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WJSC World Journal of Stem Cells

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ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

  WJSC publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryoid bodies, embryonal carcinoma stem cells, hemangioblasts, hematopoietic stem cells, lymphoid progenitor cells, myeloid progenitor cells, *etc*.

INDEXING/ABSTRACTING

The *WJSC* is now indexed in PubMed, PubMed Central, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports/Science Edition, Biological Abstracts, and BIOSIS Previews. The 2019 Edition of Journal Citation Reports cites the 2018 impact factor for *WJSC* as 3.534 (5-year impact factor: N/A), ranking *WJSC* as 16 among 26 journals in Cell and Tissue Engineering (quartile in category Q3), and 94 among 193 journals in Cell Biology (quartile in category Q2).

RESPONSIBLE EDITORS FOR THIS ISSUE

Basic Study

Cytotoxicity of nonylphenol on spermatogonial stem cells *via* phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin pathway

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Abstract
BACKGROUND
With continuous advancement of industrial society, environmental pollution has become more and more serious. There has been an increase in infertility caused by environmental factors. Nonylphenol (NP) is a stable degradation product widely used in daily life and production and has been proven to affect male fertility. However, the underlying mechanisms therein are unclear. Thus, it is necessary to study the effect and mechanism of NP on spermatogonial stem cells (SSCs).

AIM
To investigate the cytotoxic effect of NP on SSCs *via* the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway.

METHODS
SSCs were treated with NP at 0, 10, 20 or 30 μmol. MTT assay was performed to evaluate the effect of NP on the proliferation of SSCs. Flow cytometry was conducted to measure SSC apoptosis. The expression of Bad, Bcl-2, cytochrome-c, pro-Caspase 9, SOX-2, OCT-4, Nanog, Nanos3, Stra8, Scp3, GFRα1, CD90, VASA, Nanos2, KIT, PLZF and PI3K/AKT/mTOR-related proteins was observed by western blot, and the mRNA expression of SOX-2, OCT-4 and Nanog was detected by quantitative reverse transcription polymerase chain reaction.

RESULTS

Compared with untreated cells (0 µmol NP), SSCs treated with NP at all concentrations showed a decrease in cell proliferation and expression of Bcl-2, Nanog, OCT-4, SOX-2, Nanos3, Stra8, Scp3, GFRα1, CD90, VASA, Nanos2, KIT, and PLZF ($P < 0.05$), whereas the expression of Bad, cytochrome-c, and pro-Caspase 9 increased significantly ($P < 0.05$). We further examined the PI3K/AKT/mTOR pathway and found that the phosphorylation of PI3K, AKT, mTORC1, and S6K was significantly decreased by NP at all concentrations compared to that in untreated SSCs ($P < 0.05$). NP exerted the greatest effect at 30 µmol among all NP concentrations.

CONCLUSION
NP attenuated the proliferation, differentiation and stemness maintenance of SSCs while promoting apoptosis and oxidative stress. The associated mechanism may be related to the PI3K/AKT/mTOR pathway.

Key words: Spermatogonial stem cells; Nonylphenol; Cytotoxicity; Phosphatidylinositol-3-kinase; Protein kinase B; Mammalian target of rapamycin

Core tip: With continuous advancement of industrial society, environmental pollution has become more and more serious. There has been an increase in infertility caused by environmental factors. Nonylphenol is a stable degradation product widely used in daily life and production and has been proven to affect male fertility. Our study demonstrated that nonylphenol reduced the proliferation, differentiation and stemness maintenance of spermatogonial stem cells while promoting apoptosis and oxidative stress. The mechanism may be related to the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin pathway, providing a potential method for the treatment of male infertility.

INTRODUCTION

Spermatogonial stem cells (SSCs) are a class of primitive spermatogonia located at the basement membrane of convoluted seminiferous tubules that not only self-renew and maintain their population but also differentiate into spermatocytes. SSCs are transformed into primary and secondary spermatocytes, which then produce round and long spermatozoa cells, eventually forming sperm. SSCs are considered to be immortal because they can replicate themselves in adults and pass on from generation to generation.

With continuous advancement of industrial society, environmental pollution has become more and more serious. There has been an increase in infertility caused by environmental factors[1,2]. Environmental endocrine disruptors are exogenous substances that can interfere with the endocrine functions of the body, including those that mediate the synthesis, secretion, transport, binding, biological effects and clearance of hormones. This in turn leads to endocrine disorders and causes abnormal damage to body behavior, reproduction and development. Nonylphenol (NP) is a stable degradation product of alkylphenol that is widely used in plasticizers, pesticide emulsifiers and other industrial applications. Its molecular structure is similar to that of estradiol in the human body and can interfere with endocrine metabolism and damage human health[3-5]. Studies have shown that NP affects the body's hormonal balance and damages the body's reproductive functions[6,7]. NP can reduce the testicular weight and sperm production in rats without inducing pathological changes in the testis[8], but the underlying mechanisms therein are unclear. In this study, we observed the effect of different concentrations of NP on the proliferation, differentiation and apoptosis of isolated SSCs. Further molecular experiments were

performed to verify whether these effects were achieved *via* the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway.

MATERIALS AND METHODS

SSC culture and NP treatment
Ten-day-old (specific pathogen-free, disinfected with 75% ethanol) neonatal mice were provided by the Hubei Province Disease Control Center (No. 211002300042744). After anesthesia, bilateral testicles were removed from the mice and placed in sterile Petri dishes. Testicular tissue was rinsed three times with phosphate-buffered saline containing 5% penicillin-streptomycin (PAB180086, Bioswamp). Primary SSCs were isolated according to the method of Yu *et al*[9]. The rinsed testicular tissue was peeled off under a microscope, placed in a Petri dish with a small amount of high-glucose Dulbecco's modified Eagle medium (DMEM, SH30022.01B, Hyclone) and cut into 1-mm³ pieces with surgical ophthalmic scissors. The tissue block was resuspended in a solution of 0.125% trypsin and 0.1% collagenase I (1:1) (17100-017, Gibco) and incubated at 37 °C in an incubator containing 5% CO_2 for 30 min. Tissue digestion was terminated by adding an equal volume of DMEM, and the cells were centrifuged at $2500 \times g$ for 5 min. Thereafter, the cells were resuspended in culture solution and cultured in a Petri dish at 37 °C for 1 h. The cells were then seeded into a feeder-free Petri dish and passaged when they reached 85% confluence.

