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Tri-*ortho*-cresyl phosphate induces autophagy of rat spermatogonial stem cells

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Abstract

Tri-*ortho*-cresyl phosphate (TOCP) has been widely used as plasticizers, plastic softeners, and flame retardants in industry and reported to have a deleterious effect on the male reproductive system in animals besides delayed neurotoxicity. Our preliminary results found that TOCP could disrupt the seminiferous epithelium in the testis and inhibit spermatogenesis, but the precise mechanism is yet to be elucidated. This study shows that TOCP inhibited viability of rat spermatogonial stem cells in a dose-dependent manner. TOCP could not lead to cell cycle arrest in the cells; the mRNA levels of *p21*, *p27*, *p53*, and cyclin D1 in the cells were also not affected by TOCP. Meanwhile, TOCP did not induce apoptosis of rat spermatogonial stem cells. After treatment with TOCP, however, both LC3-II and the ratio of LC3-II/LC3-I were markedly increased; autophagy proteins ATG5 and beclin 1 were also increased after treatment with TOCP, indicating that TOCP could induce autophagy in the cells. Ultrastructural observation under the transmission electron microscopy indicated that autophagic vesicles in the cytoplasm containing extensively degraded organelles such as mitochondria and endoplasmic reticulum increased significantly after the cells were treated with TOCP. In summary, we have shown that TOCP can inhibit viability of rat spermatogonial stem cells and induce autophagy of the cells, without affecting cell cycle and apoptosis.

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Introduction

Tricresyl phosphate (TCP) has been widely used as plastic softeners, plasticizers, jet oil additives, and flame-retardants in industry, and tri-*ortho*-cresyl phosphate (TOCP) is one of the three isomers (i.e., *o*-, *m*-, or *p*-cresyl) (Craig & Barth 1999, Winder & Balouet 2002). Unlike most organophosphates (OPs) exerting their acute toxic effects by suppressing acetylcholinesterase, TOCP mainly induces a delayed neurodegenerative condition known as OP-induced delayed neuropathy (OPIDN), which affects both the central and the peripheral nerves in sensitive species such as humans and hens (Smith *et al*. 1930, Emerick *et al*. 2012, Song *et al*. 2012).

It has been reported that TOCP can induce reproductive toxicology (Somkuti *et al*. 1987*a*, 1991), besides neurotoxicity (Craig & Barth 1999, Zhang *et al*. 2007) and immunotoxicity (Foil *et al*. 1980, Brinkerhoff *et al*. 1981) in animals. TOCP has been shown to decrease sperm density in the cauda epididymis, inhibit testicular enzyme activities, and disrupt the seminiferous epithelium in rats and mice (Somkuti *et al*. 1987*a*, 1991, Chen *et al*. 2012). However, the precise mechanisms remain to be elusive.

Spermatogenesis is a complex process consisting of sequential and highly organized steps of undifferentiated spermatogonial stem cell proliferation and differentiation, in which functional sperm were generated in the testis (Mclean *et al*. 2002, Chen *et al*. 2011, 2012). The undifferentiated spermatogonial cells in the testes of non-primate mammals consist of type A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) germ cells (de Rooij & Russell 2000, Oatley *et al*. 2010, Chen *et al*. 2011). In general, type A_s spermatogonia have been considered the spermatogonial stem cells. Therefore, normal viability and function of spermatogonial stem cells in the seminiferous epithelium play an important role in maintaining the normal number of sperm.

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We also found that saligenin cyclic-*o*-tolyl phosphate (SCOTP), which was converted from TOCP by cytochrome P450 (CYP450), significantly inhibited viability of mice spermatogonial stem cells (Chen *et al.* 2012). However, inhibition of viability of spermatogonial stem cells might result from decrease in cell proliferation, induction of apoptosis, and autophagy.

The aim of this study is to investigate whether TOCP induces autophagy of rat spermatogonial stem cells. This study sets in motion our future investigation of the mechanisms underlying TOCP inhibition of spermatogenesis.

