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Triptolide disrupts the actin-based Sertoli-germ cells adherens junctions by inhibiting Rho GTPases expression

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ABSTRACT

Triptolide (TP), derived from the medicinal plant *Triterygium wilfordii* Hook. f. (TWHF), is a diterpene triepoxide with variety biological and pharmacological activities. However, TP has been restricted in clinical application due to its narrow therapeutic window especially in reproductive system. During spermatogenesis, Sertoli cell cytoskeleton plays an essential role in facilitating germ cell movement and cell-cell actin-based adherens junctions (AJ). At Sertoli cell-spermatid interface, the anchoring device is a kind of AJ, known as ectoplasmic specializations (ES). In this study, we demonstrate that β-actin, an important component of cytoskeleton, has been significantly down-regulated after TP treatment. TP can inhibit the expression of Rho GTPase such as, RhoA, RhoB, Cdc42 and Rac1. Downstream of Rho GTPase, Rho-associated protein kinase (ROCKs) gene expressions were also suppressed by TP. F-actin immunofluorescence proved that TP disrupts Sertoli cells cytoskeleton network. As a result of β-actin down-regulation, TP treatment increased expression of testin, which indicating ES has been disassembled. In summary, this report illustrates that TP induces cytoskeleton dysfunction and disrupts cell-cell adherens junctions *via* inhibition of Rho GTPases.

1. Introduction

Triptolide (TP), extracted from the Chinese traditional medicine named *Triterygium wilfordii* Hook.f. (TWHF) (Ying et al., 2010), is a diterpene triepoxide with variety biological and pharmacological activities, such as *anti*-inflammatory (Liu, 2011), anti-cancerogenic (Johnson et al., 2011; Liu et al., 2013), immunomodulatory(Ho et al., 2013) and pro-apoptotic activities (Liu et al., 2013), and antifertility effects (Singla and Challana, 2014). Because of the narrow therapeutic window, antifertility effects of TP usually manifest in reproductive toxicity, especially in male (Li et al., 2014).

In adult mammalian testis, Sertoli cell are the most important somatic epithelial cells that nurse and structurally support developing germ cells in the seminiferous epithelium and finally germ cells develop into spermatozoa by the process known as spermatogenesis (Lie et al., 2010). The Sertoli cells cytoskeleton contain microfilaments, microtubules and intermediate filaments (Vogl et al., 2008), which is linked physically and functionally to numerous biological processes, such as junction dynamics between Sertoli-Sertoli (tight junctions, TJ) and Sertoli–Germ (cell-cell actin-based adherens junctions, AJ) cell interface, the transport of spermatids (Tang et al., 2016) and phagocytosis function (Xiong et al., 2009). It is reported that studying Sertoli cells physiology is a good model in male reproductive toxicology (Reis et al., 2015). And Sertoli cell cytoskeleton is also reported to be one of the primary targets of toxicants in the testis(Li et al., 2016).

The cytoskeleton network and junction dynamics can be regulated by Rho GTPases. The mammalian Rho GTPase families have >10 proteins, such as Rho, Rac and Cdc42. Many researches have proved that Rho GTPases can regulate the organization of the actin cytoskeleton(Murali and Rajalingam, 2014) and junctions between Sertoli-Germ cells are relevant to abnormal spermatogenesis(Lui et al., 2003a).

During spermatogenesis, germ cells move from the basal to the adluminal compartment of seminiferous epithelium. This cellular event is accompanied with intermittent disassembly and reassembly of cellular junctions, such as TJ, AJ, and intermediate filament-based desmosome-like junctions(Siu et al., 2010). Ectoplasmic specializations (ES), as a testis-specific AJ type, should also be influenced by Rho GTPases.

Previous studies used to suggest that TP might be a potential candidate for post-testicular male contraceptive agent without affecting

Abbreviations: TP, Triptolide; AJ, adherens junctions; ES, ectoplasmic specializations; Cdc42, Cell division control protein 42; Rac1, Ras related C3 botulinum toxin substrate 1.

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Table 1
Primers used for RT-PCR in this article.

Gene	Sequence	
GAPDH	CAGGGCTGCCTTCTCTTGTG	GATGGTGATGGGTTTCCCGT
β-Actin	ACAACCTTCTTGCAGCTCCTC	CTGACCCATACCCACCATCAC
RhoA	CCAAATGTGCCCATCATCCTAGTTG	TCCGTCTTTGGTCTTTGCTGAACAC
RhoB	ATATTAGCGTGGGCACCGAG	GTAGGGGTGTAGGGAGTCGT
Cdc42	CGACCGCTAAGTTATCCACAG	GCAGCTAGGATAGCCTCATCA
Rac1	GAGTACATCCCCACCGTCTTTG	TGTCCCAGAGGCCCAGATT
Rock1	AGGCCTGTGCCAAACCTTT	TGGTCCCTGTGGGACTTAACA
Rock2	CCCGATCATCCCCTAGAACC	TTGGAGCAAGCTGTCGACTG
Testin	TTTCTTTATGTCCCAAAACGTGTG	TACAATGGGGTATGTGGAATATGT

endocrine(Lue et al., 1998; Hikim et al., 2000). However, more researches shown that TP might affect testis.

In a recent male reproductive toxicity study, the authors had showed that 100 μg/kg TP induced weight reduction in testis and epididymis. Simultaneously, apparent changes of seminiferous tubules had been observed. The authors also found that mature sperms in the epididymis were decreased (Ni et al., 2008). Germ cells degeneration and exfoliation had been found in 100 μg/kg TP treated rat testis, it may indicate that Sertoli-germ cells AJ might be disassembled.