For flow cytometric identification of SSCs, seven Eppendorf tubes were prepared and 100 µL of single cell suspension was added to each tube. Antibodies against glial cell line-derived neurotrophic factor family receptor alpha-1 (GFRα1, 1:200, ab186855, Abcam), CD90 (11-0903-82, 0.125 µg/test, Invitrogen), VASA (PA5-23378r, 1:200, Invitrogen), Nanos2 (PA5-20553r, 1:50, Invitrogen), KIT (17-1171-82, 0.125 µg/test, Invitrogen) and promyelocytic leukemia zinc finger (PLZF, 1 µg/test, 53-9320-82, Invitrogen) were individually added to each tube (with one blank tube). The tubes were incubated at 4 °C for 45 min in the dark. After the addition of 2 µL of fluorescein (FITC)-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (PAB160016, Bioswamp) to each tube, they were further incubated at 4 °C for 45 min in the dark. After adding 400 µL of flow cytometry dyeing buffer to each tube, the cells were subjected to flow cytometry (CytoFLEX S, BECKMAN), and the results were analyzed using CYEXPERT software. After SSCs have been successfully isolated and identified, they were treated with NP (N109556, Aladdin) at 0, 10, 20 and 30 µmol for 7 d based on the method of Huang *et al*[10]. SSCs were treated with mTOR agonists (MHY1485, 10 µmol) and mTOR inhibitors (AZD8055, 10 µmol) according to the references[11,12] for 24 h. SSCs were respectively divided into four groups: Control (Control group, not subjected to any activator or inhibitor), NP (NP group), OV or IV (mTOR activator group; mTOR inhibitor group), OV/IV + NP (OV/IV + NP group) groups.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay
The proliferation of SSCs was detected using an MTT assay kit (PAB180013, Bioswamp) according to the manufacturer's instructions. Cells in the logarithmic growth phase were collected and seeded at 5×10^3 cells/well in a 96-well plate, and 20 µL of MTT solution was added to each well. The cells were incubated overnight at 37 °C in an incubator containing 5% CO_2, and 150 µL of dimethyl sulfoxide (D2650, Sigma) was added to each well. The optical density was measured at 490 nm with a microplate reader (Multiskan FC, Thermo).

Flow cytometric detection of apoptosis
SSCs (1.0×10^5 cells/mL) were treated once with different concentrations of NP (0, 10, 20 and 30 µmol) in sterile tubes and cultured for 7 d, after which 1 mL of pre-cooled phosphate-buffered saline was added. The cells were centrifuged at $1000 \times g$. Then, 10 µL of Annexin V-FITC and 10 µL of propidium iodide were added. The cell samples were analyzed by flow cytometry (CytoFLEX S, Beckman Coulter), and one-step fluorescence compensation strategy was used to eliminate interference in the FITC channel. After mTOR overexpression and mTOR inhibitor AZD8055 intervention, the effect of NP on apoptosis of SSCs was also examined.

Western blot
The concentration of proteins extracted from cells was measured using a bicinchoninic acid protein assay kit (PAB180007, Bioswamp). Total protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20, incubated with specific

primary antibodies overnight at 4 °C and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (1:20000, PAB160011, Bioswamp) for 2 h at 4 °C. The following primary antibodies were used: Bad (1:1000, PAB0589, Bioswamp), Bcl-2 (1:100, PAB30041, Bioswamp), cytochrome-c (Cyt-c, 1:1000, ab90529, Abcam), pro-caspase 9 (1:500, ab135544, Abcam), Nanos3 (1:1000, ab216694, Abcam), Stra8 (1:1000, ab49602, Abcam), synaptonemal complex protein 3 (Scp3, 1:1000, ab150292, Abcam), GFRα1 (1:500, PA1-32476, Thermo), CD90 (1:1000, ab92574, Abcam), VASA (1:1000, ab209710, Abcam), Nanos2 (1:1000, ab70000, Abcam), KIT (1:1000, ab32363, Abcam), PLZF (1:1000, ab39354, Abcam), PI3K (1:1000, ab191606, Abcam), p-PI3K (1:1000, ab182651, Abcam), AKT (1:1000, ab227100, Abcam), p-AKT (1:1000, ab133458, Thermo), mTOR complex 1 (mTORC1, 1:1000, ab137341, Abcam), p-mTORC1 (1:2000, ab226957, Abcam), ribosomal protein S6 kinase (S6K, 1:1000, ab186753, Abcam), p-S6K (1:1000, ab59208, Abcam), sex determining region Y-box 2 (SOX-2, 1:1000, PAB30154, Bioswamp), octamer-binding transcription factor 4 (OCT-4, 1:1000, ab18976, Abcam), Nanog (1:1000, PAB33609, Bioswamp) and GAPDH (1:2000, PAB36264, Bioswamp). After three washes with phosphate-buffered saline/Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (1:20000, PAB160011, Bioswamp). Protein bands were visualized by enhanced chemiluminescence color detection (Tanon-5200, TANON) and analyzed using AlphaEase FC gel image analysis software.

Quantitative reverse transcription polymerase chain reaction

Whole RNA was extracted from cell samples using Trizol reagent according to the manufacturer's procedures, and cDNA was synthesized using a reverse transcriptase kit (639505, TAKARA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a real-time system (CFX-96, Bio-RAD) with the SYBR Green PCR Kit (KM4101, KAPA Biosystems). Each qPCR reaction was performed in duplicate and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers were designed and configured by Nanjing Kingsy Biotechnology Co., Ltd. and are listed in Table 1.

Statistical analysis

All data are presented as the mean ± standard deviation. The SPSS 19.0 software was used for statistical analyses and GraphPad Prism 5.0 was used to prepare the figures. The data were evaluated for statistical significance using one-way analysis of variance. Statistical significance was established at $P < 0.05$.

RESULTS

Culture and identification of mouse SSCs

We observed SSCs on the 3rd and 10th d of culture with optical microscopy. As shown in Figure 1A, the isolated SSCs were round with large and round nuclei and less cytoplasm. Compared with the 3rd d, the number of SSCs increased significantly on the 10th d. We further identified SSCs by flow cytometry. As shown in Figure 1B, the SSCs showed high expression of GFRα1, CD90, VASA, Nanos2, KIT and PLZF, suggesting that mouse SSCs were successfully isolated and cultured.

NP inhibited the proliferation of SSCs

After SSCs were treated with different concentrations of NP (0, 10, 20 and 30 μmol) for 7 d, we examined the changes in cell proliferation (Figure 2). Compared with untreated cells (0 μmol), the proliferation of cells treated with NP at 10, 20 and 30 μmol NP decreased significantly ($P < 0.05$) in a concentration-dependent manner. This suggests that NP had an inhibitory effect on SSC proliferation.