Materials and methods

Materials

Cell culture reagents were obtained from Gibco BRL. TOCP (purity >99.0%) was purchased from BDH Chemicals Co. Ltd (Poole, England). Recombinant rat epidermal growth factor (EGF) (400-25), recombinant rat GDNF (450-51), and recombinant rat fibroblast growth factor (FGF)-basic (400-29) were obtained from PeproTech (Rocky Hill, NJ, USA). Rabbit anti-LC3 polyclonal antibody (PD014), rabbit anti-Atg5 polyclonal antibody (PM050), and rabbit anti-Beclin 1 polyclonal antibody (PD017) were obtained from MBL Co. Ltd (Nagoya, Japan). Mouse anti-β-actin MAB, goat anti-mouse IgG–HRP, and goat anti-rabbit IgG–HRP were purchased from Santa Cruz Biotechnology. The ECL reagent was obtained from Pierce Biotechnology (Rockford, IL, USA). TRIzol reagent was purchased from Invitrogen Life Technologies and EasyScript First-strand cDNA Synthesis SuperMix for RT-PCR was purchased from TransGen Biotechnology (Beijing, China). Annexin V–FITC Apoptosis Detection Kit was obtained from Invitrogen Life Technologies.

Cell culture

Rat spermatogonial stem cells were isolated from male rats of 9 days according to Hamra *et al.* (2008) and Chen *et al.* (2011) with minor modifications. In brief, six pups were anesthetized and then killed by cervical dislocation. After decapsulation, the testes were minced into small pieces with scissors and incubated in DMEM containing 1 mg/ml type IV collagenase, 1 mg/ml trypsin, 1 mg/ml type I hyaluronidase, and 7 μg/ml DNAse (all from Sigma) and incubated at 32 °C for a total of 30 min. Then, germ cells were purified from somatic testis sells by simple cell culture manipulations, and spermatogonial stem cells were enriched from the germ cell fraction by selection in culture on laminin (Hamra *et al.* 2008). The enriched SSCs were cultured on STO (SIM mouse embryo-derived thioguanine and ouabain resistant) cell feeders in a serum-free medium supplemented with growth factors, such as recombinant rat EGF, recombinant rat Glial cell line-derived neurotrophic factor (GDNF), and recombinant rat FGF-basic. Incubations were carried out at 34 °C in a humidified atmosphere of 5% CO_2/95% air. The protocol was approved by the Institutional Animal Care and Use Committee of Nanchang University.

MTT reduction assay

The cells (1×10^4 cells/well) were seeded in a 96-well culture plate and were incubated with a fresh medium containing 0–1.0 mM TOCP for 48 h. TOCP were dissolved in DMSO, and the final concentration of DMSO in the culture medium was 0.1% (v/v). Forty-eight hours later, cell viability was assessed by the MTT assay. Cell medium containing 0.5 mg/ml MTT was replaced in each well and incubated at 34 °C in an atmosphere of 5% CO_2/95% for 4 h. The formed insoluble formazan was dissolved in DMSO, and the absorbance was measured in a spectrophotometer at a wavelength of 570 nm with a background reading of 660 nm.

Flow cytometric analysis

The cells were seeded at a density of 1×10^5 in 60-mm culture dishes. After 24 h, cells were treated with 0–1.0 mM TOCP for 48 h in a complete medium. At the indicated time, cells were harvested by trypsinization, centrifuged at 600 ***g*** for 5 min, washed in cold PBS, fixed overnight in 75% cold ethanol, digested with 20 μg/ml RNase A, and stained with 50 μg/ml propidium iodide (PI). The cells were then subjected to flow cytometric analysis on a FACScan (Becton Dickinson Company, Franklin Lakes, NJ, USA).

