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Research Article

Yangjing Capsule Increases Testosterone Production through SET/PI3K/Akt Pathway in Leydig Cell

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Background. Our previous works revealed Yangjing capsule (YC) could inhibit cell apoptosis and promote testosterone production in Leydig cells. SET protein has been reported to be important in regulating testosterone synthesis and apoptosis. The purpose of this study was to investigate whether the steroidogenic and antiapoptotic effects of YC are mediated in part by the SET/PI3K/Akt pathway in Leydig cells. *Methods*. MLTC-1 cells were treated with YC-medicated serum. si-RNA was used to knockdown SET expression in MLTC-1 cells. The elderly BALB/c mice were treated with different doses of YC extract. Flow cytometry and TUNEL staining were used to assess apoptosis in MLTC-1 cells and mouse testes. HE staining was conducted to detect the morphological changes in mouse testes. Testosterone levels in cell culture medium and serum were measured by ELISA kit. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and Western blot were used to detect the expression levels of key molecules. *Results*. YC-medicated serum could significantly increase the expression of SET and StAR, P450scc, HSD17B, and testosterone production in MLTC-1 cells. YC-medicated serum could increase Akt phosphorylation and Bcl-2/Bax ratio to suppress apoptosis in MLTC-1 cells. SET knockdown significantly inhibited YC-medicated serum-induced steroidogenic and antiapoptotic effects in MLTC-1 cells. In vivo experiments revealed the YC extract could enhance SET expression, trigger Akt phosphorylation, and suppress apoptosis in mouse testis to raise serum testosterone levels. *Conclusions*. A possible mechanism of promoting testosterone production induced by YC in Leydig cell was by triggering SET/PI3K/Akt pathway.

1. Introduction

Testosterone, the major circulating functional androgen secreted by Leydig cells, is essential for various physiological processes [1]. A large longitudinal study has shown that most men experience a gradual decline in serum testosterone levels with aging even in the absence of disease [2]. Approximately 30% of middle-aged or older men exhibit varying degrees of clinical symptoms, such as low sexual desire, erectile dysfunction, insomnia, and depression, known as late-onset hypogonadism (LOH) [3].

Previous studies have shown that the number of Leydig cells rarely changed with age in both rats and humans [4, 5]. These findings suggest that the age-related decline in testosterone production may be mainly related to Leydig cell dysfunction. However, recent research in human testes has observed that testes of the same weight contained fewer Leydig cells during aging, suggesting that the age-related disturbance in testosterone synthesis was more likely due to decreased Leydig cells [6]. Up to date, the molecular mechanisms associated with age-related Leydig cell loss remain elusive. With aging, chronic inflammation, and oxidative stress lead to excessive apoptosis in Leydig cells. Accumulating evidence supports the notion that apoptosis may cause a decrease in Leydig cell numbers and testosterone production [7, 8].

SET protein, as an inhibitor of protein phosphatase 2A, was first reported in a leukemia patient [9]. An earlier study showed that SET protein was highly expressed in mouse

Leydig cells [10]. A subsequent study showed that SET overexpression or knockdown could significantly promote or decrease testosterone production [11]. These results confirmed the relationship between SET and testosterone production in Leydig cells. The activated PI3K/Akt pathway can trigger the phosphorylation of various substrates or enhance the activity of numerous enzymes to control cell cycle and apoptosis [12]. In many human malignancies, SET can bind to PP2A to play an important role in cell apoptosis through the PI3K/Akt and Bcl-2 survival pathways [13, 14]. However, the specific mechanism by which SET that regulates testosterone synthesis in Leydig cells remains unclear.

Yangjing capsule (YC), which has been used clinically for many years, is a compound preparation composed of herbs including Epimedii Brevicornus, Placenta Hominis, *Rehmannia glutinosa*, *Astragalus mongholicus*, *Lycium barbarum*, and other components [15]. Our previous studies showed that the YC can promote testosterone production through upregulating the expression of steroidogenic enzymes and reducing cell apoptosis in Leydig cells [16, 17]. However, the mechanism underlying YC to reduce cell apoptosis and upregulate the expression of steroidogenic enzymes to promote testosterone production in Leydig cells remains unclear.

In this paper, based on previous studies, we propose that YC can regulate SET both in vitro and in vivo to induce phosphorylation of the PI3K/Akt pathway and then reduce cell apoptosis. These findings may explain by which YC promotes testosterone production, and provide strong evidence for the treatment effect of YC on LOH.

2. Materials and Method

2.1. Preparation of YC-Medicated Serum. Experiments with animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publications Number 85–23, revised 1996), and were approved by the Ethics Committee of Southeast University (Nanjing, China).

Forty Sprague-Dawley rats (250–300 g) were purchased from Charles River (Beijing, China). All animal experiments in this study were in accordance with the Chinese Guide for the Care and Use of Laboratory Animals. They were randomly divided into four groups (10 rats per group): YC low-dose group (received intragastric administration of 7.14 g/kg YC extract), YC medium-dose group (received intragastric administration of 14.28 g/kg YC extract), YC high-dose group (received intragastric administration of 28.56 g/kg YC extract), and control group (received the same volume of physiological saline). The administrations were carried out twice a day for 7 days. Rats were anesthetized with 10% pentobarbital sodium 2 hr after the last administration. Blood was collected from the abdominal aorta and centrifuged at 3000 rpm for 15 min at 4°C. Serum samples were collected, filtered with 0.22 μm filters, inactivated at 56°C for 30 min and then stored at −80°C.

2.2. Culture and Treatment of Leydig Cell Line. MLTC-1 cells were obtained from American Type Culture Collection (ATCC). They were cultured in RPMI-1640 (Gibco, NY, USA) containing 10% FBS (Sciencell, San Diego, USA), 1% penicillin/streptomycin (Beyotime, Shanghai, China) in 5% CO2 at 37°C. MLTC-1 cells were cultured at a density of 3×10^5/well in six-well plates and divided into four groups, control serum (CS), low-dose YC-medicated serum (LS) group, medium-dose YC-medicated serum (MS) group, and high-dose YC-medicated serum (HS) group. When cell density reached 70%, they were treated with medium containing 10% CS, LS, MS, and HS, respectively, for 48 hr.

