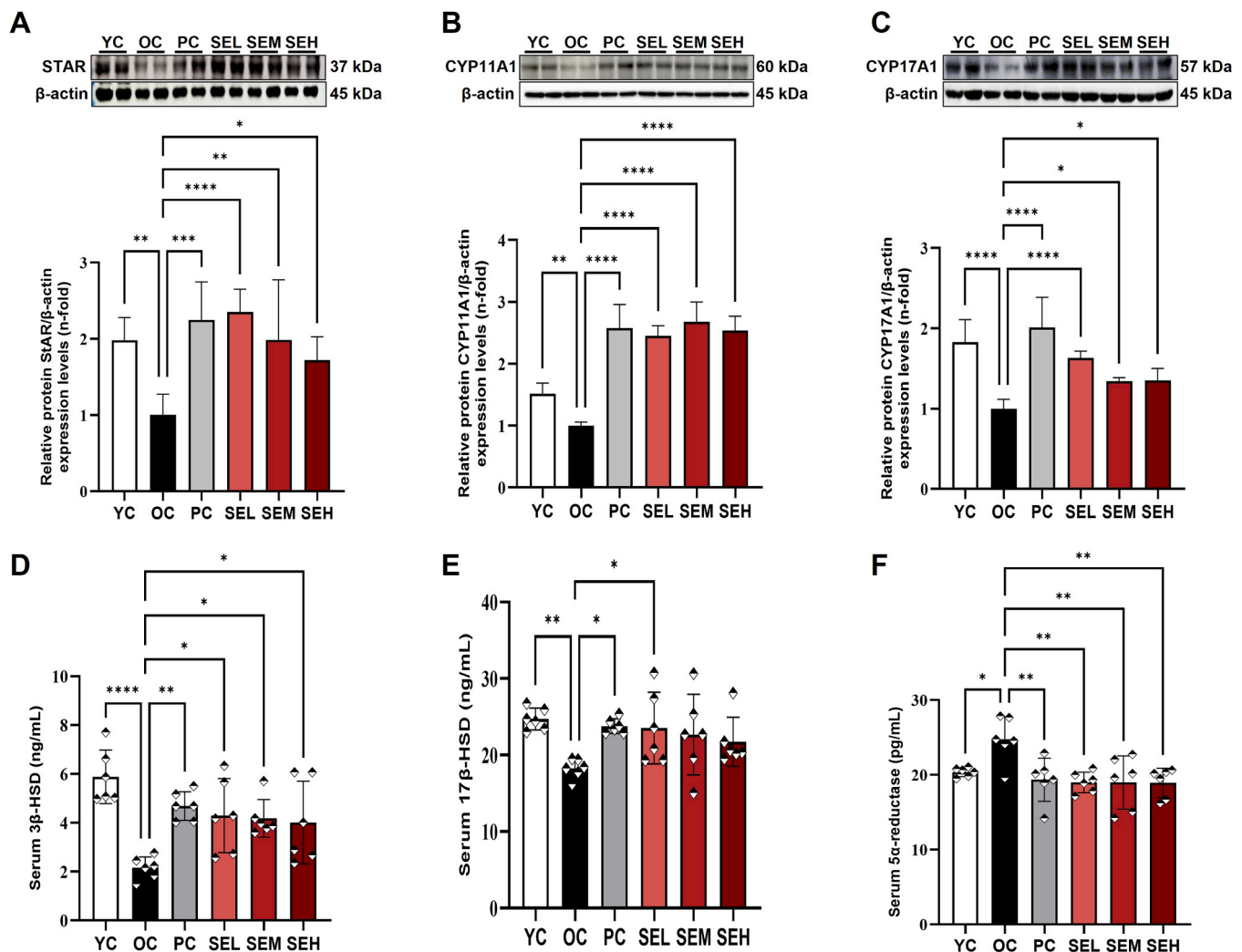


Fig. 4. SME regulates steroidogenic pathway-associated markers *in vivo*. SD rats were divided into six groups and given oral administration for 6 weeks as follows: YC (young control, 8 weeks old, vehicle administration), OC (old control, aging control, 36 weeks old, vehicle administration), PC (positive control, 36 weeks old, Testofen 300 mg/kg administration), SEL (SME low-dose, 36 weeks old, 50 mg/kg administration), SEM (SME medium-dose, 36 weeks old, 75 mg/kg administration), and SEH (SME high-dose, 36 weeks old, 100 mg/kg administration). The protein expression levels of STAR (A), CYP11A1 (B), and CYP17A1 (C) were assessed using western blot analysis ($n = 6$). Serum levels of 3 β -HSD (D), 17 β -HSD (E), and 5 α -reductase (F) were measured using specific ELISA kits ($n = 6$). SME, *Salvia miltiorrhiza* extract; STAR, steroidogenic acute regulatory; CYP11A1, cytochrome P450 cholesterol side-chain cleavage enzyme; CYP17A1, cytochrome P450 17 α -hydroxylase/C17–20 lyase; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase. Data are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

concentration was used for subsequent experiments. SME was nontoxic and showed cytoprotective effects at all selected concentrations. The results were consistent with previously reported *Morinda officinalis* extract, as it also protected the TM3 cells against H_2O_2 -induced cytotoxicity [50]. OS is associated with decreased testosterone levels and impaired Nrf2/HO-1 signaling might be an essential target for andropause. H_2O_2 impairs Nrf2/HO-1 signaling, as the mRNA and protein expression levels of Nrf2, HO-1, SOD, CAT, and GPx are downregulated after H_2O_2 treatment, consistent with previous studies [50,51]. In addition, the old-age control group (OC) showed downregulated expression of Nrf2, HO-1, SOD, CAT, and