RT-PCR

The cells were plated at a density of 5×10^5 in 60-mm culture dishes and were incubated with a fresh medium containing 0–1.0 mM TOCP for indicated time. Total RNA from cultured cells was isolated using TRIzol reagent according to the manufacturer's instructions and quantified spectrophotometrically. Total RNA (1 μg) of each sample was reverse transcribed using the EasyScript First-strand cDNA Synthesis SuperMix in a final volume of 20 μl, according to the manufacturer's instructions. The PCR analyses were then performed on the aliquots of the cDNA preparations to detect the cyclin D1, *p21*, *p27*, *p53*, and actin gene expression using a thermal cycler, Mastercycler personal (Eppendorf AG 22331, Hamburg, Germany). The reactions were carried out in a volume of 25 μl containing (final concentration) 2.5 U of Taq DNA polymerase, 0.2 mM dNTPs, 1× reaction buffer, and 100 pmol of specific 5′ and 3′ primers. All primers for cyclin D1, *p21*, *p27*, *p53*, and actin sequences are listed in Table 1. According to the PCR conditions indicated in Table 1, for each indicated gene amplification, the denaturation cycle at 94 °C for 3 min was followed by 30 cycles and an elongation cycle at 72 °C for 5 min at the end of amplification. Using 2% agarose gel electrophoresis and ethidium bromide staining, the PCR products were detected with Chemi-DocXRS (Bio-Rad) under u.v. irradiation. The relative mRNA levels of these genes were quantified by densitometry using the Quantity One Software program (Hercules, CA, USA).

Annexin V–FITC/PI apoptosis assay

The apoptosis assay was analyzed by double staining the cells with FITC-labeled Annexin V and PI, using an Annexin V–FITC

Table 1 Sequence of primers used in PCR, amplicon sizes, and annealing temperatures.

Target accession no.	Primer sequences	Cycling parameters	Amplicon size (bp)
Cyclin D1, D14014	F: 5′-GGAGCAGAAGTGCGAAGA-3′ R: 5′-TGGAGGGTGGGTTGGA-3′	94 °C 30 s, 46 °C 15 s, and 72 °C 30 s	398
p21, U24174	F: 5′-GCAAAGTATGCCGTCGTCT-3′ R: 5′-CAAAGTTCCACCGTTCTCG-3′	94 °C 30 s, 46 °C 15 s, and 72 °C 30 s	111
p27, D83792	F: 5′-GGACGCCAGACAAACCG-3′ R: 5′-TCTCCACCTCCTGCCACTC-3′	94 °C 30 s, 46 °C 15 s, and 72 °C 30 s	194
p53, NM_030989	F: 5′-TGCTGAGTATCTGGACGACA-3′ R: 5′-CAGGCACAAACACGAACC-3′	94 °C 30 s, 46 °C 15 s, and 72 °C 30 s	225
Actin, NM_031144	F: 5′-CGTGCGTGACATTAAAGAG-3′ R: 5′-CTGGAAGGTGGACAGTGAG-3′	94 °C 30 s, 45 °C 15 s, and 72 °C 30 s	436

Apoptosis Detection Kit, according to the manufacturer's instructions. Briefly, rat spermatogonial stem cells treated with 0–1.0 mM TOCP were collected and washed twice with PBS. Then, the cells were resuspended with the Annexin V binding buffer, and transferred to test tubes containing FITC-labeled Annexin V and PI. The cells were then incubated in the dark for 15 min at room temperature, and analyzed by flow cytometry using the FACS Calibur System (BD Biosciences, San Jose, CA, USA). The excitation wavelength was 488 nm and the emission wavelength was 530 nm. A total of 10 000 cells were acquired. Flow cytometric data were analyzed using the FlowJo 7.6 Software (Tree Star, Ashland, OR, USA) and displayed in dot plot of Annexin V/FITC (*y*-axis) against PI (*x*-axis). The normal healthy cells were Annexin V–FITC and PI double negative, whereas the late apoptotic or secondary necrotic cells were double positive. The early apoptotic cells were only Annexin V–FITC positive, whereas the isolated nuclei or cellular debris were only PI positive (van Engeland *et al.* 1998).