NP promoted apoptosis in SSCs

To study the effect of NP on SSCs apoptosis, flow cytometry and western blot were performed. As the NP concentration increased, the percentage of SSC apoptosis increased gradually (Figure 3A). Examination of the expression of apoptosis-related proteins revealed that compared to untreated SSCs (0 μmol NP), those treated with 10, 20 and 30 μmol NP showed a significant increase in the expression of Bad, Cyt-c and pro-Caspase 9 ($P < 0.05$), while the expression of Bcl-2 decreased significantly ($P < 0.05$) (Figure 3B). We next observed the cell apoptosis after mTOR activator or inhibitor intervention, the results showed that the apoptosis rate of NP group decreased significantly after increased mTOR activity, while the apoptosis rate of SSCs in the NP group increased significantly after inhibiting mTOR activity (Figure 3C). The expression levels of apoptosis-related proteins are similar to the above trend ($P < 0.05$) (Figure 3D).

Table 1 Primer sequences	
Primer	**Sequence, 5'-3'**
OCT-4-F	CACTCACATCGCCAATC
OCT-4-R	AAGGTGTCCCTGTAGCC
SOX-2-F	AGCGGCGTAAGATGGC
SOX-2-R	CGGTCTCGGACAAAAGT
Nanog-F	AGCCCTGATTCTTCTACC
Nanog-R	TGAAACCTGTCCTTGAGT
GAPDH-F	CCACTCCTCCACCTTTG
GAPDH-R	CACCACCCTGTTGCTGT

NP inhibited stemness maintenance and differentiation of SSCs

As shown in Figure 4, the mRNA and protein expression of stemness maintenance markers Nanog, OCT-4 and SOX-2 in SSCs treated with 10, 20 and 30 µmol NP was decreased compared with those in untreated cells ($P < 0.05$), and the decrease was directly proportional to the NP concentration. Further detection of SSC differentiation markers showed that the protein expression of Nanos3, Stra8, Scp3, GFRα1, CD90, VASA, Nanos2, KIT and PLZF in SSCs treated with NP was decreased significantly ($P < 0.05$) compared with that in untreated cells (Figure 5). These results indicated that NP inhibited the stemness maintenance and differentiation of SSCs.

Effect of NP on PI3K/AKT/mTOR pathway

To investigate the influence of NP on PI3K/AKT/mTOR signaling in SSCs, we measured the protein expression of p-PI3K, p-Akt, p-mTORC1 and p-S6K using western blot (Figure 6). We observed that compared with untreated cells (0 µmol), the expression of p-PI3K, p-Akt, p-mTORC1 and p-S6K was significantly decreased by NP at all concentrations ($P < 0.05$), with 30 µmol NP exerting the greatest effect among all concentrations. We further observed the effect of NP on the PI3K/AKT/mTOR pathway in SSCs after activation or inhibition of mTOR. The results showed that after activation of mTOR, the expression of PI3K, AKT and mTORC1 protein in the NP group decreased significantly compared with the control group ($P < 0.05$), while that in the NP + OV group increased significantly ($P < 0.05$). When mTOR was inhibited, the trend reversed ($P < 0.05$) (Figure 7).

DISCUSSION

Numerous self-renewal systems exist in male mammals, but in terms of species continuity, the most important process is spermatogenesis, which is crucial in gene transmission for the generation and evolution of male gametes. A large number of sperm is produced *via* spermatogenesis through orderly and strict cell proliferation and differentiation, and SSCs form the basis of this system. NP is an important fine chemical raw material and intermediate that is mainly used in the production of surfactants, antioxidants, lubricant additives, pesticide emulsifiers, resins and rubber stabilizers and is known as a "sperm killer"[13]. NP has certain toxic effects on sperm, which are differentiated from SSCs. Shaliutina *et al*[14] found that NP induced oxidative stress in fish sperm, reduced sperm activity and destroyed the integrity of the sperm cell membrane. Meng *et al*[15] found that NP decreased the number and activity of sperm and increased the rate of sperm deformity with significant NP concentration-dependent response. Though the impact of NP on sperm health has been demonstrated, whether NP has similar toxic effects on SSCs is unclear.

While SSCs can maintain their relative number by self-proliferation, they also undergo mitosis and meiosis to form spermatocytes, which eventually form sperm. In addition, they have the potential to maintain self-renewal and proliferation to cope with possible damage[16]. The lifetime amplification and immortality of SSCs guarantee sperm production. In this experiment, we treated SSCs with different concentrations of NP for 7 consecutive days. We found that with the increase in NP concentration, the proliferation of SSCs decreased while apoptosis increased gradually. We detected apoptosis-related proteins and found that the pro-apoptotic proteins Bad, Cyt-c and pro-Caspase 9 were upregulated significantly, while the anti-apoptotic protein Bcl-2 was downregulated, suggesting that NP promoted apoptosis and inhibited SSC proliferation by inducing oxidative stress.
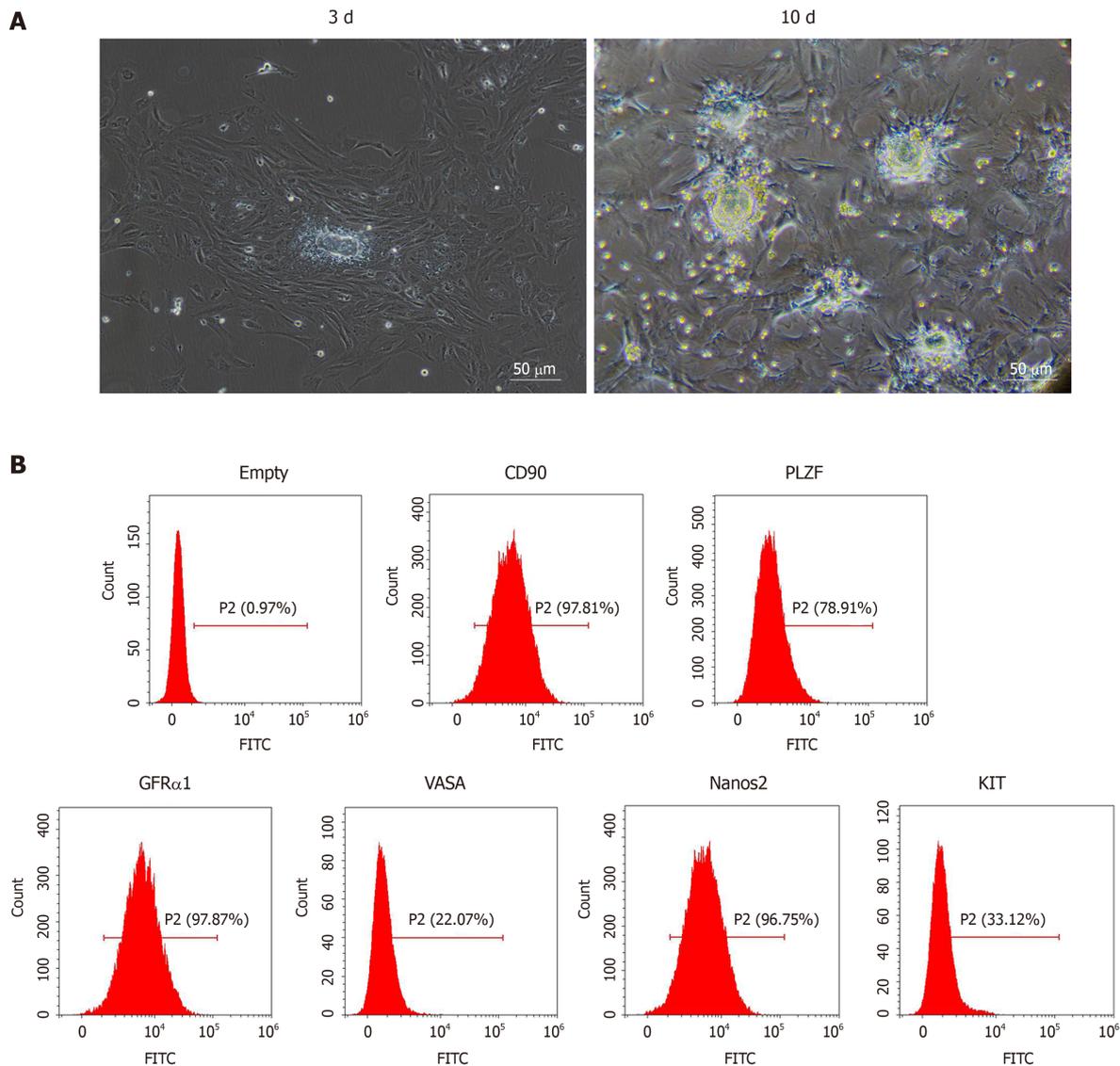
Figure 1 Culture and identification of spermatogonial stem cells. A: Light microscopy of spermatogonial stem cells cultured for 3 d and 10 d (scale bar = 50 μm); B: Identification of spermatogonial stem cells by flow cytometry.