2.3. si-RNAs and Transfection. si-RNAs and riboFECT™ CP were obtained from RIBOBIO (Guangzhou, China). The sequences of si-RNAs for SET are shown as follows: si-SET-1 GGT TTA CTG ACC ATT CTG A, si-SET-2 CGC AGG TGC TGA TGA GTT A, and si-SET-3 GTC ATC AAA GAT GAC ATC T. They were mixed to transfect cells to ensure efficiency. MLTC-1 cells were divided into four groups, CS group, HS group, CS + SET knockdown (CS + si-SET) group, and HS + SET knockdown (HS + si-SET) group. MLTC-1 cells were cultured at a density of 2×10^5/well in six-well plates. When the cell density reached 50%, cells in CS + si-SET and HS + si-SET were treated with si-RNAs. After 36 hr, cells in the CS and CS + si-SET groups were treated with medium containing 10% CS, while cells in the HS and HS + SET groups were treated with medium containing 10% HS for another 48 hr.

2.4. RNA Isolation and Real-Time Polymerase Chain Reaction (PCR). Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA). The concentration and purity of the extracted RNA were then measured by spectrometry (NanoDrop, USA). PrimeScript RT Master Mix (TaKaRa, Dalian, China) was used to synthesize cDNA from 1 mg of total RNA. Primers used in this study were SET, forward: 5′-GTC CAC CGA AAT CAA ATG GAA ATC-3′, and reverse: 5′-GTC CAC CGA AAT CAA ATG GAA ATC-3′, and reverse: 5′-GCA CCT GCA TCA GAA TGG TCA-3′; StAR, forward: 5′-CCA CCT GCA TGG TGC TTC A-3′, and reverse: 5′-TTG GCG AAC TCT ATC TGG GTC TG-3′; P450scc: forward: 5′- AGG TCC TTC AAT GAG ATC CCT T-3′, and reverse: 5′- TCC CTG TAA ATG GGG CCA TAC-3′; HSD17B: forward: 5′- TCT CTG CCA TGT GGA TAA CCC-3′, and reverse: 5′- GGT CGG TAG CGT ATT TGG AAG-3′; β-actin, forward: 5′-GCT ACA GCT TCA CCA CCA CAG-3′, and reverse: 5′-GGT CTT TAC GGA TGT CAA CGT C-3′. cDNA amplification was performed out at 95°C for 30 s and 60°C for 60 s for 5 min, followed by 40 cycles. The final step was the melt curve analysis. The relative abundance of the target gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method.

2.5. Protein Extraction and Western Blot Analysis. After the cells were treated as described above, the total cell lysate was collected by RIPA buffer containing 1% Halt™ protease inhibitor cocktail (Thermo, Shanghai, China). The cell lysate was placed at 4°C for 15 min, and then centrifuged at 12,000 rpm for 15 min to retain the supernatant. The same volume of 2× loading buffer (BIO-RAD, USA) was added to the supernatant, and then denatured the protein was denatured at 95°C for 10 min. Equal amounts of total protein

(40 μg) were separated via SDS-PAGE and then transferred onto polyvinylidene flouride (PVDF) membranes (BIO-RAD, USA). After the transfer, 5% nonfat milk was used to block the binding sites of the membranes for 1 hr, and then the membranes were incubated with primary antibodies against SET (Abcam, ab181990, 1:10000), Bcl-2 (Abcam, ab182858, 1:2000), Bax (Proteintech, 60267-1-Ig, 1:5000), StAR (Proteintech, 12225-1-AP, 1:1000), P450scc (Proteintech, 67264-1-Ig, 1:5000); HSD17B (Proteintech, 14854-1-AP, 1:500); β-actin (Proteintech, 20536-1-AP, 1:1000), Akt (CST, C67E7, 1:1000), p-Akt (CST, D9E, 1:1000), and caspase-3 (CST, D3R6Y, 1:1000) on a shaking table at 4°C overnight. The membranes were incubated for 1 hr at room temperature with goat antirabbit IgG (Abcam, ab6721, 1:4000) or goat antimouse IgG (Proteintech, SA00001-1, 1:2000). The membranes were exposed by an enhanced chemiluminescence solution (Thermo, Shanghai, China), and band intensities were semiquantified using Image J software. In addition, β-actin was selected to normalize relative protein expression.

2.6. Flow Cytometry. After cells were treated as described above, they were harvested and washed with Phosphate Buffered Saline (PBS). The Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, Nanjing, China) was then used to detect cell apoptosis according to the protocol. Apoptosis rates were measured by flow cytometry. Cells exhibiting FITC+/PI− were considered early apoptotic cells, while those exhibiting FITC+/PI + were considered late apoptotic cells.

2.7. Animals and YC Extract Intragastric Administration. Fifty 18-month-old and ten 3-month-old male BALB/c mice were purchased from Charles River (Beijing, China). These mice were randomly divided into five groups (10 mice per group): low-dose group (mice received intragastric administration of 0.63 g/kg of YC extract), medium-dose group (mice received intragastric administration of 1.26 g/kg of YC extract), high-dose group (mice received intragastric administration of 2.52 g/kg of YC extract), sham group and control group (mice received the same volume of physiological saline). The administrations were carried out twice a day for 30 days. The mice were anesthetized with 10% pentobarbital sodium 2 hr after the last administration. Blood was then collected from the medial canthus and centrifuged at 3,000 rpm for 15 min at 4°C. Serum samples were collected and stored at −80°C. Testes were collected after the mice were killed. One testis was placed in tissue fixative and the other was stored at −80°C.

2.8. Hormone Assessment Assays. The mouse testosterone ELISA kit was purchased from Jining Biotechnology (Shanghai, China). Cell culture medium and serum were collected from different groups described above for testosterone level detection using mouse testosterone ELISA kit following protocol.

2.9. HE Staining. Testicular tissue was routinely embedded with paraffin. The wax blocks were sliced into 4–8 μm sections. The tissue sections were placed on the glass slide, stretched in warm water at 40°C, and then dried in an oven at 80°C for 1 hr. Paraffin was dissolved from the tissue sections with xylene, and then the sections were fully hydrated with ethanol. After hydration, the sections were stained hematoxylin, and then differentiated them using 1% hydrochloric acid ethanol. The differentiated sections were then stained with an eosin dye solution. After staining, the sections were completely dehydrated with ethanol and patched with neutral gum. Finally, the tissue sections were observed and photographed under a microscope.

2.10. Immunohistochemical Staining. Tissue section preparation, dewaxing, and hydration were described above. 0.01 M sodium citrate buffer solution, pH 6.0, was heated to boiling in a microwave oven for 4 min. The sections were then placed in the boiled buffer solution for 15 min to repair the antigen. The sections were then washed 3 times with PBS. Nonspecific binding sites were blocked with serum at 37°C for 30 min. 20μl anti-SET (Abcam, ab181990, 1:250) was added to the tissue on the section and incubated overnight at 4°C. The sections were then washed 3 times with PBS. A second antibody (20 μl) was added to the tissue on the section and incubated at 4°C for 1 hr. The sections were stained with DAB-H_2O_2 for 10 min and then rinsed with distilled water. Slices were dehydrated and dewaxed with ethanol and xylene, respectively, and then patched with neutral gum. Finally, the tissue sections were observed under the microscope and photographed.