Western blotting analysis

The cells were trypsinized, washed twice with ice-cold PBS, and harvested in cell lysis buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 5 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, and 0.5% NP40) containing the protease inhibitor cocktail (Huatesheng Biotech, Fushun, Liaoning, China). Cell lysates were briefly ultrasonicated and clarified by centrifugation at 12 000 ***g*** for 10 min at 4 °C. The supernatants were collected for further experiments. Protein concentration was determined according to the method described by Lowry *et al.* (1951) using BSA as a standard. The protein samples were separated by SDS–PAGE with a 5% stacking gel and 8% separating gel and transferred onto PVDF membrane (Millipore Corporate, Billerica, MA, USA). Following transfer, membranes were blocked with 1× Tris-buffered saline buffer containing 0.05% Tween-20 and 5% non-fat milk for at least 1 h at room temperature, then incubated with primary antibodies (diluted 1:1000), and finally incubated with HRP-conjugated goat anti-rabbit IgG (diluted 1:5000). Immunoreactive bands were detected using a ChemiDoc XRS System (Bio-Rad) and standard ECL reagents.

Transmission electron microscopy analyses

The cells were incubated in normal DMEM or in Hanks' solution for 3 h or treated with TOCP at 1.0 mM for 48 h. At the end of incubation, the cell monolayers were washed with PBS, and scraped gently with a plastic cell scraper. Then, the harvested cells were pelleted by centrifugation at 100 ***g*** for 10 min and fixed in ice-cold 2.5% glutaraldehyde for 2 h. Afterwards, samples were post-fixed in 1% OsO_4 for 1 h, dehydrated through ethanol series, and embedded in epoxy resin, then ultra-thin sections (60 nm) were double stained with uranyl acetate and lead citrate. Representative areas were examined under a transmission electron microscope (TEM, Hitachi H800).

Statistical analysis

Values are expressed as means ± S.E.M. Data were evaluated by one-way ANOVA with a Newman–Keuls multiple range test. For each test, P values <0.05 were considered statistically significant.

Results

TOCP inhibits cell proliferation of rat spermatogonial stem cells

Rat spermatogonial stem cells were isolated from a male rat according to the protocol in our laboratory (Chen *et al.* 2011). The purity of the spermatogonial stem cells is ∼90% (data not shown). We treated rat spermatogonial stem cells with 0–1.0 mM TOCP for 48 h. As shown in Fig. 1, we found that TOCP inhibited cell viability in a dose-dependent manner.

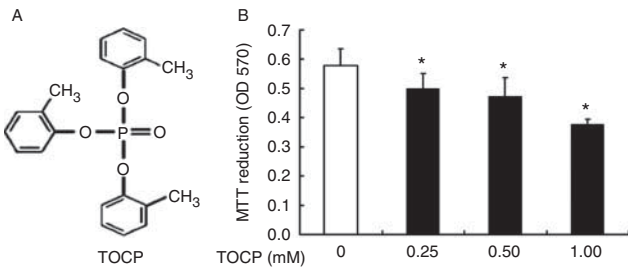

Figure 1 TOCP inhibits viability of rat spermatogonial stem cells. (A) Chemical structure of tri-*ortho*-cresyl phosphate (TOCP). (B) Rat spermatogonial stem cells were treated with 0–1.0 mM TOCP for 48 h. Then, cell viability was observed by MTT assay. The experiment was performed in triplicate and repeated three times. Data were analyzed by one-way ANOVA. *$P<0.05$.

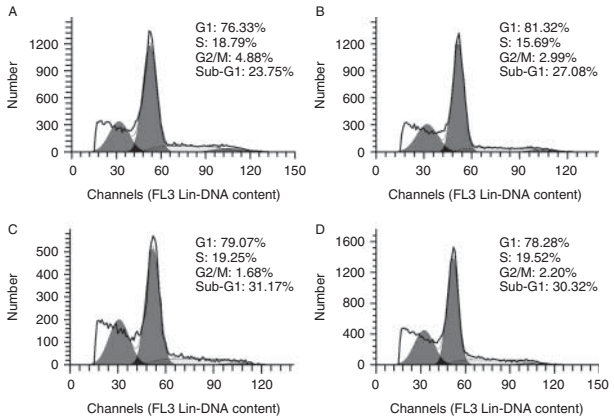

Figure 2 Effect of TOCP on the cell cycle of rat spermatogonial stem cells. Rat spermatogonial stem cells were treated with 0 mM (A), 0.25 mM (B), 0.5 mM (C), or 1.0 mM (D) TOCP for 48 h. Then, cell cycle was observed by FCM assay. The experiment was performed in triplicate and repeated three times. Data were analyzed by one-way ANOVA.