Stemness maintenance and differentiation potential are important characteristics of SSCs, and the continuous differentiation of SSCs is necessary for spermatogenesis. We showed that NP decreased the expression of markers of SSC stemness maintenance and differentiation. Stra8, which can be specifically activated by retinoic acid, is expressed in mouse embryonic ovary and adult testis, as well as before meiosis in mammalian germ cells[17,18]. Male Stra8-knockout mice were sterile and had severe defects in spermatogenesis and decreased testicular volume, suggesting that Stra8 plays a vital role in spermatogenesis[19]. Anderson *et al*[20] confirmed that Stra8 is not necessary for DNA replication in pre-filament spermatocytes but is essential for chromosome condensation and DNA homologous recombination in pre-filament spermatocytes during meiosis. Scp3 is an essential component of synaptonemal complex formation that plays an important role in male germ cell meiosis[21]. Nanos3 is a homologous gene of *Drosophila* Nanos that is specifically expressed in mouse germ cells. It is also an endogenous factor that initiates the process of male germ cell differentiation and plays a critical role in germ cell meiosis[22,23]. In this study, NP downregulated the expression of Stra8, Nanos3 and Scp3, suggesting that NP may inhibit the differentiation and stemness maintenance of SSCs by regulating pre-meiotic factors.

The PI3K/AKT/mTOR signaling pathway is essential for the proliferation of SSCs. The AKT/mTOR signaling pathway plays an important role in spermatogenesis by regulating the growth and transport of sperm cells in the testis and maintaining the normal function of SSCs[24,25]. The mTOR signal is directly involved in the late differentiation of SSCs. Inhibition of mTOR expression can lead to the accumulation of
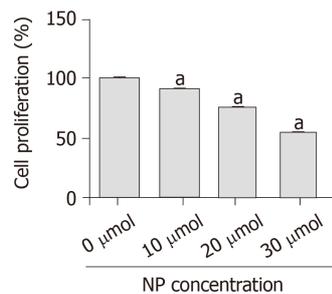
Figure 2 MTT assay of cell proliferation. The results are presented as the mean ± standard deviation, *n* = 3. ^a*P* < 0.05 *vs* 0 μmol nonylphenol. NP: Nonylphenol.

a large number of SSCs[26,27], and the promotion of mTOR expression can promote the proliferation of SSCs[28,29]. Duan *et al*[30] observed that NP inhibits the expression of mTOR and mediates autophagy, apoptosis and necrosis of sperm cells. Duan *et al*[31] also revealed that NP inhibits the activity of mTOR-p70S6K/4EBP1 signaling pathway and inhibits sperm differentiation and proliferation. Ma *et al*[32] found that Lin28a, a conserved RNA-binding protein, is involved in SSC development, pluripotency, stemness maintenance, proliferation and self-renewal through the PI3K/AKT/mTOR pathway. Huang *et al*[33] observed that exposure to NP during pregnancy led to endocrine dysfunction in male offspring, inhibition of AKT/mTOR signaling and induction of apoptosis and autophagy in testicular tissue. We showed that NP significantly reduced the expression of p-PI3K, p-Akt, p-mTORC1 and p-S6K, suggesting that the effect of NP may be exerted through the PI3K/AKT/mTOR signaling pathway.

NP is produced by biodegradation of NP polyoxyethylene ether and is soluble in water. NP polyoxyethylene ether is widely used in industry and household. NP is widely found in rivers and silts in Japan, the United States, Germany and other countries. In heavily polluted rivers in the United Kingdom, the concentration of NP is as high as 534 μg/L[34-36]. Similar findings have been found in some rivers in China, and the pollution of NP in China's current water system has significantly exceeded that in foreign countries. Compared with the significant increase in the concentration of NP in water, the reproduction rate of wild animals and humans is greatly reduced[37,38]. More and more families are childless, and women have been criticized for their inability to reproduce. Gender discrimination has become increasingly serious worldwide. Male oligospermia or azoospermia is also one of the important reasons for the lack of offspring, which is often overlooked. In this experiment, we confirmed that NP can not only reduce the number and activity of sperm but also affect the proliferation and differentiation of SSCs. The mechanism may be related to the PI3K/AKT/mTOR pathway. Therefore, we speculate that promoting the activity of the PI3K/AKT/mTOR pathway may help relieve male oligozoospermia caused by NP.