GPx, indicating age-associated OS. However, treatment with SME improved overall survival by increasing the mRNA and protein expression levels of various antioxidants. Histological analysis of testicular tissues revealed the potential of SME to protect the testes from age-associated OS.

Depletion in antioxidant GSH is involved in reducing testosterone synthesis [18], and OS is associated with impaired steroidogenic pathways by reducing STAR, cytochrome P450, and cAMP production [10]. The reduction in 3 β -HSD, SOD, CAT, and glutathione-S-transferase

activity was observed in OS-induced testicular macrophages [14,15]. Previously, *Taraxacum officinale* extract induced mRNA and protein expression levels of CYP11A1, CYP17A1, and STAR, and testosterone levels in Leydig's TM3 cells [52]. In addition, a report published on *Acorus gramineus* extract showed improved transcriptional levels of STAR, CYP11A1, CYP17A1, and 17 β -HSD, increased serum total testosterone and free testosterone levels, and reduced PSA, and total cholesterol levels upon *A. gramineus* extract administration in old age rats [53]. Similarly, in the present study, we attempted to assimilate the steroidogenic effects of SME both *in vitro* and *in vivo*. H_2O_2 reduced the transcriptional and translational levels of important steroidogenic pathway-associated markers such as STAR, CYP11A1, 3 β -HSD, CYP17A1, and 17 β -HSD, while inducing expression levels of 5 α -reductase. These results were most pronounced in old rats, revealing an impaired steroidogenic pathway. However, SME application, both *in vitro* and *in vivo*, improved the steroidogenic pathway by inducing the transcriptional and translational levels of significant steroidogenic pathway-associated markers, such as STAR, CYP11A1, 3 β -HSD, CYP17A1, and 17 β -HSD, and reducing the expression levels of