TOCP has no effect on cell cycle of rat spermatogonial stem cells

To determine the mechanism underlying TOCP-induced growth suppression, the rat spermatogonial stem cells were treated with indicated concentrations of TOCP (0, 0.25, 0.5, and 1.0 mM) for 48 h. The G1, S, and G2/M phases were determined by flow cytometry. Compared with control group, there was no change in cell cycle in the cells treated with the indicated concentration of TOCP (Fig. 2).

TOCP has no effect on mRNA expressions of cell cycle-related proteins

To further investigate whether TOCP affects cell cycle of rat spermatogonial stem cells, the mRNA levels of certain cyclins and cyclin-dependent kinase inhibitor (CKI) family members such as *p21*, *p27*, *p53*, and cyclin D1, which were crucially required for the regulation of cell cycle progression, were observed by RT-PCR after the cells were treated with 0–1.0 mM TOCP for 48 h. As shown in Fig. 3, TOCP did not affect the mRNA levels of *p21*, *p27*, *p53*, and cyclin D1 in the cells, compared with the control group.

Effect of TOCP on apoptosis in rat spermatogonial stem cells

As shown in Fig. 2, we did not find the apoptotic cell death marker sub-G1 peak from cell cycle distribution in all of the tested cells. Therefore, it has been suggested that the anti-proliferative effect of TOCP might not result from the induction of apoptotic cell death.

In order to further confirm whether TOCP can induce apoptosis of rat spermatogonial stem cells, we counted the number of Annexin V-positive/PI-negative and Annexin V-positive/PI-positive staining cells after the cells were treated with different concentrations of TOCP for 48 h. As shown in Fig. 4, TOCP did not induce apoptosis of rat spermatogonial stem cells.

TOCP induces autophagy in rat spermatogonial stem cells

To evaluate whether TOCP induces autophagy in rat spermatogonial stem cells, the cells were treated with an indicated concentration of TOCP for 48 h, and the autophagy protein LC3, a widely used marker of mammalian autophagy, was analyzed by western blot (Kabeya *et al.* 2000). As shown in Fig. 5, after treatment with TOCP, both LC3-II and the ratio of LC3-II/LC3-I were markedly increased; autophagy proteins ATG5 and beclin 1 were also increased after treatment with TOCP, indicating that TOCP could induce autophagy in the cells.

To further evaluate whether TOCP induces autophagy in rat spermatogonial stem cells, autophagic vesicles in the cells treated with TOCP were visualized by TEM and it was found that autophagic vesicles in the cytoplasm containing extensively degraded organelles such as mitochondria and endoplasmic reticulum increased significantly after the cells were treated with TOCP (Fig. 6C and D) or starvation (Fig. 6E and F). However, control cells had relatively few autophagosomes in the cytoplasm (Fig. 6A and B). These results indicated that TOCP induced autophagy of rat spermatogonial stem cells.

Discussion

In this study, we provided evidence that TOCP inhibited viability of rat spermatogonial stem cells and induced autophagy, while having no effect on cell cycle progression and apoptosis.

TOCP, one of the three TCP isomers, has been widely used in the industry (Craig & Barth 1999, Winder &

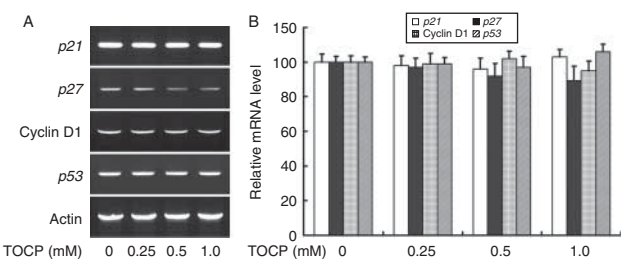

Figure 3 Effect of TOCP on the mRNA levels of cell cycle-related proteins in rat spermatogonial stem cells. Rat spermatogonial stem cells were treated with 0–1.0 mM TOCP for 48 h. Then, the mRNA levels of *p21*, *p27*, *p53*, and cyclin D1 were observed by RT-PCR; actin was used as an internal control (A). The relative mRNA levels were quantified by densitometry and expressed as the percentage of the control cells (B). The experiment was performed in triplicate and repeated three times. Data were analyzed by one-way ANOVA.

