



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.

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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	CAGGGCTGCCTTCTCTTG	GATGGTGATGGGTTCCCGT
β -Actin	ACAACCTTCTTGAGCTCCTC	CTGACCCATACCCACCATCAC
RhoA	CAAATGTGCCATCATCTAGTTG	TCCGTCITTTGGCTTTGCTGAACAC
RhoB	ATATTAGCGTGGGCACCGAG	GTAGGGGTGTAGGGAGTCTGT
Cdc42	CGACCCGCTAAGTTATCCACAG	GCAGCTAGGATAGCCTCATCA
Rac1	GAGTACATCCCCACCGTCTTTG	TGTCCAGAGGCCAGATT
Rock1	AGGCCTGTGCCAACCTTT	TGGTCCCTGTGGGACTTAACA
Rock2	CCCGATCATCCCTAGAACC	TTGGAGCAAGCTGTGCACTG
Testin	TTTCTTTATGTCCCAAACGTGTG	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 μ g/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 μ g/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/cm²), 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 testin 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 μ g/kg body weight) for 4 weeks (Singla and Challana, 2014), 0 μ g/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/cm² (Grima et al., 1998), and were incubated in a humidified atmosphere of 95% air, 5% CO₂ 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

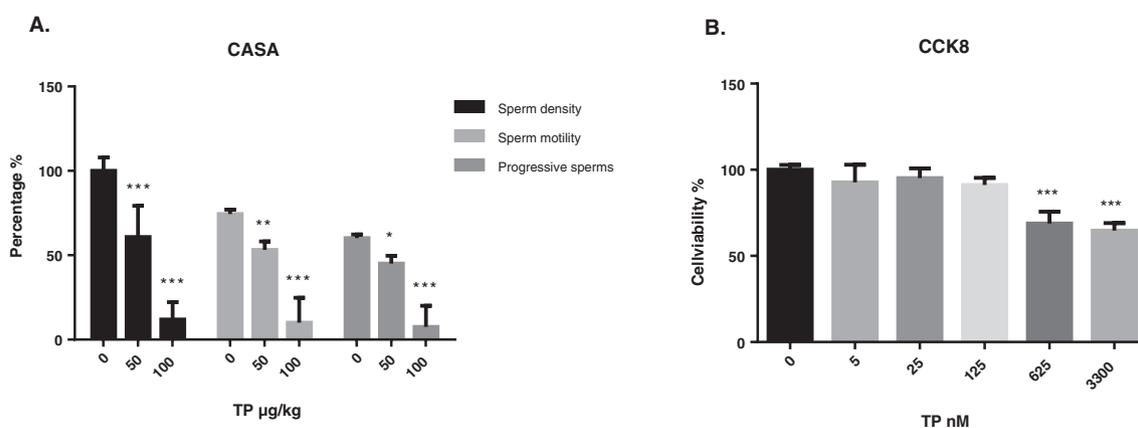


Fig. 1. Antifertility effects and cytotoxicity of TP. A. Percentage reductions in values of different sperm parameters in TP treated groups from that of control group. Animals were treated with different concentrations of TP (0, 50, 100 μ g/kg) for 28 days. B. Exposure to increasing doses of TP (625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