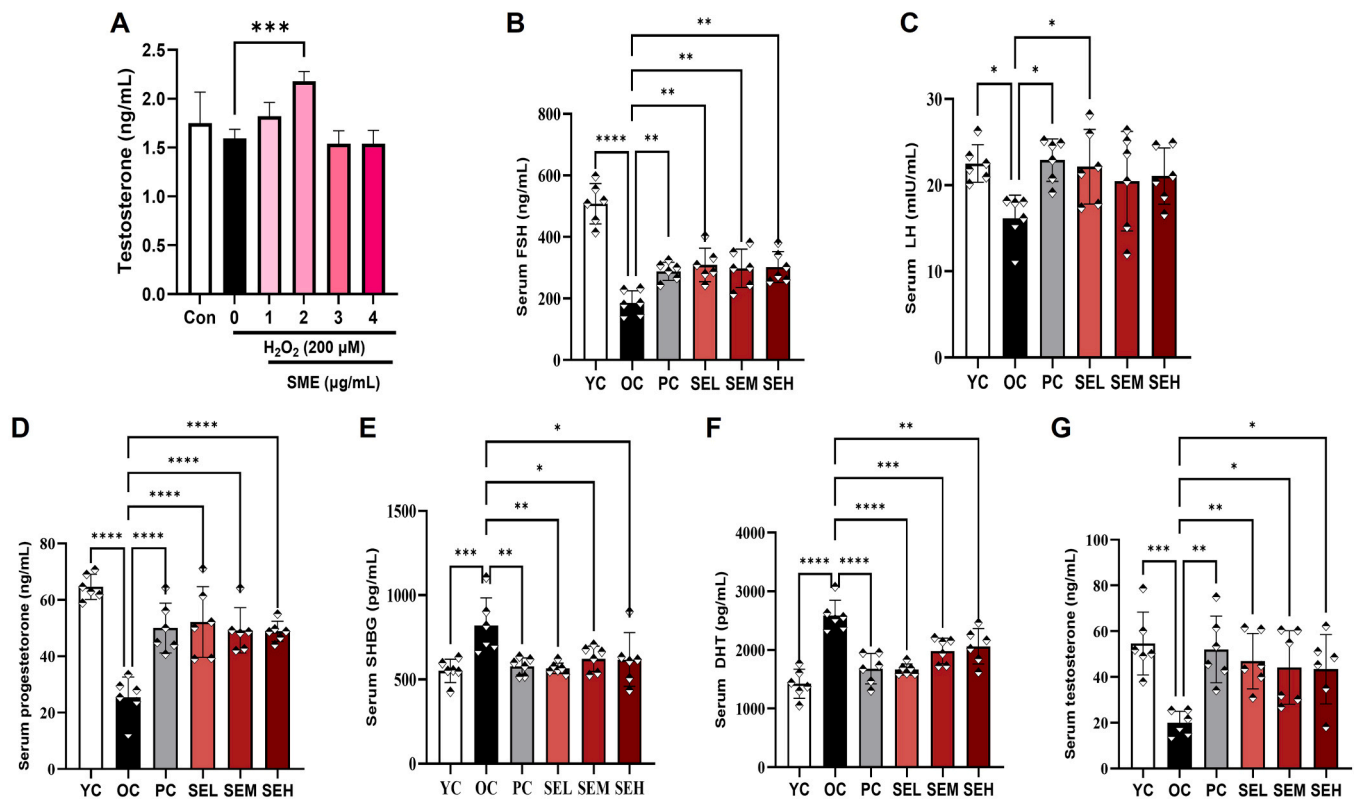


Fig. 5. SME regulates hormone levels both *in vitro* and *in vivo*. To observe the effects of SME on testosterone levels *in vitro*, mouse Leydig TM3 cells were exposed to 200 μ M H_2O_2 in the presence of SME (0, 1, 2, 3, and 4 μ g/mL) for 12 h. (A) Testosterone levels were measured using a specific enzyme-linked immunosorbent assay (ELISA) kit. For observing the effects of SME on hormone levels *in vivo*, the SD rats were divided into six groups and given oral administration for 6 weeks as follows: YC (young control, 8 weeks old, vehicle administration), OC (old control, aging control, 36 weeks old, vehicle administration), PC (positive control, 36 weeks old, Testofen 300 mg/kg administration), SEL (SME low-dose, 36 weeks old, 50 mg/kg administration), SEM (SME medium-dose, 36 weeks old, 75 mg/kg administration), and SEH (SME high-dose, 36 weeks old, 100 mg/kg administration). The serum levels of FSH (B), LH (C), progesterone (D), SHBG (E), DHT (F), and testosterone (G) were measured using specific ELISA kits ($n = 6$). SME, *Salvia miltiorrhiza* extract; H_2O_2 , hydrogen peroxide; SHBG, sex hormone-binding globulin; DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone. Data are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

