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Saligenin cyclic-o-tolyl phosphate (SCOTP) 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, which can be metabolized to High-toxic saligenin cyclic-*o*-tolyl phosphate (SCOTP). Our previous results found that TOCP could disrupt the seminiferous epithelium in the testis and induce autophagy of rat spermatogonial stem cells. Little is known about the toxic effect of SCOTP on rat spermatogonial stem cells. The present study showed that SCOTP decreased viability of rat spermatogonial stem cells in a dose-dependent manner. Both LC3-II and the ratio of LC3-II/LC3-I were significantly increased; autophagy proteins atg5 and Beclin 1 were also markedly increased after treatment with SCOTP, indicating SCOTP could induce autophagy of the cells. Ultrastructural observation under the transmission electron microscopy (TEM) indicated that there were autophagic vacuoles in the cytoplasm in the SCOTP-treated cells. However, cell cycle arrest was not observed by flow cytometry; and the mRNA levels of p21, p27, p53 and cyclin D1 in the cells were also not affected by SCOTP. Meanwhile, SCOTP didn't induce apoptosis of the cells. In summary, we showed that SCOTP could induce autophagy of rat spermatogonial stem cells, without affecting cell cycle and apoptosis.

1. Introduction

Tri-*ortho*-cresyl phosphate (TOCP) is one of the three isomers of tricresyl phosphate (i.e. *o*-, *m*-, or *p*-cresyl), which has been widely used as plastic softeners, plasticizers, jet oil additives, and flame-retardants in industry [1,2]. TOCP was first reported to induce organophosphate-induced delayed neuropathy (OPIDN), which is characterized by paralysis of the lower limbs due to degeneration of long axons in the spinal cord and peripheral nerves in sensitive species such as humans and hens [1,3–6]. TOCP was shown to induce autophagy in undifferentiated SH-SY5Y cells [7]. We also found that TOCP can induce autophagy and degrade cytoskeletal components in differentiating SH-SY5Y cells, and subsequently led to neurite outgrowth inhibition in the cells [8].

Recent years, TOCP has been shown to inhibit testicular enzyme activities, disrupt the seminiferous epithelium, and inhibit spermatogenesis in rats and mice [9–11]. Spermatogenesis is a complex process, in which undifferentiated spermatogonial stem cells proliferate and differentiate sequentially in the testis. The undifferentiated spermatogonial cells mainly consist of type A_{single} (A_s), A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) germ cells [11–14]. Type A_s spermatogonia have been generally considered the spermatogonial stem cells. Therefore, sequential proliferation and differentiation of the cells play a crucial role in generating functional sperm and maintaining normal number of sperm in male animals [11,14,15]. 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 [16].

TOCP can be metabolized to saligenin cyclic-o-tolyl phosphate (SCOTP) by cytochrome P450 (CYP450), which will be more toxic *in vivo* [17,18]. We found that SCOTP significantly decreased viability of mice spermatogonial stem cells [11]. However, little is known whether inhibition of viability of spermatogonial stem cells might result from cell cycle arrest, induction of apoptosis or autophagy.

The aim of the present study is to investigate the toxic effect of SCOTP on rat spermatogonial stem cells, which will set in motion our future investigation of the underlying mechanisms of SCOTP inhibiting spermatogenesis.

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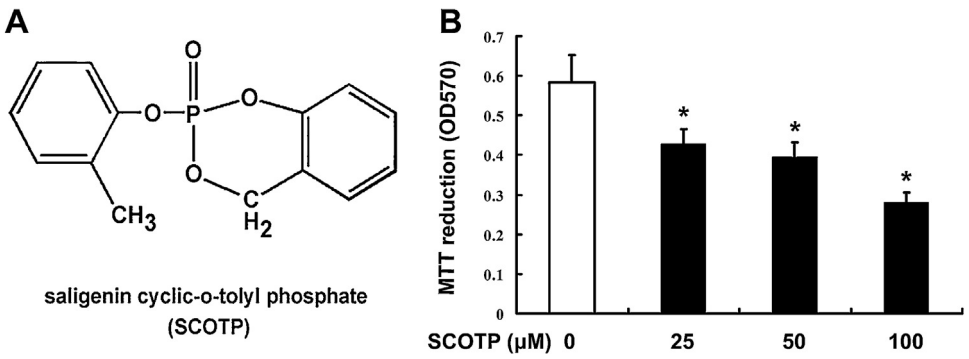

Fig 1. SCOTP inhibits viability of rat spermatogonial stem cells. (A) Chemical structure of saligenin cyclic-o-tolyl phosphate (SCOTP). (B) Rat spermatogonial stem cells were treated with 0–100 μM SCOTP for 48 h. Then, cell viability was observed by MTT assay. The experiment was done in triplicate and repeated three times. Data were analyzed by one-way ANOVA. $^*P < 0.05$.