2.11. TUNEL Staining. TUNEL BrightRed Apoptosis Detection Kit (Vazyme, Nanjing, China) was used to detect cell apoptosis in tissue sections according to the protocol. Scanning and imaging with the confocal microscope. Semiquantitative analysis of fluorescence intensity using Image Pro Plus 6 software.

2.12. Statistical Analysis. Data are presented as the mean ± SD of values from at least three independent experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD). $P < 0.05$ were considered statistically significant.

3. Results

3.1. YC-Medicated Serum Upregulated SET Expression and Promoted Testosterone Production in MLTC-1 Cells. To evaluate the effects of YC-medicated serum on the expression of SET and changes in testosterone production, MLTC-1 cells were treated with 10% control serum, low, medium, and high doses of YC-medicated serum, respectively. As shown in Figure 1(a), testosterone production was significantly increased in the MS and HS groups ($P < 0.05$). SET expression was rapidly induced by YC-medicated serum both at the mRNA (Figure 1(b)) and protein (Figures 1(c) and 1(d)) levels in the HS group ($P < 0.05$). In addition, (Figure 1(b), 1(c), and 1(d)) showed the mRNA and protein expression levels of StAR, P450scc, and HSD17B were significantly increased in the HS group ($P < 0.05$).

3.2. YC-Medicated Serum Reduces Apoptosis in MLTC-1 Cells through PI3K/Akt Signal Pathway. Flow cytometry analysis (Figures 2(a) and 2(b)) revealed that the apoptosis of MLTC-1 cells was significantly decreased by YC-medicated serum in a dose-dependent manner, and the difference was significant in the HS group compared with the CS group ($P < 0.05$). Western blot (Figures 2(c) and 2(d)) showed that the protein

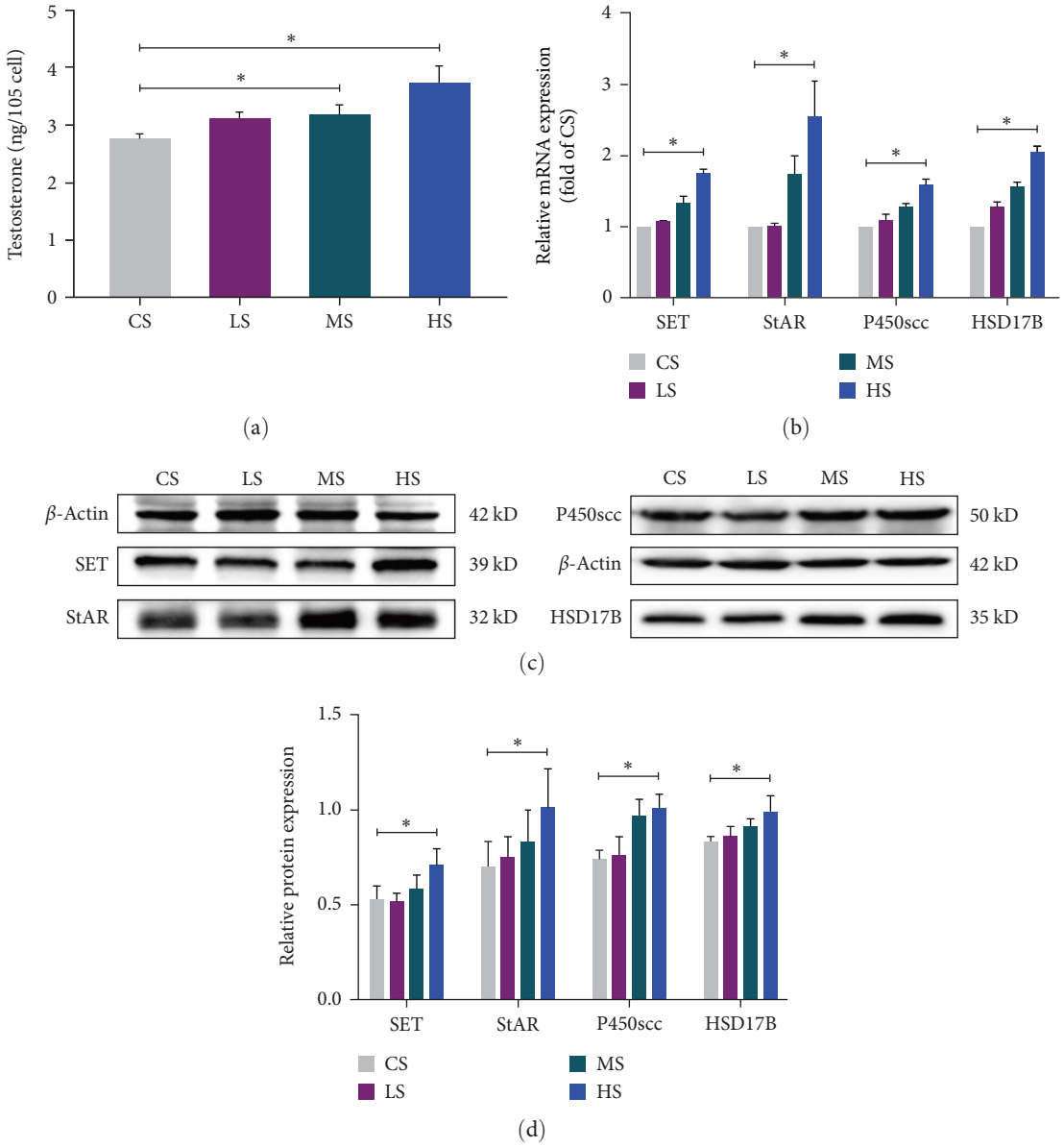

Figure 1: YC-medicated serum upregulates SET expression and promotes steroidogenesis in MLTC-1 cells. MLTC-1 cells were treated with 10% control serum (CS), low (LS), medium (MS), and high (HS) doses of YC-medicated serum, respectively for 48 hr. The testosterone levels of the culture medium in each group were determined by ELISA (a). SET, StAR, P450scc, and HSD17B mRNA and protein expression levels were determined by real-time PCR and Western blot, respectively (b), (c) and (d). $^*P<0.05$.

expression of P-Akt and Bcl-2 were significantly increased, while cleaved-caspase-3 and Bax were significantly reduced in the HS group ($P<0.05$).