5 α -reductase. SME also enhanced testosterone levels *in vitro* and *in vivo*, consistent with previous studies [29,51–53]. In addition, the testosterone precursor hormone, progesterone, increased after the application of SME in old rats. SHBG levels increase with age, associated with a progressive decline in bioavailable testosterone with aging [54], and 5 α -reductase is involved in converting testosterone to DHT, contributing to andropause [55]. SME administration reduced the age-associated increase in SHBG and DHT levels, revealing regulatory control of the steroidogenic pathway. Age-associated andropause could also be caused by low production of LH and FSH [56]. Rutin improves LH and FSH levels, enhancing hypogonadism in CCl₄-induced albino rats [57]. SME followed the pattern of previous studies, showing improvements in LH and FSH levels in old age rats, reflecting its promising role in enhancing sexual development and reproduction. SME regulates testosterone production at various levels, including controlling the steroidogenic pathway and regulating essential hormones such as LH and FSH.

Liver injury markers, such as AST, TG, and TC, and the lipid peroxidation marker MDA, were enhanced in an old-age control group, mimicking age-associated andropause characteristics [58]. However, oral administration of SME reversed age-associated liver injury by decreasing AST, TG, TC, and MDA levels. An imbalance between LDL and HDL levels is believed to be associated with testosterone decline [59]. The present study also found an increase in LDL and a decrease in HDL levels in old rats; however, SME treatment improved HDL levels and decreased LDL levels. These results are consistent with those of previously published reports on the protective effects of dehydroepiandrosterone against male hypogonadism [60]. Elevated levels of PSA, a

protein produced by the prostate gland, are a marker for prostate cancer and were reduced after SME administration compared to the OC group. This reveals the protective effects of SME against age-associated prostate diseases.

Recent studies have shown the importance of gut microbiota in regulating testicular function and androgen production by releasing endotoxins or their metabolites [61]. Several bacterial strains metabolize androgens; for example, *Comamonas testosteroni* utilizes testosterone [62]. Additionally, feeding *Lactobacillus reuteri* reversed the age-associated testosterone decline in old male mice [63]. *Bifidobacterium breve* in high abundance is a sign of a healthy gut [62]; in the present study, it was abundant in the YC group. The *Desulfovibrio oxamici* is associated with liver diseases [64] and was abundant in the feces of the OC group, indicating age-associated gut dysbiosis. However, SME-treated old age rats exhibited an increased abundance of *Succinispira mobilis*, an anaerobic succinate-decarboxylating bacterium that stimulates propionate production [65]. The *S. mobilis* abundance and changes in testosterone levels upon SME's application highlight potentially novel interactions between gut microbiota and endocrine function, though the underlying mechanisms are yet to be studied. Propionate, produced by members of the *Succinispira* genus, could be a possible mediator of this relationship. The SCFAs regulate cholesterol metabolism, fatty acid synthesis, hepatic lipogenesis, energy homeostasis, and apoptosis [66]. SCFAs, particularly propionate, can exert systemic effects via activation of G-protein-coupled receptors such as GPR41 and GPR43, which are expressed in various tissues, including the gastrointestinal tract, immune cells, and endocrine system components.