In conclusion, our study demonstrated that NP reduced the proliferation, differentiation and stemness maintenance of SSCs while promoting apoptosis and oxidative stress, and the mechanism may be related to the PI3K/AKT/mTOR pathway. In future studies, we will investigate whether promoting PI3K/AKT/mTOR activity has therapeutic effects on NP-induced oligospermia.
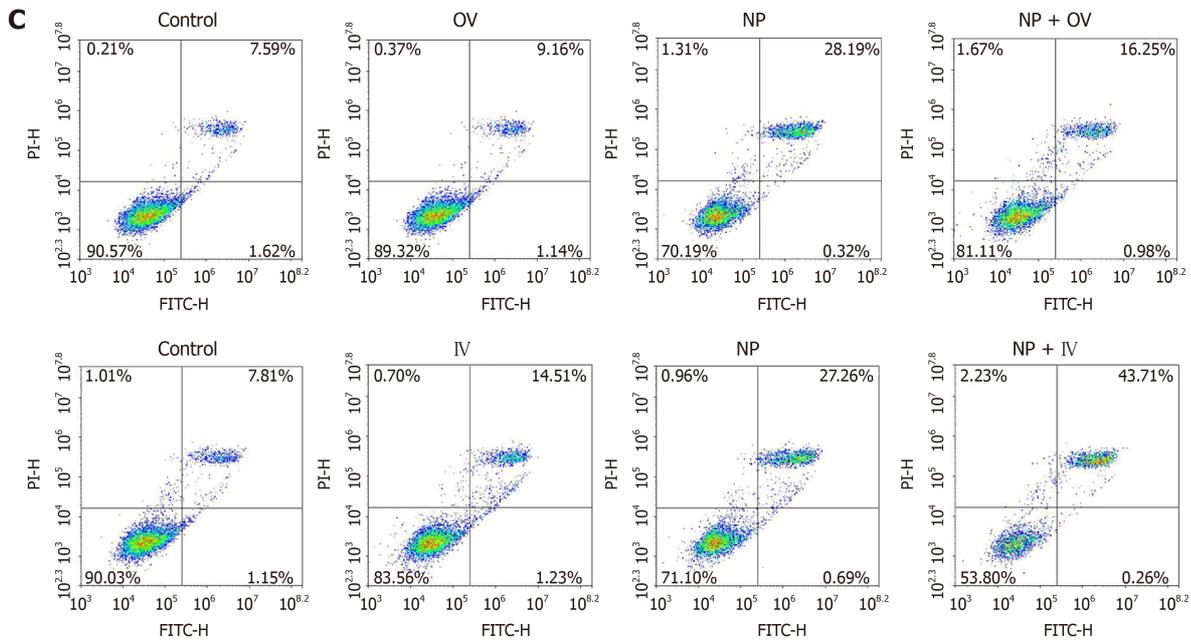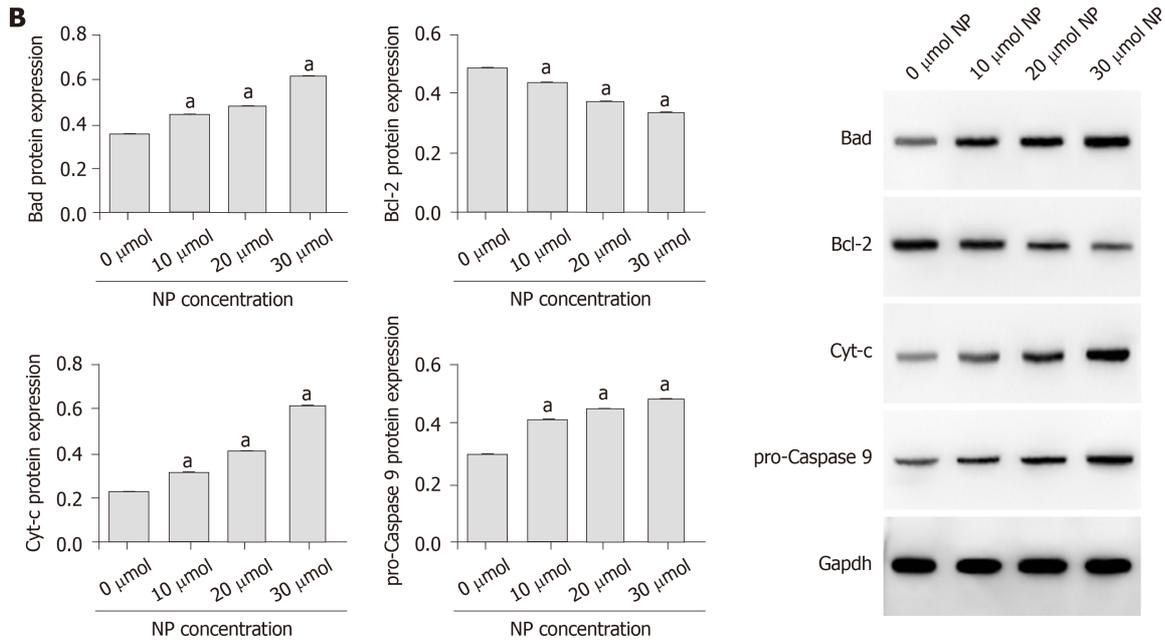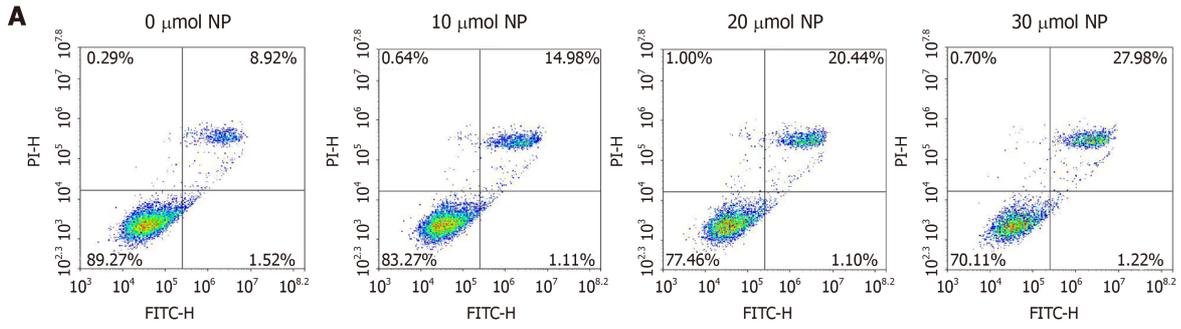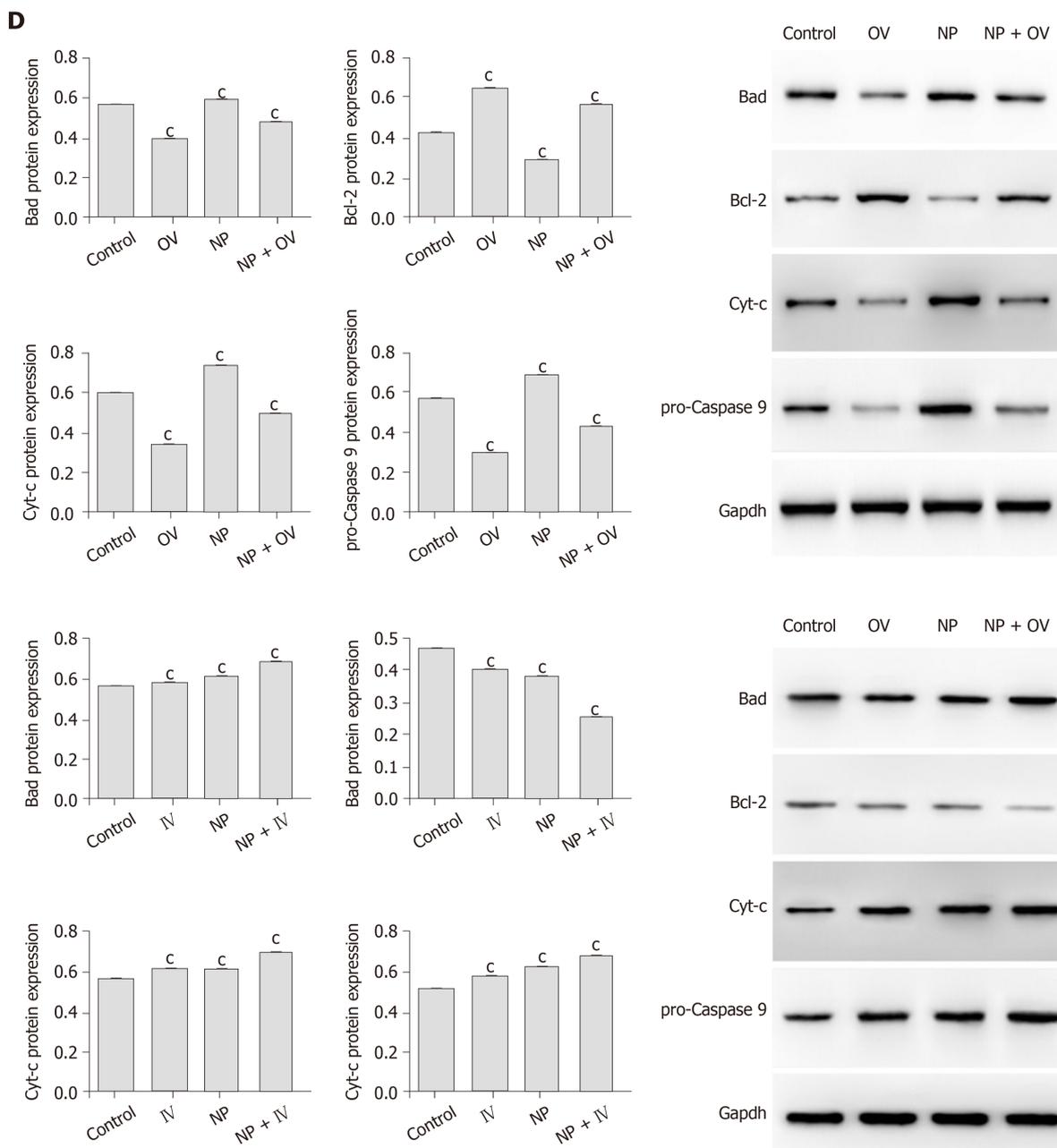
Figure 3 Effect of nonylphenol on apoptosis and oxidative stress in spermatogonial stem cells. A: Detection of apoptosis by flow cytometry; B. Western blot of the protein expression of Bad, Bcl-2, Cyt-c, and pro-Caspase 9. The results are presented as the mean ± standard deviation, *n* = 3. [a]*P* < 0.05 *vs* 0 µmol NP; C: Effect of nonylphenol on spermatogonial stem cell apoptosis after mammalian target of rapamycin activation or inhibition; D: Expression