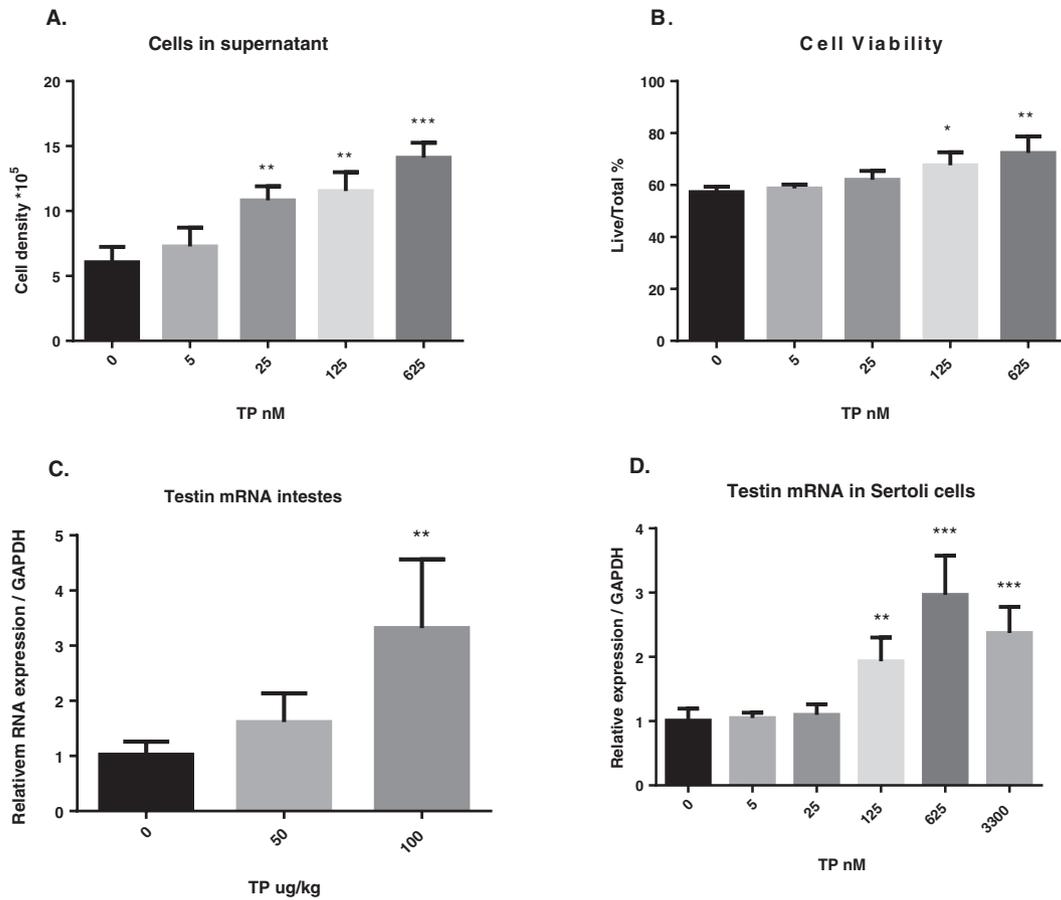


Fig. 2. TP disturbs Sertoli-germ cells adherence junctions. A. Cell counting of suspending cells in the supernatant. B. Cell viability of suspending cells in the supernatant. C. and D. Testin gene expression in testes and Sertoli cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

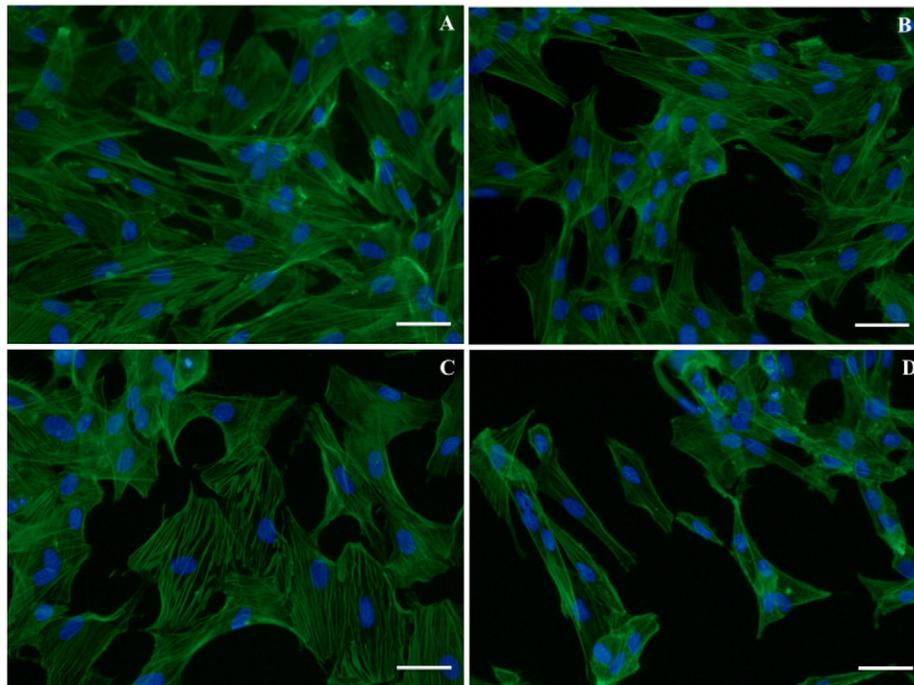


Fig. 3-1. Analysis of actin cytoskeletal rearrangement was performed after 24 h of TP treatment by immunofluorescence staining with FITC Phalloidin. Scale bar: 25 μm . A. Normal control of cytoskeleton; B. treated with 125 nM TP; C. treated with 625 nM TP; D. treated with 3300 nM TP. The immunofluorescence analysis was repeated at least three times using different batches of Sertoli cells. Results were similar each time and data from a representative experiment are presented. Negative control was used to establish the necessary experimental conditions including changes in phenotypes were excluded from this analysis. All the samples used to compare the treatment groups versus the corresponding controls were processed in a single experimental session to avoid inter-experimental variation.