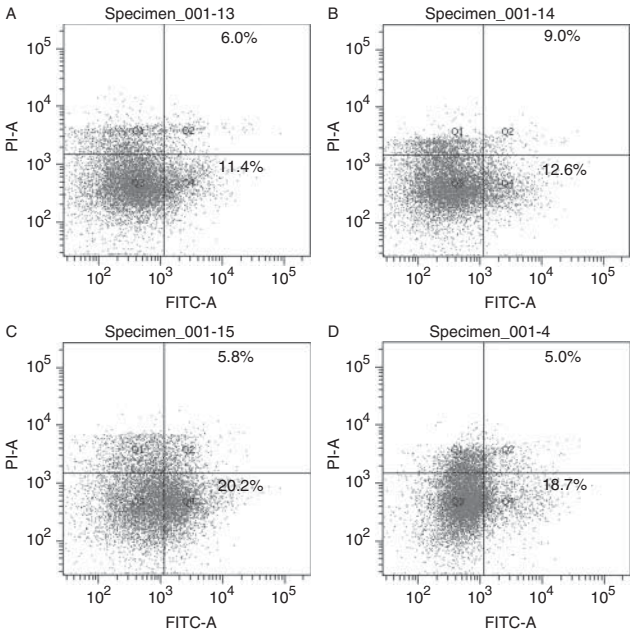

Figure 4 Effect of TOCP on apoptosis in rat spermatogonial stem cells. Rat spermatogonial stem cells were treated with 0 mM (A), 0.25 mM (B), 0.5 mM (C), or 1.0 mM (D) TOCP for 48 h. Then, cell apoptosis was observed by FCM assay. The experiment was performed in triplicate and repeated three times. Data were analyzed by one-way ANOVA.

Balouet 2002, Chen *et al*. 2012). TOCP can induce neurotoxicity (Craig & Barth 1999, Zhang *et al*. 2007) and immunotoxicity (Foil *et al*. 1980, Brinkerhoff *et al*. 1981) in animals.

In addition, TOCP had adverse effects on male reproductive system. TOCP can inhibit sperm motility and decrease sperm number in both roosters (Somkuti *et al*. 1987*b*) and rats (Somkuti *et al*. 1991, Latendresse *et al*. 1994). TOCP can also decrease the fertility index in Swiss (CD-1) mice and lead to decrease in the number of liveborn pups per litter (Chapin *et al*. 1988).

Spermatogonial stem cells have the ability of self-renewal and maintain their normal number in the seminiferous epithelium; and they can also finally differentiate into sperm. Therefore, spermatogonial stem cells play an important role in spermatogenesis (Mclean *et al*. 2002, Chen *et al*. 2011). TOCP was shown to decrease the sperm density in the epididymis and inhibit viability of mice spermatogonial stem cells (Chen *et al*. 2011). In the current study, we found that TOCP inhibited cell proliferation of rat spermatogonial stem cells in a dose-dependent manner. However, as far as the SH-SY5Y cells are concerned, the viability of the cells can be inhibited by TOCP with a higher concentration or a longer time (Chang *et al*. 2006, Long & Wu 2008, Chen *et al*. 2012).

The inhibition effect of TOCP might result from the cell cycle arrest (Long & Wu 2008). The cell cycle is a series of highly regulated events that takes place in a cell leading to its division into two genetically identical daughter cells. The cell cycle consists of three periods: gap phases (G1 and G2 phases), during which the cell accumulates nutrients to grow or prepare for cell division, DNA replication phase (S phase), during which the cell accurately duplicates large amounts of DNA in chromosomes, and the mitotic phase (M phase), during which the cell segregates duplicated chromosomes precisely into two daughter cells (Pardee 1989, Goto *et al*. 2013). To evaluate this hypothesis, rat spermatogonial stem cells were observed by flow cytometry. In this study, TOCP with an indicated concentration had no effect on the population of cells in the G1 phase, S phase, or G2/M phase.