of apoptosis-related protein detected by western blot. The results are presented as the mean ± standard deviation, *n* = 3. [c]*P* < 0.05 *vs* control. NP: Nonylphenol; Control: Control group, not subjected to any activator or inhibitor; NP: Nonylphenol group; OV: mammalian target of rapamycin activator group; OV + NP: OV + NP group.
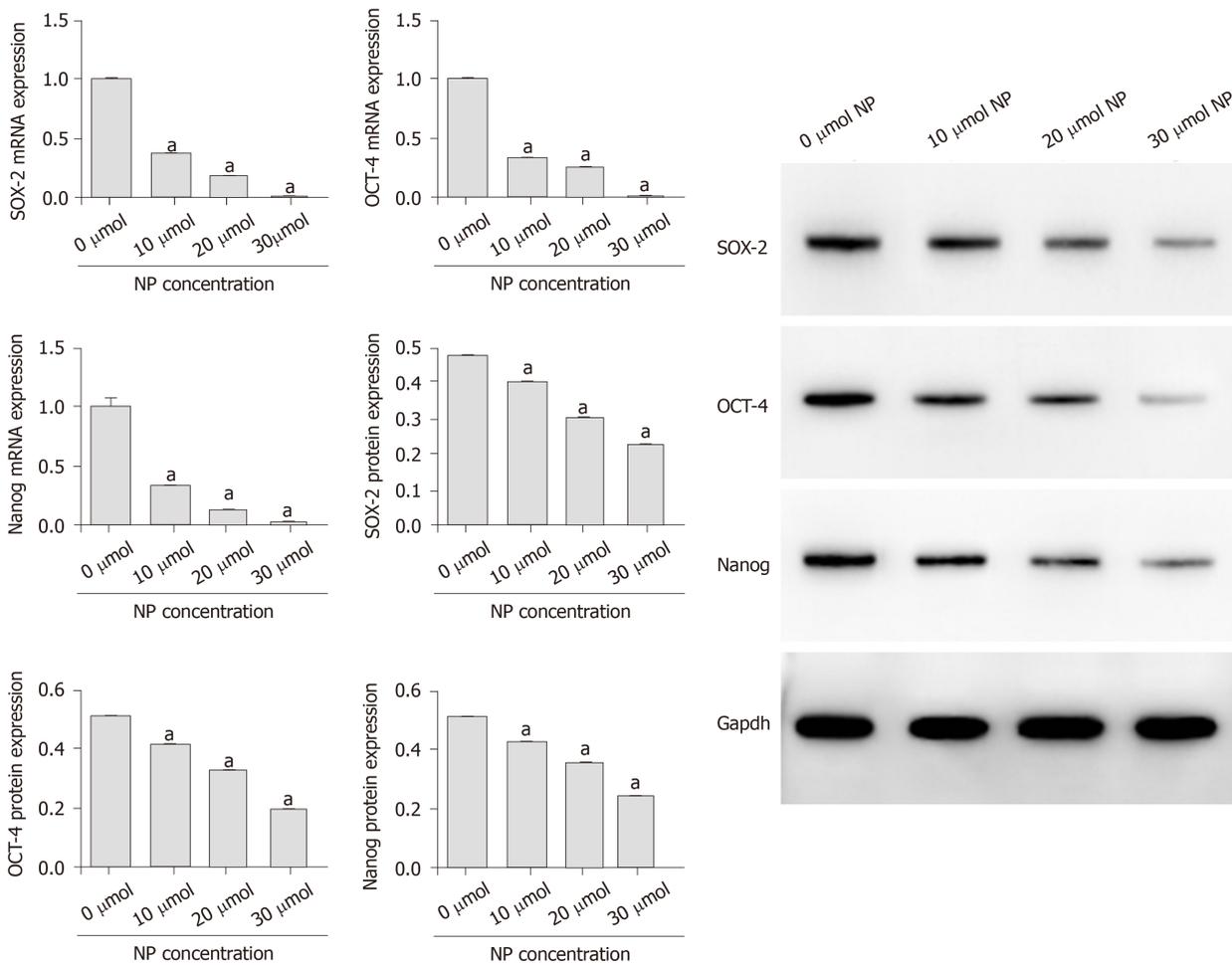
Figure 4 Effect of nonylphenol on stemness maintenance of spermatogonial stem cells. mRNA and protein expression of SOX-2, OCT-4 and Nanog were measured by quantitative reverse transcription polymerase chain reaction and western blot, respectively. The results are presented as the mean ± standard deviation, *n* = 3. [a]*P* < 0.05 *vs* 0 µmol nonylphenol. NP: Nonylphenol.
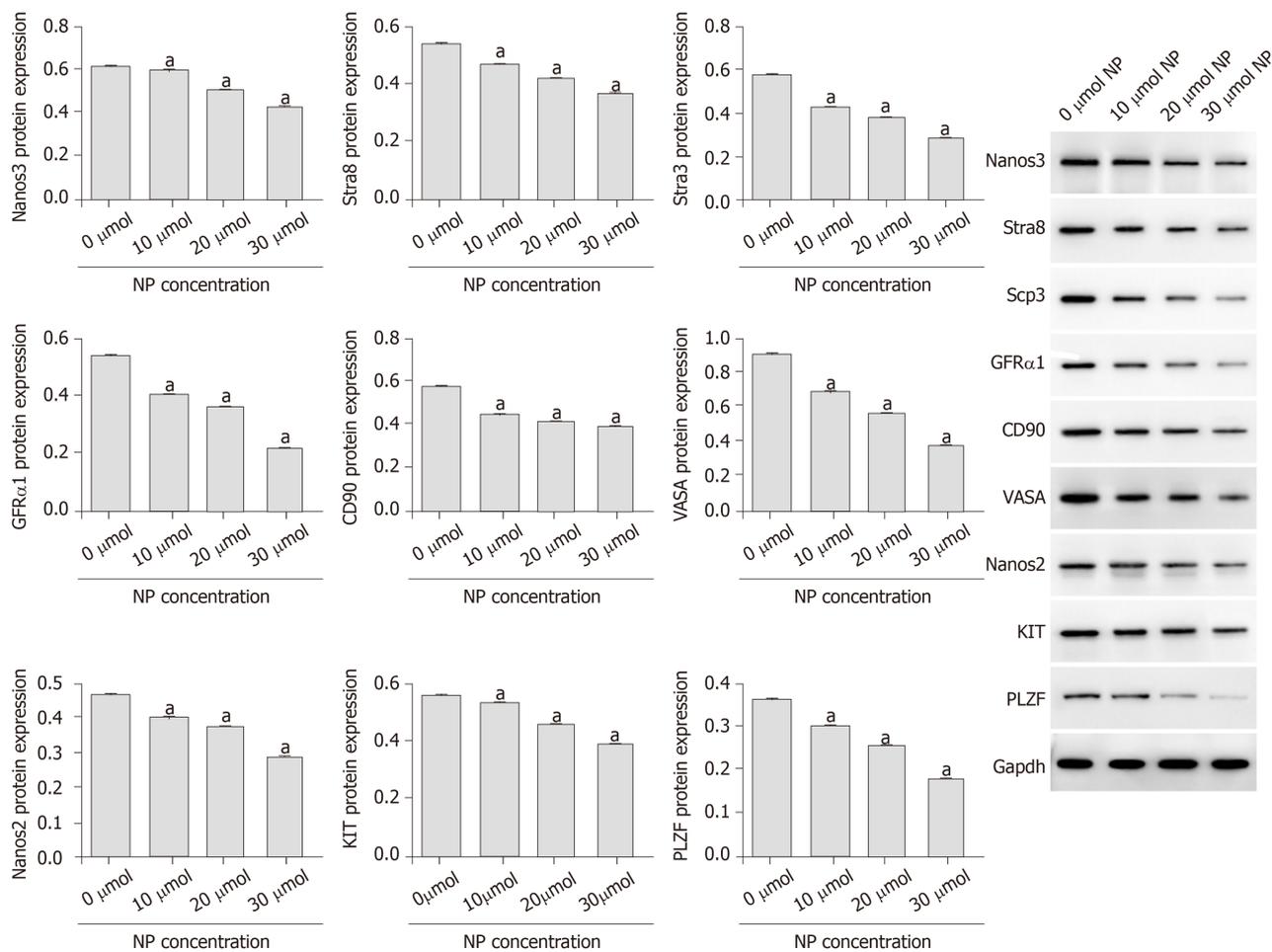
Figure 5 Effect of nonylphenol on differentiation of spermatogonial stem cells. Protein expression of Nanos3, Stra8, Scp3, GFRα1, CD90, VASA, Nanos2, KIT and PLZF was measured by western blot. The results are presented as the mean ± standard deviation, $n = 3$. [a]$P < 0.05$ *vs* 0 μmol nonylphenol. NP: Nonylphenol.