Earlier studies have shown that Sertoli cells cultured *in vitro* in monolayer at low cell density (about $0.5 * 10^5$ cells/cm^2), where specialized tight junctions did not form, actively synthesize and secrete a novel protein designated testin (Grima et al., 1995). Testin is secreted by Sertoli cells, in which mRNA level of testis can reflect the integrity of inter-testicular cell junctions (Grima et al., 1997). After that, the authors also proved that testin expression correlates with the disruption of Sertoli-germ cell junction but not the inter-Sertoli tight junction (Grima et al., 1998). Therefore, testin was reported an important marker in monitoring Sertoli-germ cells interactions throughout spermatogenesis (Cai et al., 2012).

The objectives of our present study were to assess whether TP possesses Rho GTPases inhibition properties both *in vivo* and *in vitro*, and to determine the effect of TP on actin-based ES. These finding supports our hypotheses that TP induced ES disassembly *via* inhibition of Rho GTPases, eventually result in spermatogenesis dysfunction.

2. Materials and methods

2.1. Materials and antibodies

Triptolide (>98% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Antibodies to RhoA, RhoB, Cdc42, Rac1/2/3 and p-Rac1/Cdc42 were purchased from Cell Signaling Technology (Rho-GTPase Antibody Sampler Kit #9968, CST, MA, USA).

2.2. Animals and treatments

Sprague Dawley (SD) rats were purchased from QingLongShan Laboratory Animal Company (Nanjing, China). Animals were maintained in a temperature- and humidity-controlled facility at Jiangsu Provincial Center for Disease control and Prevention (Nanjing, China).

Triptolide was dissolved in 10% pentadiol, then diluted in 0.5% sodium carboxymethylcellulose (Sigma, MO, USA). 90 days SD rats were continuously given an oral administration of different dosage of TP (0, 50, 100 μg/kg body weight) for 4 weeks (Singla and Challana, 2014), 0 μg/kg group as control group.

After 28 days, rats were euthanasia by an injection of sodium pentobarbital (100 mg/kg). Testes and epididymis were collected and one epididymis was prepared for sperm counting.

All the measures taken for the rats were in accordance with the Guidelines on the Care and Use of Laboratory Animals (Chinese Council on Animal Research and the Guidelines of Animal Care). The study was approved by the Ethical Committee of China Pharmaceutical University.

2.3. Isolation of Sertoli cells, germ cells and co-culture of Sertoli-germ cells

Sertoli cells were isolated from testis of 18–20 day-old male SD rats (Mruk and Cheng, 2011a) as described previously (Li and Han, 2012; Gao et al., 2016) with modifications.

Briefly, rats testes were removed, decapsulated and rinsed twice in phosphate buffered saline (PBS). The seminiferous tubules were dispersed gently using ophthalmic forceps and then transferred into 50 ml plastic tubes. The loosened seminiferous tubules were digested in 0.25% trypsin at 37 °C in a rocking incubator for 30 min to remove Leydig cells and other interstitial tissue. The isolated testicular fragments were centrifuged at 800 rpm and washed twice in PBS before further digestion in 0.1% collagenase I for 30 min at 37 °C to remove the peritubular cells. The homogenate was filtered through a 100-mesh stainless steel filter, and cells were collected for centrifugation at 800 rpm for 5 min. Cells were washed three times in PBS, then resuspended in complete DMEM-F12 medium (Invitrogen, MA, USA). Finally, dispersed cells were seeded on cell culture dishes at a density of $0.5 * 10^5$ cells/cm^2 (Grima et al., 1998), and were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. After cultured for 48 h, Sertoli cells became attached to the bottom of dishes with tiny dendrites protruding, but most of the germ cells were suspended in the medium and can be removed by changing the medium(Hu et al., 2014). Then Sertoli cells cultures were treated with 20 mM Tris, pH 7.4 at 22 °C to

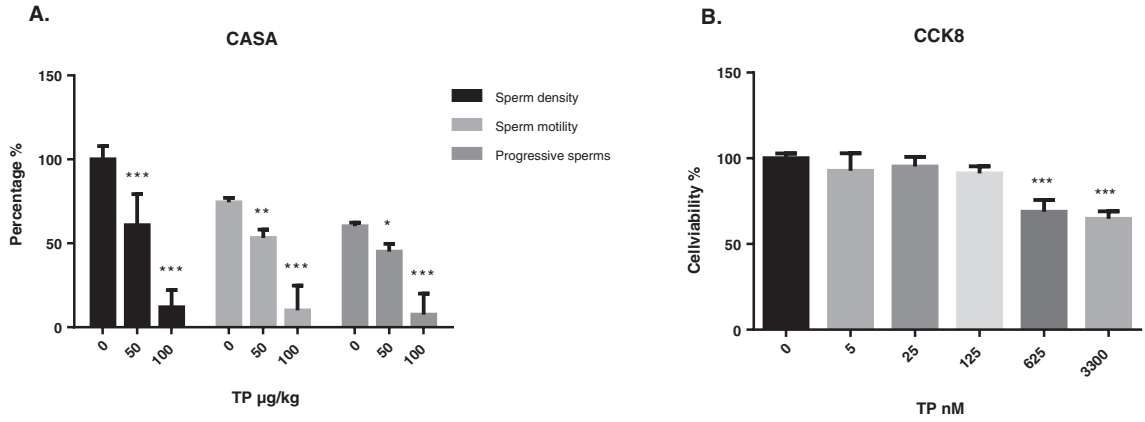

Fig. 1. Antifertility effects and cytotoxicity of TP. A. Percentage reductions in values of different sperm parameters in TP treated groups from that of control group. Animals were treated with different concentrations of TP (0, 50, 100 μg/kg) for 28 days. B. Exposure to increasing doses of TP (625 nM, 3300 nM) led to significant inhibition of cell proliferation. *P < 0.05, **P < 0.01, ***P < 0.001.