Cell cycle is controlled by cyclins and cyclin-dependent kinases (CDKs; Chang *et al*. 2004). Cyclin D1 is well known as one of the major proteins that can increase cell proliferation by enhancing cell cycle progression from the G1 phase to the S phase (Shimura *et al*. 2013). In addition, the activity of CDK/cyclin complexes is negatively regulated by binding to CKIs, such as p21 and p27 (Lim & Kaldis 2013). The tumor suppresser gene *p53* acts as a transcription factor by modulating the transcriptional activity of target genes, which increases the expression of p21 and p27 (Lanza & Bi 1995).

To further investigate the effect of TOCP on cell cycle, the mRNA levels of cell cycle-related proteins, such as cyclin D1, p21, p27, and p53, were observed by RT-PCR. We found that TOCP did not affect the mRNA levels of cyclin D1, *p21*, *p27*, or *p53* in rat spermatogonial stem cells. These results indicated that TOCP inhibited the viability of rat spermatogonial stem cells not by affecting cell cycle, which is different from SH-SY5Y cells.

As shown in Fig. 2, the sub-G1 peak, a marker of cell death, had not been affected in TOCP-treated rat spermatogonial stem cells, indicating that the inhibition of cell viability by TOCP did not result from the induction of apoptosis. To further investigate whether TOCP can induce apoptosis of rat spermatogonial stem

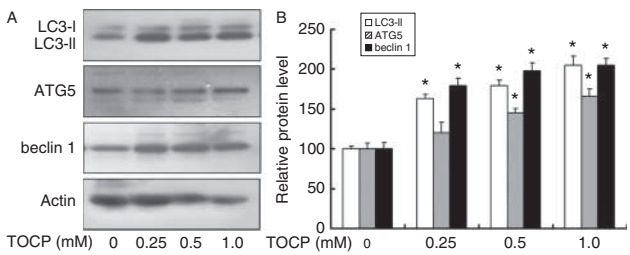

Figure 5 TOCP induces autophagy of rat spermatogonial stem cells. Rat spermatogonial stem cells were treated with 0–1.0 mM TOCP for 48 h. Then, the protein levels of LC3, ATG5, and beclin 1 were observed by western blot; actin was used as an internal control (A). The relative protein levels were quantified by densitometry and expressed as the percentage of the control cells (B). The experiment was performed in triplicate and repeated three times. Data were analyzed by one-way ANOVA. $*P<0.05$.

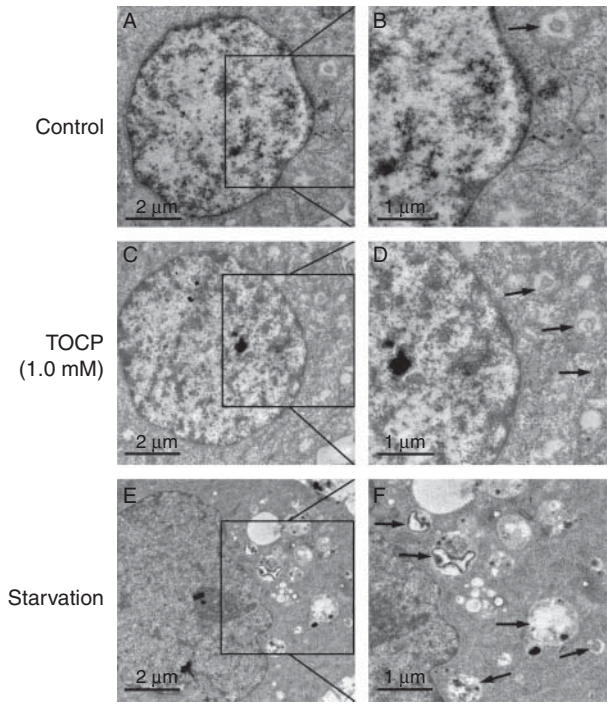

Figure 6 Ultrastructural evidence of autophagy in rat spermatogonial stem cells treated with TOCP. Rat spermatogonial stem cells were treated with DMSO (A and B) or 1.0 mM TOCP for 48 h (C and D). Then, autophagic vesicles in the cells were visualized by transmission electron microscopy (TEM), and starvation-treated cells were used as a positive control (E and F). The autophagic vacuoles are indicated by arrows.

cells, Annexin V–FITC and PI dual staining was used to detect apoptosis after the cells were treated with TOCP. As shown in Fig. 4, TOCP did not induce apoptosis of rat spermatogonial stem cells.