lyse residual germ cells and to yield Sertoli cells with a purity of >93%. Sertoli cells purity was analysed by Oil red O (Sigma, MO, USA) and Feulgen (Senbeijia, Nanjing, China) staining. Under these conditions, specialized tight junctions were not formed when assessed by various criteria as described previously.

Germ cells were isolated from 90-day-old SD rat testes by a mechanical procedure without any enzymatic treatment (Aravindan et al., 1996). Rat testes were decapsulated and minced in PBS. After centrifugation (100 g, 1 min), the supernatant was collected and the loose pellet was washed twice in PBS and the supernatants were pooled. Pooled supernatant was filtered through 20-mesh filter and then through glass wool to remove elongate spermatids and spermatozoa. Finally, we got a mixture consisted of spermatogonia, spermatocytes, and a majority of round spermatids. Germ cells were cultured in DMEM-F12 medium as well as a standard condition.

In the Sertoli-germ cell co-culture system, Sertoli cells were cultured along for 5 days to form the intact epithelium (Siu et al., 2005). Then isolated germ cells were added onto the Sertoli cell epithelium on day 6, and co-cultured at a Sertoli/germ cell ratio of 1:1 to permit ES assembly.

2.4. Cell treatment

Sertoli cells were incubated with TP at an increasing concentration (0, 5, 25, 125, 625, 3000 nM). The stock solutions of TP (100 mM) were made in DMSO to increase their solubility. Solvents at the highest concentration (0.1%DMSO) in working solutions were not cytotoxic. After 24 h (Lui et al., 2003b) of incubation, cells were prepared for further studies.

2.5. Cell viability assay

Sertoli cells were seeded in a 96-well black microplate at a density of 0.5×10^5 cells/cm². Treat cells with TP as described above, the viability of Sertoli cells were assessed by the CCK8 Cell Viability Assay (Invitrogen, CA, USA) according to manufacturer's instructions.

2.6. Adherence assay of Sertoli-germ cells

Sertoli cells were planted in a 6-well plate and cultured alone for 5 days. On day 6, Sertoli cells were treated with TP for 24 h. Then cells