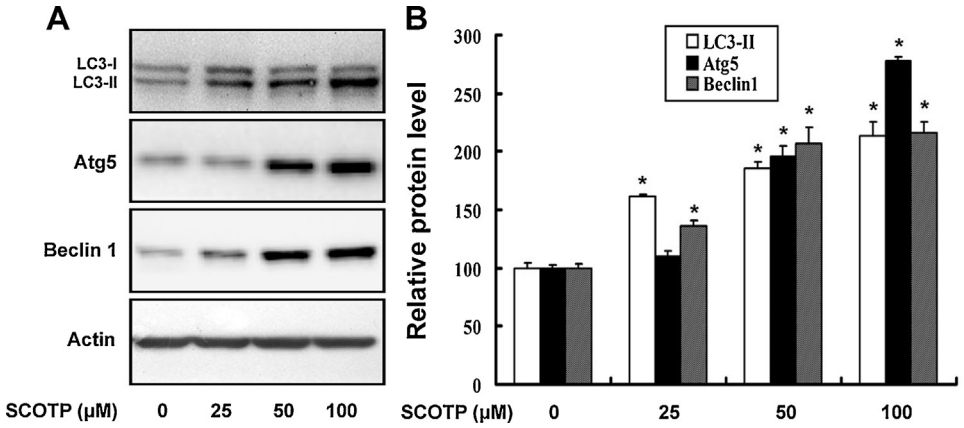

Fig. 2. SCOTP induces autophagy of rat spermatogonial stem cells. Rat spermatogonial stem cells were treated with 0–100 μM SCOTP for 48 h. Then, the protein levels of LC3, Atg5 and Beclin1 were observed by Western blot; the Actin was used as an internal control (A). The relative protein levels were quantified by densitometry and expressed as percentage of the control cells (B). The experiment was done in triplicate and repeated three times. Data were analyzed by one-way ANOVA. $^*P < 0.05$.

2. Materials and methods

2.1. Materials

SCOTP was synthesized in our laboratory according to Eto et al. and Nomeir and Abou-Donia [17,19]. Cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA). Recombinant rat GDNF (450–51), recombinant rat EGF (400–25), and recombinant rat FGF-basic (400–29) were obtained from Pepro-Tech (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 monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) reagent was obtained from Pierce Biotechnology (Rockford, IL, USA). TRIzol reagent was purchased from Invitrogen Life Technologies (Groningen, The Netherlands) and *EasyScript*™ First-strand cDNA Synthesis SuperMix for RT-PCR reaction was purchased from TransGen Biotechnology (Beijing, China). Annexin V-FITC Apoptosis Detection Kit was obtained from Invitrogen Life Technologies (Oregon, USA).

2.2. Cell culture

Rat spermatogonial stem cells were isolated from male rats of 9 days according to Liu et al. [16] and Hamra et al. [20]. Briefly, the rat spermatogonial stem cells were isolated and enriched, and then cultured on STO (SIM mouse embryoderived thioguanine and ouabain resistant) cell feeders in serum-free medium supplemented with growth factors, such as recombinant rat EGF, recombinant rat 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.

2.3. MTT reduction assay

The cells (1×10^4 cells/well) were seeded in a 96-well culture plate and were incubated with fresh medium containing 0–100 μM SCOTP for 48 h. SCOTP was dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO in the culture medium was 0.1% (v/v). Forty-eight hours later, cell medium containing 0.5 mg/mL MTT was replaced to each well and incubated at 34 °C in 5% CO2/95% air for 4 h. The formed insoluble formazan was dissolved in DMSO, and the absorbance was measured in a spectrophotometer at 570 nm with a background reading of 660 nm.

2.4. Western blotting analysis

The cells were plated at a density of 5×10^5 in 60-mm culture dishes and were treated with 0–100 μM SCOTP for 48 h. The cells were then 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, 0.5% NP40) containing the protease inhibitor cocktail (Huatesheng Biotech, Fushun, Liaoning, China). Cell lysates were briefly ultra-sonicated, and clarified by centrifugation at $12{,}000 \times g$ for 10 min at 4 °C. The supernatants