Macroautophagy, hereafter referred to as autophagy, is an evolutionarily conserved mechanism in all eukaryotes. Autophagy is a major catabolic pathway for delivery of proteins and organelles to lysosomes, where they are degraded and recycled in response to nutrient starvation (Kim *et al*. 2013, Wong *et al*. 2013). Generally, autophagy has been considered to be a cytoprotective response during amino acid deprivation or under stressful conditions such as neurodegenerative diseases, pathogen infections, and chemotherapy (Shintani & Klionsky 2004, Nikoletopoulou *et al*. 2013, Sharma *et al*. 2014). Autophagy can protect the cells against the accumulation of damaged organelles or protein aggregates. In certain cellular settings, however, autophagy can also lead to cell death (Scarlatti *et al*. 2009, Denton *et al*. 2012). Some chemotherapeutic agents or drugs in combination with radiation promote autophagic cell death (Sharma *et al*. 2014). Long *et al*. (2014) showed that TOCP significantly increased the ectopic accumulation of LC3-immunopositive puncta, beclin 1, and LC3-II/LC3-I levels in SH-SY5Y cells in a dose-dependent manner. Furthermore, the cytoplasm was occupied by autophagosomes in TOCP-treated cells and this was observed by TEM, which indicates that TOCP can induce autophagy in SH-SY5Y cells.

To evaluate whether TOCP induces autophagy in rat spermatogonial stem cells, the autophagy protein LC3, a widely used marker of mammalian autophagy, was analyzed by western blot. During the induction of autophagy, cytosolic form of LC3 (LC3-I) covalently conjugates with phosphatidylethanolamine and forms the faster SDS–PAGE migrating form (LC3-II), which is recruited and bound to the autophagosome membrane (Kabeya *et al*. 2000, Chen *et al*. 2013). The conversion of LC3-I into LC3-II was considered as a marker for the initiation of autophagy (Mizushima 2004). The amount of the LC3-II is related to the extent of autophagosome formation (Kabeya *et al*. 2000). We found that TOCP could significantly increase both LC3-II and the ratio of LC3-II/LC3-I; autophagy proteins ATG5 and beclin 1 were also increased after treatment with TOCP, indicating that TOCP could induce autophagy in the cells. Finally, autophagy was further observed by TEM, which is the 'golden' standard method (Mizushima *et al*. 2004). We found that autophagic vesicles in the cytoplasm containing extensively degraded organelles such as mitochondria and endoplasmic reticulum increased significantly after the cells were treated with TOCP. However, there might be different in other kinds of animals; Song *et al*. (2012, 2014) showed that the number of autophagosomes was markedly increased in the myelinated and unmyelinated axons of hen spinal cords after treatment of hens with TOCP, while the protein levels of LC3, beclin 1, and ATG5 were significantly decreased, which suggested that the administration of TOCP resulted in a disruption of autophagy-regulated machinery in hens.

As we know, TOCP can be metabolized into SCOTP by CYP450, which will be more toxic *in vivo*. Levallet *et al*. (1998) showed that Leydig cells, Sertoli cells, and germ cells all highly express functional CYP450 in mature rat testes. However, it is not sure whether or not SSCs express functional CYP450, hence the toxicity might result from TOCP itself or/and SCOTP.

In summary, we have shown that TOCP can inhibit the viability of rat spermatogonial stem cells and induce autophagy of the cells, without affecting cell cycle and apoptosis. The main toxic effect of TOCP on animals is the inhibition of cholinesterase with the subsequent cholinergic syndrome; however, cholinergic inhibitory effect of TOCP might not play an important role in toxic effects of rat spermatogonial stem cells, which suggests that there might have been other molecular mechanisms responsible for male reproductive toxicity. This study sets in motion our future investigation of the molecular mechanisms underlying TOCP toxicity of male reproductive system.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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