3.3. SET Knockdown Blocks YC-Medicated Serum-Induced Testosterone Production and Upregulated Steroidogenic Enzymes in MLTC-1 Cells. Our results presented above demonstrated that YC-medicated serum could upregulate SET expression to trigger the PI3K-Akt signaling pathway, reduce MLTC-1 cells apoptosis, and then promote testosterone production. To obtain further evidence for our inference, SET expression was knocked down to evaluate the effects of YC-medicated serum. As shown in Figure 3(a), testosterone production was markedly suppressed when SET was knocked down compared to the control group ($P<0.05$). SET knockdown significantly inhibited YC-medicated serum-induced testosterone production ($P<0.05$). PCR and Western blot results (Figures 3(b), 3(c), and 3(d)) showed that the relative mRNA and protein expression levels of StAR and HSD17B were reduced when SET knockdown was performed compared to the control group ($P<0.05$). Additionally, SET knockdown significantly suppressed YC-medicated serum-induced upregulated mRNA and protein expressions of StAR and HSD17B.

3.4. SET Knockdown Blocks YC-Medicated Serum-Induced Akt Phosphorylation and antiapoptotic Effect in MLTC-1 Cells. Flow cytometry analysis (Figures 4(a) and 4(b)) revealed that the apoptosis rate of SET-knockdown MLTC-

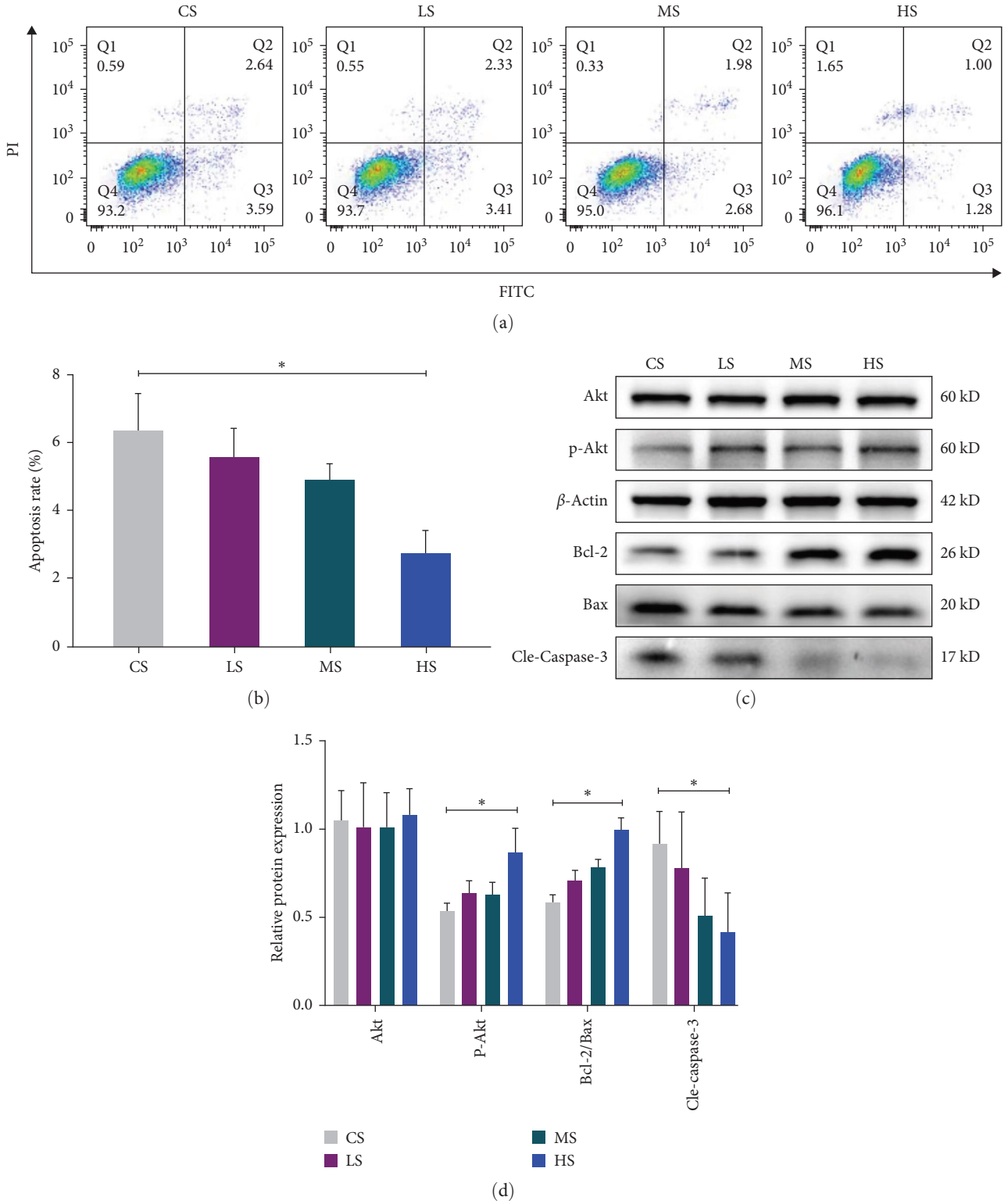

Figure 2: YC-medicated serum reduces apoptosis in MLTC-1 cells through the PI3K-Akt signaling pathway. MLTC-1 cells were treated with 10% control serum (CS), low (LS), medium (MS), and high (HS) doses of YC-medicated serum, respectively, for 48 hr. Apoptosis rates of each group were assessed by flow cytometry (a) and (b). The protein expression levels of Akt, P-Akt, Bcl-2, Bax, and cleaved-caspase-3 were evaluated by Western blot (c) and (d). $^*P<0.05$.