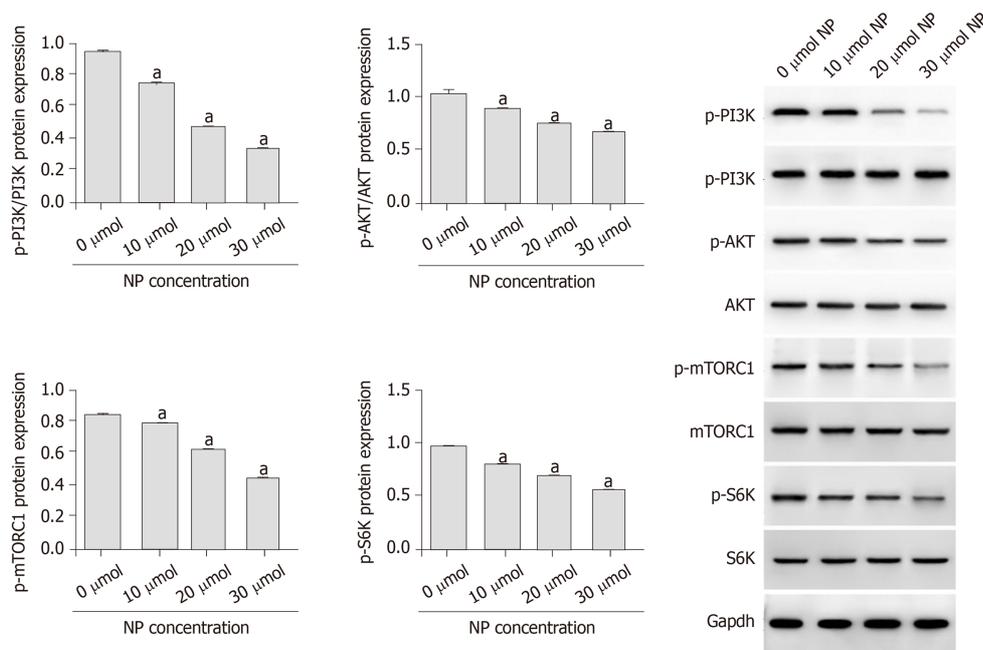
Figure 6 Effect of nonylphenol on the expression of proteins associated with phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin signaling. The results are presented as the mean ± standard deviation, $n = 3$. [a]$P < 0.05$ *vs* 0 μmol nonylphenol. NP: Nonylphenol.
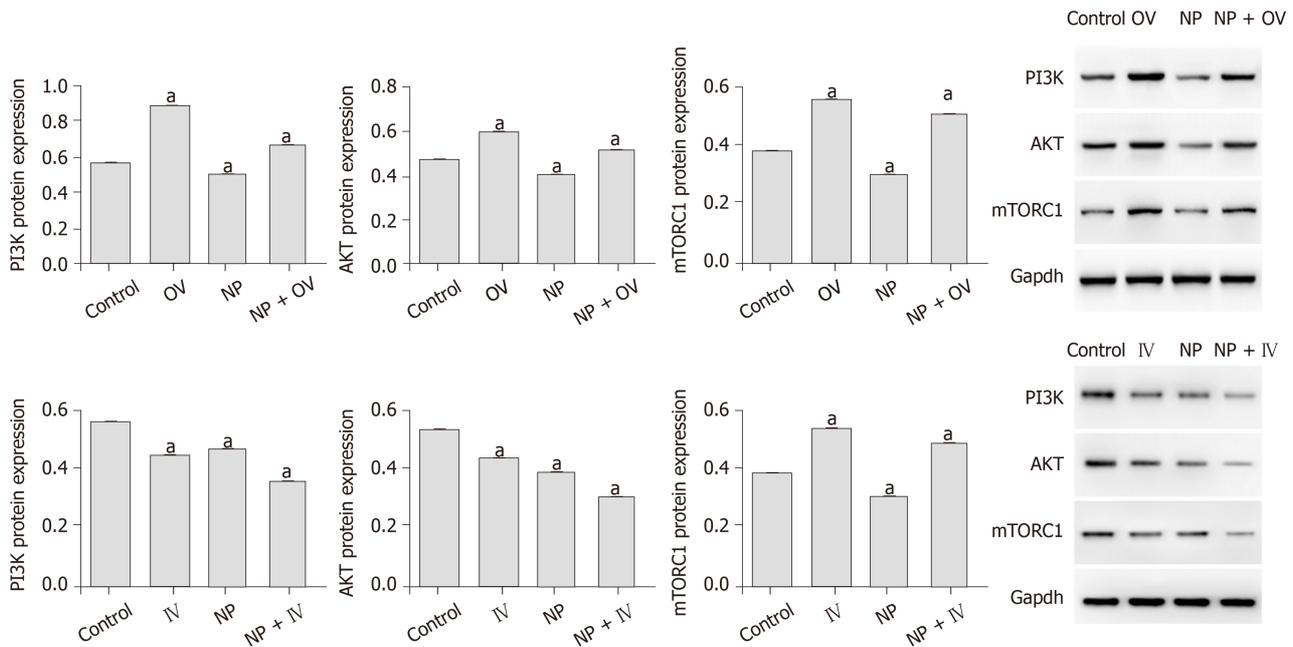
Figure 7 Effects of nonylphenol on phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway after mammalian target of rapamycin activation or inhibition. The results are presented as the mean ± standard deviation, *n* = 3. [a]*P* < 0.05 *vs* Control. Control: Control group, not subjected to any activator or inhibitor; NP: Nonylphenol group; OV: Mammalian target of rapamycin activator group; IV: Mammalian target of rapamycin inhibitor group; OV + NP: OV + NP group; IV + NP: IV + NP group.