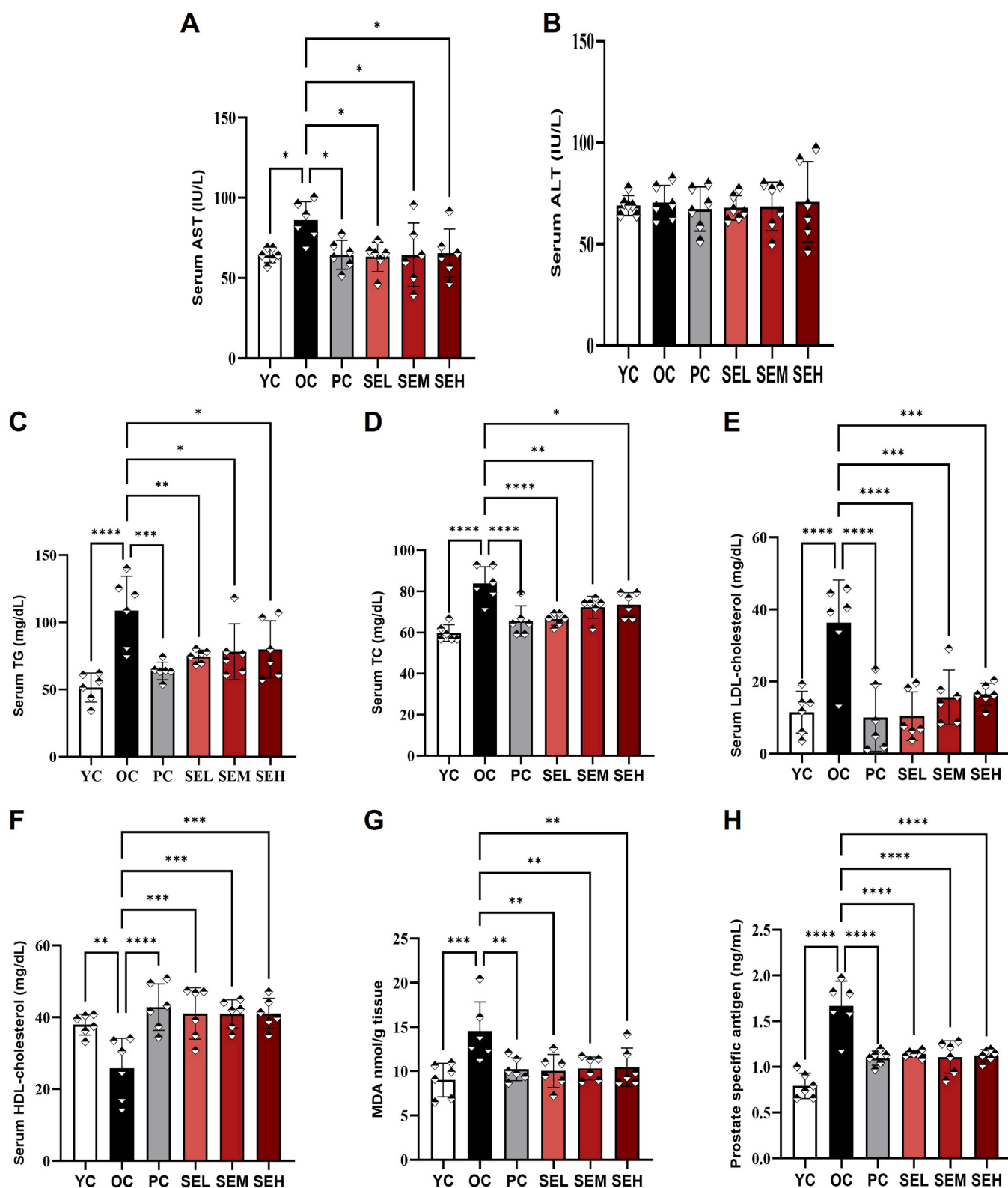


Fig. 6. SME regulates liver injury-associated markers and PSA. SD rats were divided into six groups and given oral administration for 6 weeks as follows: YC (young control, 8 weeks old, vehicle administration), OC (old control, aging control, 36 weeks old, vehicle administration), PC (positive control, 36 weeks old, Testofen 300 mg/kg administration), SEL (SME low-dose, 36 weeks old, 50 mg/kg administration), SEM (SME medium-dose, 36 weeks old, 75 mg/kg administration), and SEH (SME high-dose, 36 weeks old, 100 mg/kg administration). (A-G) Liver injury markers, including serum AST (A), serum ALT (B), serum TG (C), serum TC (D), serum LDL (E), serum HDL (F), MDA (G), and serum PSA (H) were measured ($n = 6$). SME, *Salvia miltiorrhiza* extract; AST, aspartate transaminase; ALT, alanine transaminase; TG, triglycerides; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MDA, malondialdehyde; PSA, prostate-specific antigen. Data are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