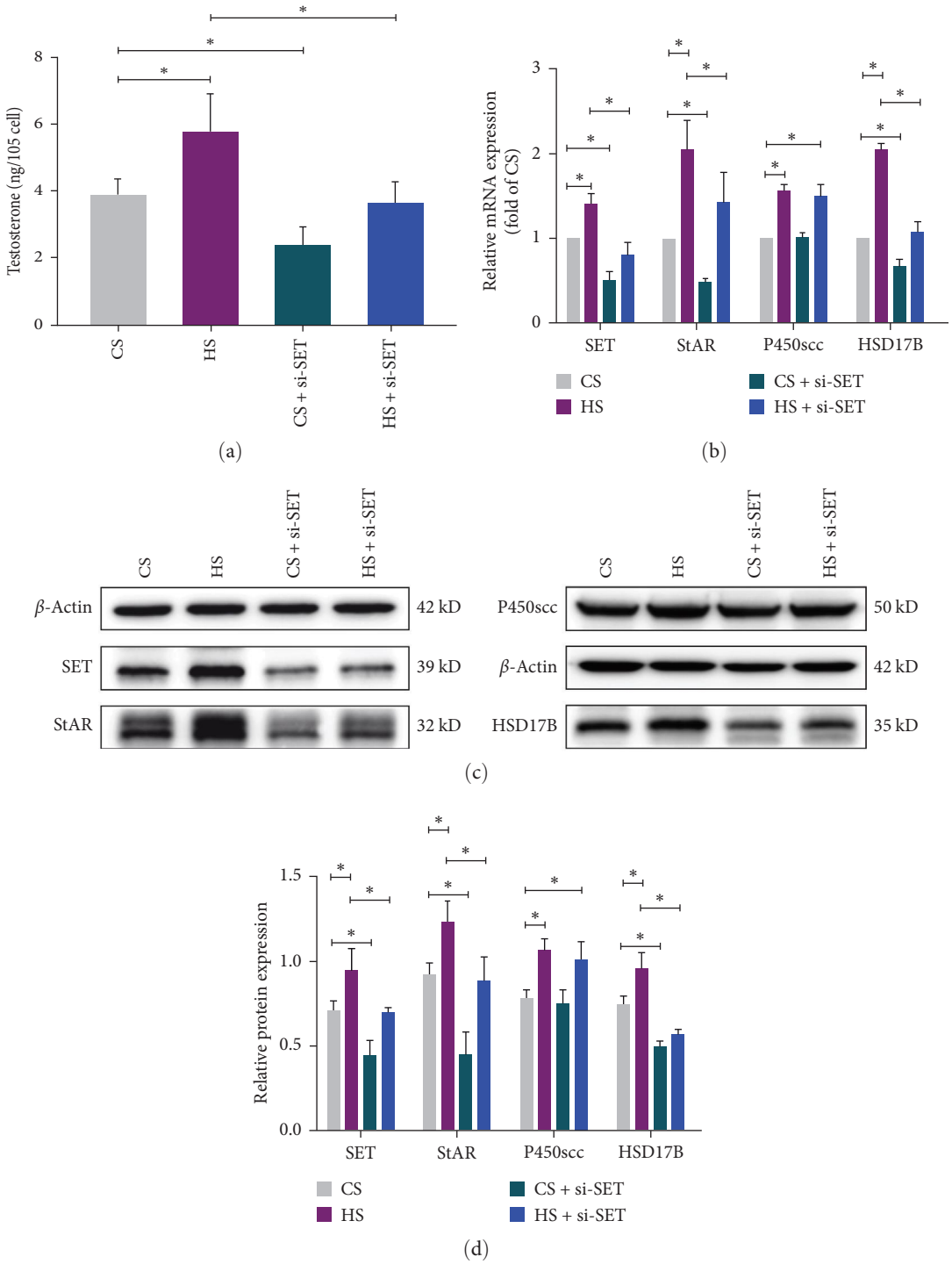

FIGURE 3: SET knockdown blocks YC-medicated serum-induced testosterone production and upregulated steroidogenic enzymes in MLTC-1 cells. MLTC-1 cells were divided into four groups, control serum (CS) group, high-dose YC-medicated serum (HS) group, CS + SET knockdown (CS + si-SET) group, and HS + SET knockdown (HS + si-SET) group. Testosterone levels in culture medium (a), mRNA expression (b), and protein expression (c) and (d) of SET, StAR, P450scc, and HSD17B in MLTC-1 cells were detected by ELISA, real-time RT-PCR, and Western blot, respectively. $^*P < 0.05$.

1 cells was significantly increased compared to the control group ($P < 0.05$). Meanwhile, SET knockdown significantly inhibited the antiapoptotic effect induced by YC-medicated serum in MLTC-1 cells ($P < 0.05$). Western blot (Figures 4(c) and 4(d)) revealed that SET inhibition suppressed Akt phosphorylation and the expression of Bcl-2, increased the expression of Bax and cleaved-caspase-3 compared to the control group ($P < 0.05$). Additionally, SET knockdown