ARTICLE HIGHLIGHTS

Research background

Infertility has become a social problem that needs to be solved urgently in the world. Apart from the infertility caused by female causes, infertility caused by male oligozoospermia has gradually been valued. Previous studies have confirmed that nonylphenol (NP) widely used in daily life can reduce male sperm counts, but the underlying mechanism is still unclear. Studying the specific mechanism of NP-induced oligospermia could provide some ideas for the treatment of NP-induced oligospermia.

Research motivation

NP has been shown to affect sperm activity, but the mechanism is currently unknown. Spermatogonial stem cells (SCCs) can eventually differentiate into sperm. We aim to study whether NP can affect the proliferation, differentiation and potential mechanism of SSCs in order to provide ideas for clinical treatment of male oligospermia caused by NP.

Research objectives

To study the effect and potential mechanism of NP on SSCs.

Research methods

SSCs were treated with NP at 0, 10, 20 or 30 μmol. MTT was used to detect the effect of NP on the proliferation of SSCs. Flow cytometry, reverse transcription polymerase chain reaction and western blot were used to detect the effect of NP on the proliferation, apoptosis, oxidative stress and stemness maintenance of SSCs. The effects of NP on phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway was also measured by western blot.

Research results

Different concentrations of NP (10, 20 or 30 μmol) could inhibit the proliferation of SSCs, reduce the expression of cell differentiation and stem maintenance related factors and promote apoptosis and the release of oxidative stress factors. We further examined the effect of NP on the PI3K/AKT/mTOR pathway, and the results showed that NP can significantly inhibit the activity of the PI3K/AKT/mTOR pathway. Among all NP concentrations, 30 μmol had the greatest effect.

Research conclusions

NP reduced the proliferation, differentiation and stemness maintenance of SSCs while promoting apoptosis and oxidative stress, and the mechanism may be related to the PI3K/AKT/mTOR pathway, providing a potential method for the treatment of male infertility.

Research perspectives

In this study, we demonstrated *in vitro* that NP could promote apoptosis and oxidative stress of SSCs and reduce the proliferation, differentiation and stem maintenance of SSCs, and the mechanism may be related to the PI3K/AKT/mTOR pathway. Therefore, we speculate that promoting the activity of the PI3K/AKT/mTOR pathway may help relieve male oligozoospermia caused by NP, and we will use PI3K/AKT/mTOR pathway agonist to verify our conjecture in the following studies *in vivo*.

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