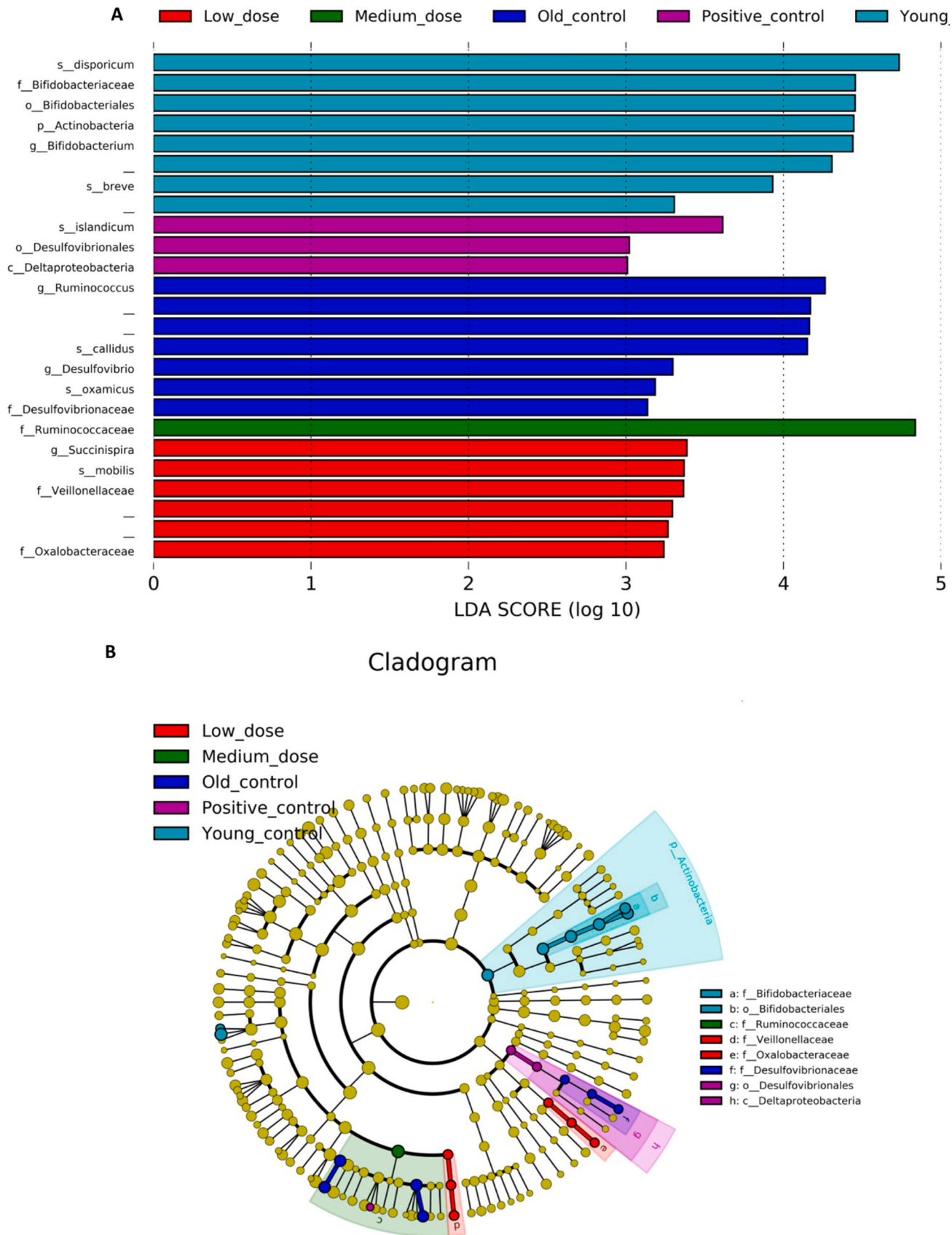


Fig. 7. Differential abundance of species. (A) Histogram of LDA scores for differential abundance of bacterial taxa. (B) The cladogram was calculated by LEfSe and displayed according to effect size. Only taxa meeting the LDA significant threshold of 2 are shown.