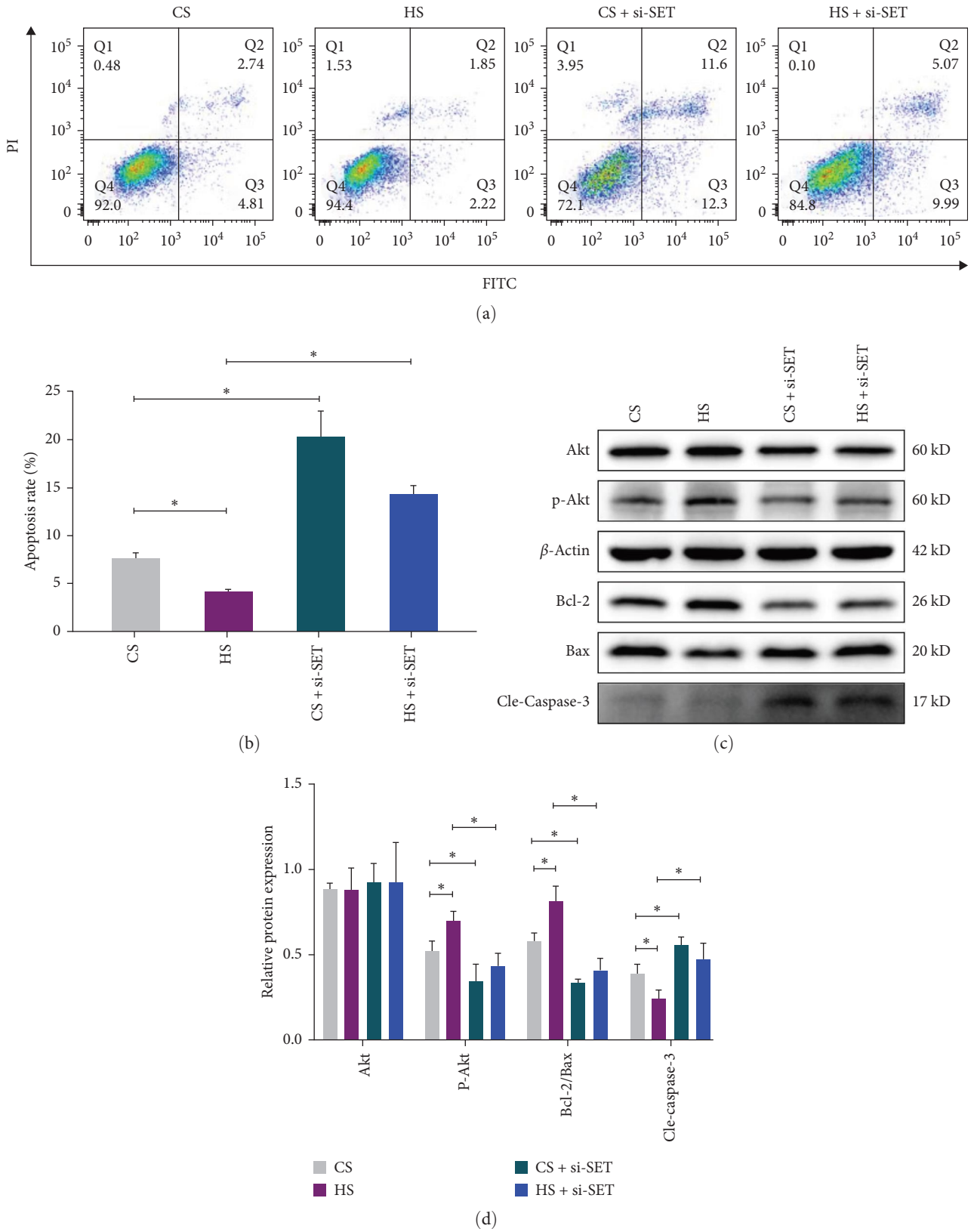

Figure 4: SET knockdown inhibited YC-medicated serum-induced Akt phosphorylation and decreased apoptosis rate in MLTC-1 cells. MLTC-1 cells were divided into four groups, control serum (CS) group, high-dose YC-medicated serum (HS) group, CS + SET knockdown (CS + si-SET) group, and HS + SET knockdown (HS + si-SET) group. Apoptosis rates of each group were assessed by flow cytometry (a) and (b). The protein expression levels of Akt, P-Akt, Bcl-2, Bax, and cleaved-caspase-3 were evaluated by Western blot (c) and (d). $^*P<0.05$.

significantly suppressed YC-medicated serum-induced Akt phosphorylation, upregulated the expression of Bcl-2, and decreased expression of Bax and cleaved-caspase-3.

3.5. YC Extract Increased Serum Testosterone Levels and Upregulated Testicular Steroidogenic Enzymes in Mice. HE staining was used to examine the histologic structures of the testes. The testes in the sham group exhibited complete seminiferous tubules and normal interstitial space. Compared with sham mice, control mice showed a reduced testis size, increased vacuolation in seminiferous tubules and testicular interstitial, and a reduced number of spermatogenic and Leydig cells. Abnormalities in the testis size, testicular structures, and number of spermatogenic and Leydig cells in the YC extract-treated groups showed varying degrees of amelioration compared with control mice (Figure 5(a)). Consistent with previous findings, immunohistochemical staining revealed that SET was mainly expressed in spermatogenic and Leydig cells in the testis. The immunohistochemical staining intensity of SET in the control group was significantly reduced compared with that in the sham group. YC extract could upregulate the staining intensity SET in the testis (Figure 5(b). Serum ELISA data showed that testosterone levels of control mice were significantly reduced compared to sham mice. Serum testosterone levels were induced by YC extract in a dose-dependent manner and significantly increased in high-dose mice compared to control mice (Figure 5(c)). Western blot results showed that the relative protein expression levels of SET, StAR, P450scc, and HSD17B were reduced in control testes compared to sham testes. YC extract could upregulate the expression of SET, StAR, P450scc, and HSD17B in testicular tissue compared to control mice (Figure 5(d)–5(f)).

3.6. YC Extract Reduces Apoptosis of Leydig Cells in Mice through PI3K/Akt Signal Pathway. Cell apoptosis in the testis was detected by TUNEL staining. DAPI-stained nuclei (blue) colocalized with TUNEL (red) in apoptotic cells. TUNEL staining results revealed that positive staining was mainly located in Leydig cells in the testicular interstitial and the spermatogenic cells in the periphery of the seminiferous tubules (Figure 6(a)). The average fluorescence intensity (AFI) was significantly increased in high-dose mice compared with control mice. YC extract could alleviate AFI in the treated groups to varying degrees (Figure 6(b). Western blot results showed that the relative protein expression levels of P-Akt and Bcl-2 were decreased, while Bax and cleaved-caspase-3 expression were increased in control testes compared with sham testes. YC extract could upregulate the expression of P-Akt and Bcl-2, and suppress the expression of Bax and cleaved-caspase-3 in testicular tissue compared to control mice (Figures 6(c) and 6(d)).

4. Discussion

YC has been used clinically for years in patients with male infertility, LOH, and sexual dysfunction. It may act to improve sexual and reproductive function in part by promoting steroidogenesis [18]. However, the cell-inrinsic mechanisms by which YC promotes steroidogenesis in Leydig cells are poorly understood. In the present study, we found that YC could decrease apoptosis by upregulating SET expression to stimulate testosterone production in Leydig cells.

SET is reported to be a phosphorylated protein that is ubiquitously expressed in various tissues, particularly in steroidogenic cells within the adrenal gland and gonad, suggesting that it may be closely assiciated with steroidogenesis [19, 20]. Previous work has shown that human chorionic gonadotropin (hCG) increased SET mRNA and protein expression through the cAMP/PKA signaling pathway to stimulate steroidogenic enzyme expression, which then promotes testosterone production in Leydig cells [11]. In the present study, we found that YC could paly a similar role to hCG in upregulating the expression of SET, StAR, P450scc, and HSD17B to stimulate steroidogenesis in Leydig cells both in vitro and in vivo. It is well known that the LH/LHR signaling pathway is crucial for steroidogenesis in Leydig cells. Binding of LH or hCG to LHR has both acute and chronic impacts on Leydig cells. The acute effect means LH or hCG can stimulate the cAMP/PKA cascade to promote testosterone synthesis. In its chronic action, LH exposure can upregulate steroidogenic enzymes expression via the cAMP/PKA signaling pathway [21]. In our previous study, H89, an inhibitor of the PKA pathway, was used to observe its effect on YC-induced steroidogenesis. The results showed that H89 could significantly reduce the expression of steroidogenic enzymes and testosterone production induced by YC extract in Leydig cells [15]. Polysaccharide, as a structural molecular form, is involved in intermolecular recognition and connection. Some structural polysaccharides of YC share the similar ability to LH/hCG to activate LH/LHR signaling pathway in Leydig cells. These investigations indicated that YC-induced steroidogenesis is due in part to regulating SET expression via the cAMP/PKA signaling pathway in Leydig cells.

The relationship between aging and apoptosis remains unclear, but some features like oxidative stress, DNA damage, and chronic inflammation associated with the aging process have been confirmed to induce apoptosis [22–24]. Apoptosis is partially controlled by Bcl-2 family proteins. Bcl-2, as the important antiapoptotic regulators, can function as homodimers to promote cell survival via maintaining mitochondrial stability, while proapoptotic members like Bax can increase mitochondrial permeability to induce apoptosis [25]. As a key serine/threonine phosphatase, the PP2A complex is involved in a variety of cellular processes, including apoptosis [26]. There is some evidence that PP2A can dephosphorylate Bcl-2, thereby increasing mitochondrial permeability, and leading to cell apoptosis [27]. The binding of SET to PP2A inhibits approximately 50% of the phosphorylation activity of PP2A, thereby exerting an antiapoptotic effect [13, 19, 28]. In the current study, we found that the YC-medicated serum significantly increased the ratio of Bcl-2 to Bax and reduced the number of apoptotic cells which was accompanied with by upregulated SET expression in the MLTC-1 cell line. In addition, we also demonstrated YC extract could also stimulate SET expression, reduce the positive rate of TUNEL staining and increase the ratio of Bcl-

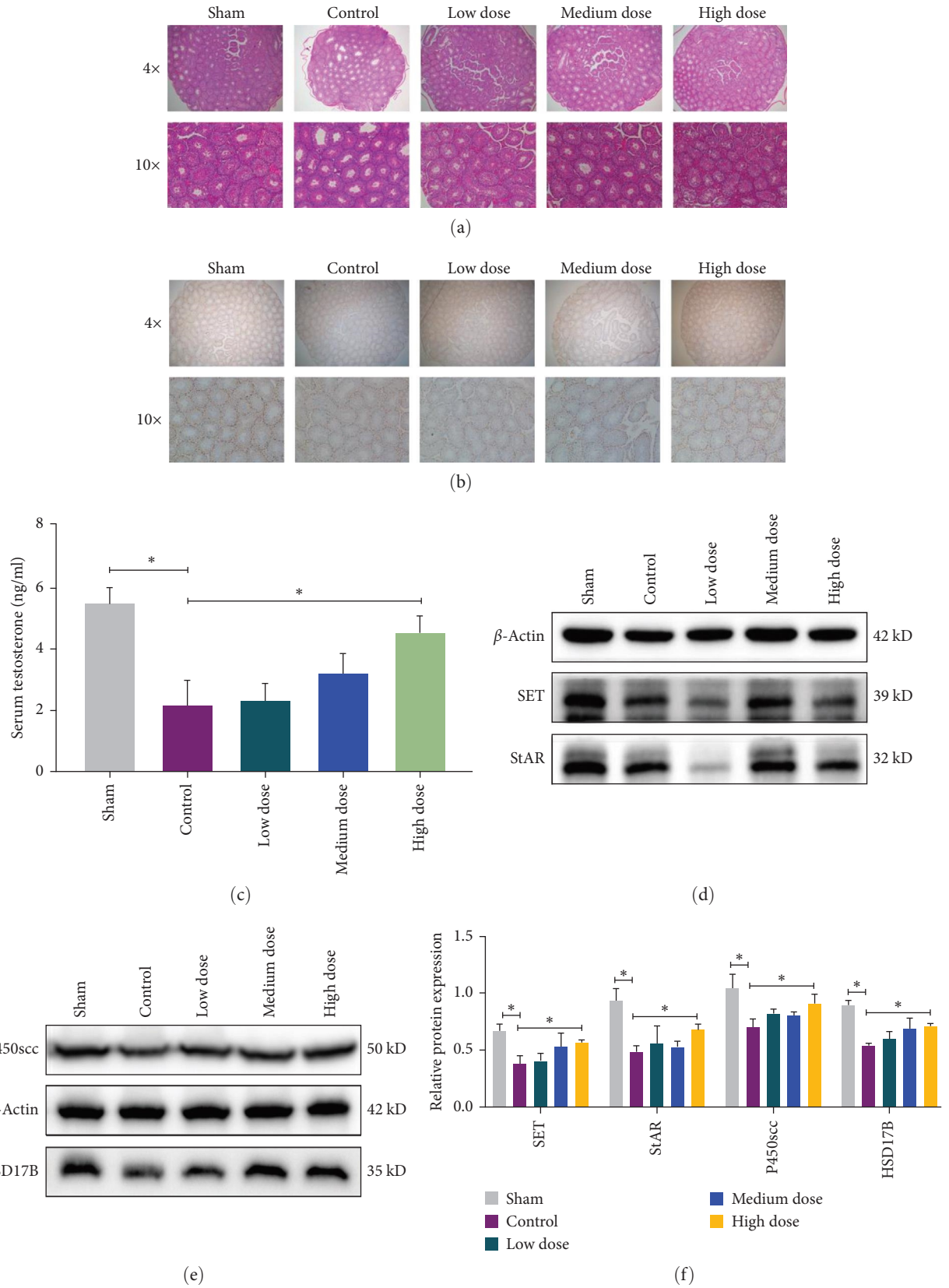

FIGURE 5: YC extract promoted testosterone production through upregulation of SET in Leydig cells in mice. The elder mice (aged 18 months) were randomly divided into four groups including old control, low dose (0.63 g/kg YC), medium dose (1.26 g/kg YC), and high dose (2.52 g/kg YC). Young mice aged 12 weeks were allocated in the sham group. HE staining was used to assess the histological structures of the testes slices of each group (a). Immunohistochemical staining showing expression of SET testis slices of each group (b). Serum testosterone levels of each group were determined by ELISA (c). Protein expression levels of SET, StAR, P450scc, and HSD17B in testis of each group were assessed by Western blot (d), (e), and (f). $^*P<0.05$.