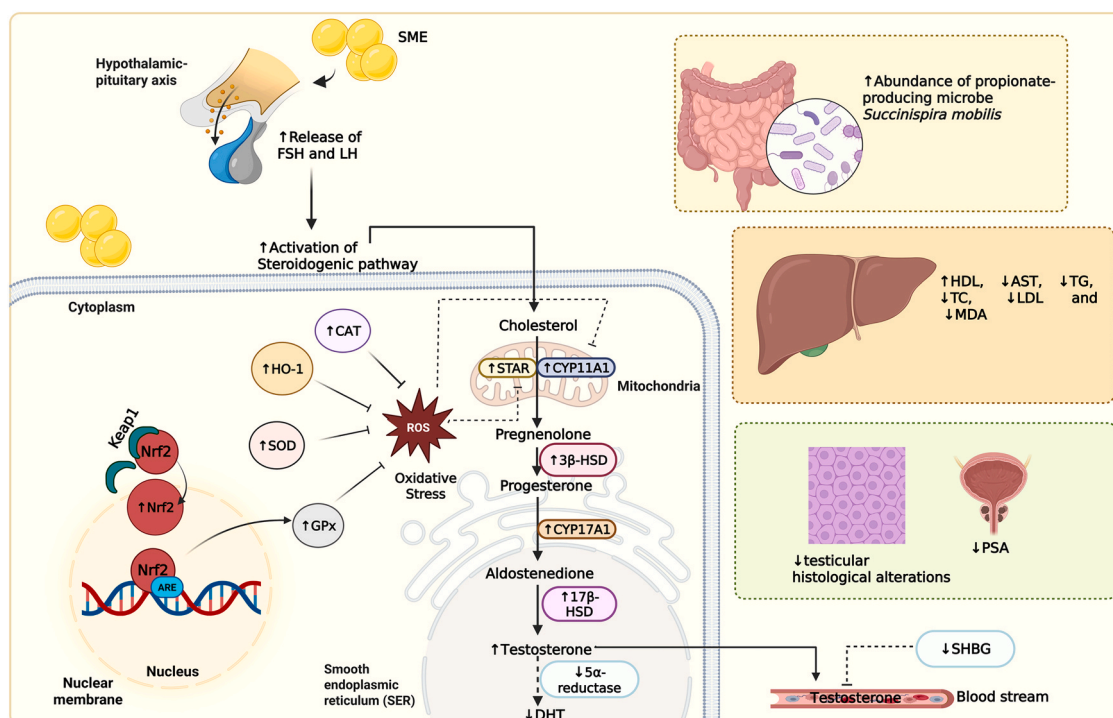


Fig. 8. SME ameliorates oxidative stress via activating Nrf2/HO-1 signaling, restores steroidogenesis, and improves gut microbiome. SME stimulates the production of FSH and LH from the hypothalamus-pituitary axis, which can further stimulate testosterone production by activating STAR, CYP11A1, 3β-HSD, CYP17A1, and 17β-HSD. In addition, SME activates the Nrf2/HO-1 signaling to enhance the expression levels of antioxidants such as GPx, SOD, and CAT, which further attenuate ROS and their inhibitory effects on age-associated STAR and CYP11A1. The effects of 5α-reductase, DHT, and SHBG are also mitigated by SME, enhancing the testosterone levels in the bloodstream. Furthermore, SME increases the abundance of the propionate-producing microbe *Succinispira mobilis* in the gut. The liver injury-associated markers, such as HDL, AST, TG, LDL, and MDA, are also modulated by SME. At last, SME improves histological alterations in testicular tissue and reduces the prostate cancer marker PSA levels. SME, *Salvia miltiorrhiza* extract; CYP11A1, cytochrome P450 cholesterol side-chain cleavage enzyme; CYP17A1, cytochrome P450 17α-hydroxylase/C17–20 lyase; STAR, steroidogenic acute regulatory; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme-oxygenase-1; CAT, catalase; SOD, superoxide dismutase; and GPx, glutathione peroxidase; AST, aspartate transaminase; ALT, alanine transaminase; TG, triglycerides; TC, total cholesterol; MDA, malondialdehyde; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PSA, prostate-specific antigen; SHBG, sex hormone-binding globulin; DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Activation of these receptors may indirectly modulate hormone synthesis by influencing inflammatory signaling pathways and regulating energy homeostasis [67]. Moreover, microbial metabolites have been shown to impact the HPG axis, potentially affecting the secretion of gonadotropins such as LH and FSH, which regulate testosterone production [68]. Immune modulation is another plausible pathway, as gut microbiota can influence cytokine profiles [69], which have been implicated in regulating Leydig cell function and steroidogenesis [70]. The increased abundance of *Succinispira mobilis* in the fecal samples of the SME-treated group suggests that SME may confer protective effects against age-associated gut dysbiosis and improve steroidogenesis. While these mechanisms are supported by emerging literature, direct evidence linking *S. mobilis* to testosterone synthesis is currently lacking. Therefore, future studies incorporating metagenomic, metabolomic, and functional assays are needed to clarify the causal pathways and confirm the biological relevance of these findings. SME contains a variety of bioactive constituents, including danshensu, flavonoids, salviolic acids, and tanshinones [71]. Among these, tanshinone IIA, a prominent fat-soluble compound, is one of the primary components with well-documented biological efficacy. It exhibits various pharmacological activities, including anti-inflammatory, cardioprotective, and neuroprotective effects [72]. These are particularly relevant to male climacteric symptoms such as metabolic disorders, inflammation, benign prostatic hyperplasia, and cognitive decline. Moreover, tanshinone IIA has demonstrated potential in alleviating anxiety- and depression-like behaviors [73], which are commonly associated with andropause. Recent studies also suggest that tanshinone IIA may elevate