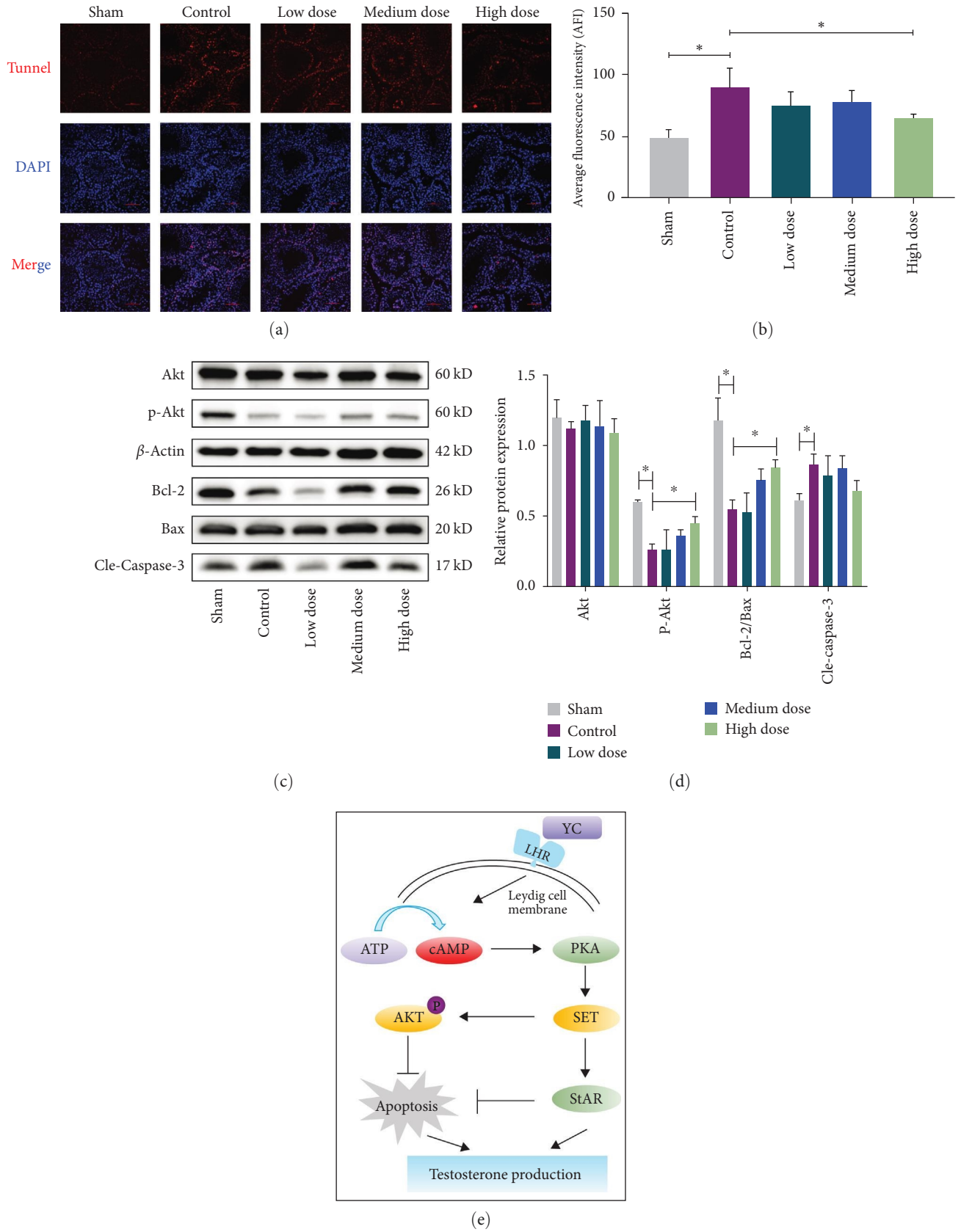

Figure 6: YC extract reduces apoptosis of Leydig cells in mice. The elder mice (aged 18 months) were randomly divided into four groups including old control, low dose (0.63 g/kg YC), medium dose (1.26 g/kg YC), and high dose (2.52 g/kg YC). Young mice aged 12 weeks were allocated in the sham group. TUNEL staining was used to assess cell apoptosis in testis sections of each group (a) and (b). Protein expression levels of Akt, P-Akt, Bcl-2, Bax, and cleaved-caspase-3 in testes of each group were assessed by Western blot (c) and (d). The mechanism of YC regulation of PI3K/Akt signaling pathway to reduce Leydig cell apoptosis (e). $^*P<0.05$.

2 to Bax in mouse testis tissue. Our work showed that the YC-induced antiapoptotic effect was partially achieved by upregulating the expression of SET both in vitro and in vivo.

In the PI3K/Akt signaling pathway, phosphorylation of Akt at T-308 and S-473 has been demonstrated to promote proliferation via suppressing apoptosis in numerous cell types [29]. Therefore, we further investigated whether the PI3K/Akt signaling pathway is involved in the YC-induced antiapoptotic effect in Leydig cells and found that the YC-medicated serum significantly increased Akt phosphorylation with upregulated SET expression in MLTC-1 cell line. In rat testis tissue, YC extract could also stimulate SET expression and increase Akt phosphorylation. Evidence indicated that PP2A, as an important Akt phosphatase, exerted an inhibitory effect on Akt phosphorylation at T-308 [30]. Binding to SET could relieve the inhibitory effect of PP2A on Akt phosphorylation [14]. Our results demonstrated that the PI3K/Akt signaling pathway was involved in the YC-induced antiapoptotic effect in Leydig cell.

Considering that YC upregulated SET expression, we further investigated whether SET knockdown affected the steroidogenic and antiapoptotic effects of YC. si-RNAs were used to knockdown the SET expression in Leydig cells. Consistent with previous reports, Akt phosphorylation, steroidogenic enzyme expression, and testosterone production were significantly suppressed in MLTC-1 cells with SET knockdown; SET knockdown decreased the ratio of Bcl-2 and Bax and induced a significant increase in cell apoptosis. Furthermore, SET knockdown suppressed the YC-induced Akt phosphorylation, inhibition of apoptosis, expression of steroidogenic enzymes, and testosterone production, suggesting that the steroidogenic and antiapoptotic effects of YC were exerted in part via the regulation of SET expression.

5. Conclusions

In conclusion, the findings from our study provide evidence that YC can effectively upregulate the expression of SET. This, in turn, leads to the inhibition of Akt dephosphorylation, resulting in an increase in the levels of phosphorylated Akt. As a consequence, there is a reduction in cell apoptosis, which further promotes steroidogenesis (Figure 6(e)). These results significantly contribute to the understanding of YC's potential clinical application as a therapeutic intervention.

Data Availability

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

Ethical Approval

Animal care guidelines (NIH Publications Number 85–23, revised 1996) were followed in this study, and the Ethics Committee of Southeast University (Nanjing, China) approved its conduct (Number: 20170309006).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

D. X. and B. F. J. designed the experiments. B. F. J. funded this study. D. X., Y. Y. L., S. D. L. and D. D. W. performed the experiments and analyzed the data. D. X. wrote the manuscript. B. C., Y. H. J., and B. F. J. revised the manuscript. All authors reviewed and approved the final manuscript.

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