testosterone levels in heat-stressed mouse models [74]. In the current study, HPLC/MS analysis confirmed the presence of tanshinone IIA in SME and the serum of experimental groups (SEL, SEM and SEH), suggesting that this compound may be a key contributor to the observed biological effects. While emerging evidence supports the role of tanshinone IIA in mediating SME's protective actions, a comparative study evaluating SME against its bioactive components is essential to delineate the specific contributions of each constituent to its overall therapeutic efficacy. The current study suggests the interdependence between OS and steroidogenesis. However, further in-depth investigations are warranted to examine the interactions between various antioxidants and steroidogenic enzymes, to better understand the mechanisms underlying OS-mediated impairment of steroidogenesis. Although aged rat models are valuable for studying age-related physiological changes, potential confounding factors, such as pre-existing comorbidities and dietary variability, may influence hormonal and immune parameters. Future studies should aim to control for these variables through rigorous health screening and standardized feeding protocols. Also, while 36-week-old rats exhibit early signs of age-related hormonal and metabolic changes, they do not represent fully senescent animals. As such, they may not adequately model the full spectrum of physiological, cellular, and functional declines observed in advanced aging. This limits the ability to generalize findings to later stages of senescence, where age-associated pathologies and systemic deterioration are more pronounced. Additionally, bioinformatic tools may offer deeper insights into the broader impact of SME on age-associated impairments, enabling the exploration of its influence across diverse molecular and cellular

pathways.

In conclusion, SME demonstrated the ability to attenuate OS by modulating Nrf2/HO-1 signaling and restored steroidogenesis by improving steroidogenic enzyme activity. In addition, SME increased the abundance of propionate-producing *Succinispira mobilis* in the gut, indicating its potential in regulating immune and endocrine functions (Fig. 8). Nevertheless, comprehensive studies are still needed to fully elucidate SME's therapeutic potential and underlying mechanisms in the context of andropause-associated disorders. The current findings highlight SME as a promising therapeutic candidate for mitigating andropause-associated pathologies, warranting further investigation to validate its clinical applicability.

Consent for publication

The graphical abstract and Fig. 8 were prepared using BioRender. We have been granted licenses (YK28708K9U and VI28708QZ0) to use the BioRender content.

Ethics approval

All the experiments were conducted in accordance with the Care and Use of Laboratory Animals guidelines approved by Gachon University (GU1–2024-IA0034–01).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118297.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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