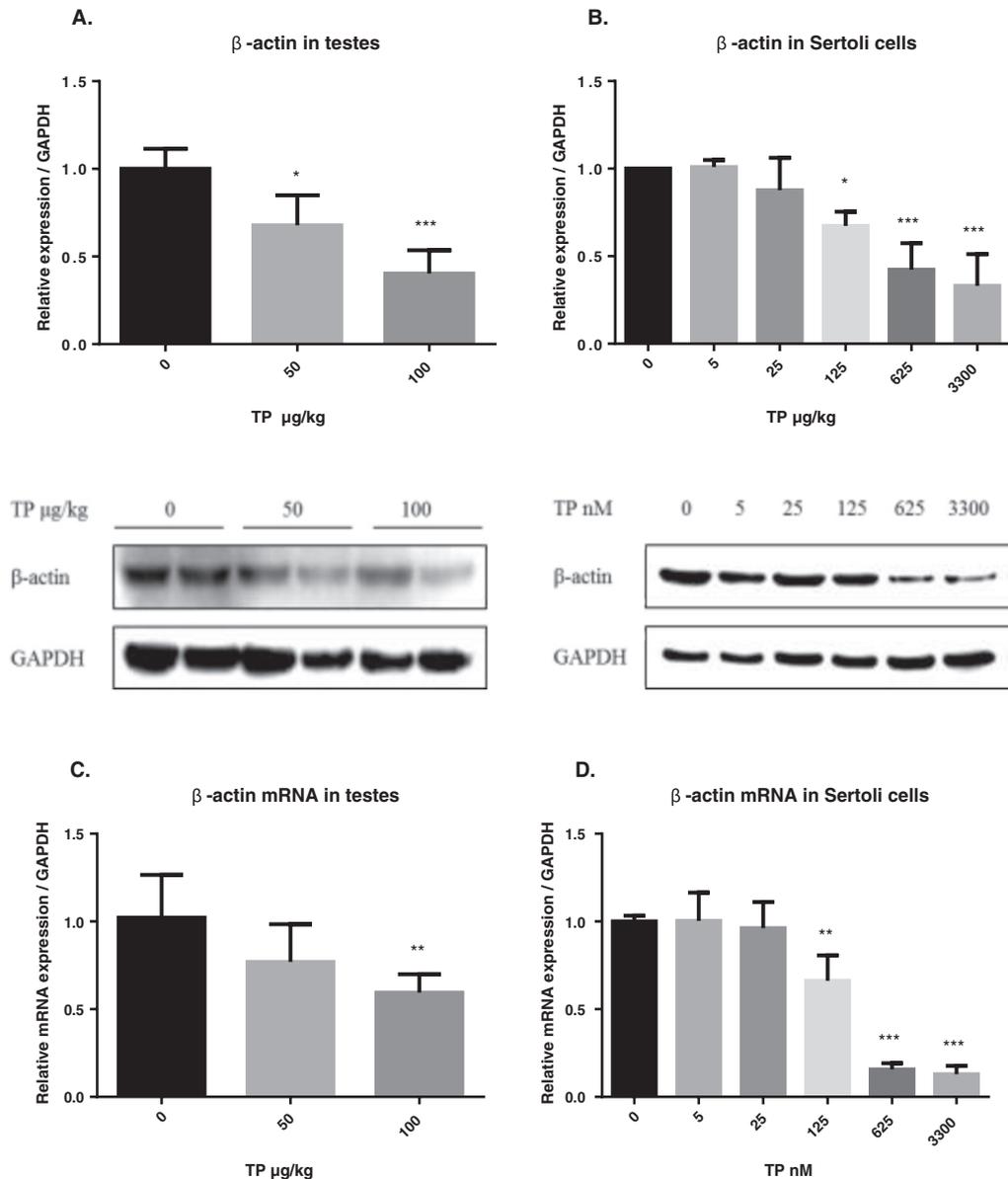


Fig. 3-2. β -actin expression has been suppressed by TP both *in vivo* and *in vitro*. A. protein expression in testes; B. protein expression in Sertoli cells; C. and D. mRNA level in testes and Sertoli cells. Statistical significance relative to vehicle control and western bolt data from a representative experiment are presented. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

were washed by PBS and germ cells were loaded onto the Sertoli cell epithelium. Sertoli-germ cells were co-cultured in standard condition for 48 h to permit ES assembly. At terminal time, the supernatant (Cao et al., 2008) was collected and cells in the supernatant were counted by Countess® Automated Cell Counter (Invitrogen, CA, USA) according to manufacturer's instructions.

2.7. Immunofluorescence analysis

For immunofluorescence analysis, Sertoli cells were planted in a 6-well plate with each well containing 2 ml DMEM-F12 medium with 10% FBS.

After treatment of TP, cells were fixed in 4% paraformaldehyde (w/v) in PBS at room temperature for 20 min, followed by incubation with 0.1% Triton X-100 for 20 min. Then F-actin filaments were stained by FITC-labeled Phalloidin (YEASEN, Shanghai, China) for 45 min. After three times wash with PBS, cells were incubated in hoechst 34580 (Invitrogen, CA, USA) for 5 min.

Images were captured using a Fluorescence microscope (OLYMPUS, Japan) with 400 × objectives, acquired using the Image-Pro Plus 6.0 software, and compiled in Image J (NIH, USA).

2.8. Real-time PCR

Total RNA from testes and Sertoli cells were extracted using Total RNA Extraction Reagent (Vazyme, Nanjing, China) according to the manufacturer's instructions.

The concentration and purity of RNA were measured by spectrophotometric analysis with GeneQuant Pro spectrophotometer (Amersham Biosciences, USA).

Total RNA (1 µg) was reverse-transcribed to cDNA by using the HiScript™ Q RT SuperMix (Vazyme, Nanjing, China) in a total volume of 20 µl. qRT-PCR was performed on an iCycler iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) with SYBR® Green Master Mix (Vazyme, Nanjing, China). The sequences of primers used for qRT-PCR analyses are shown in Table 1.

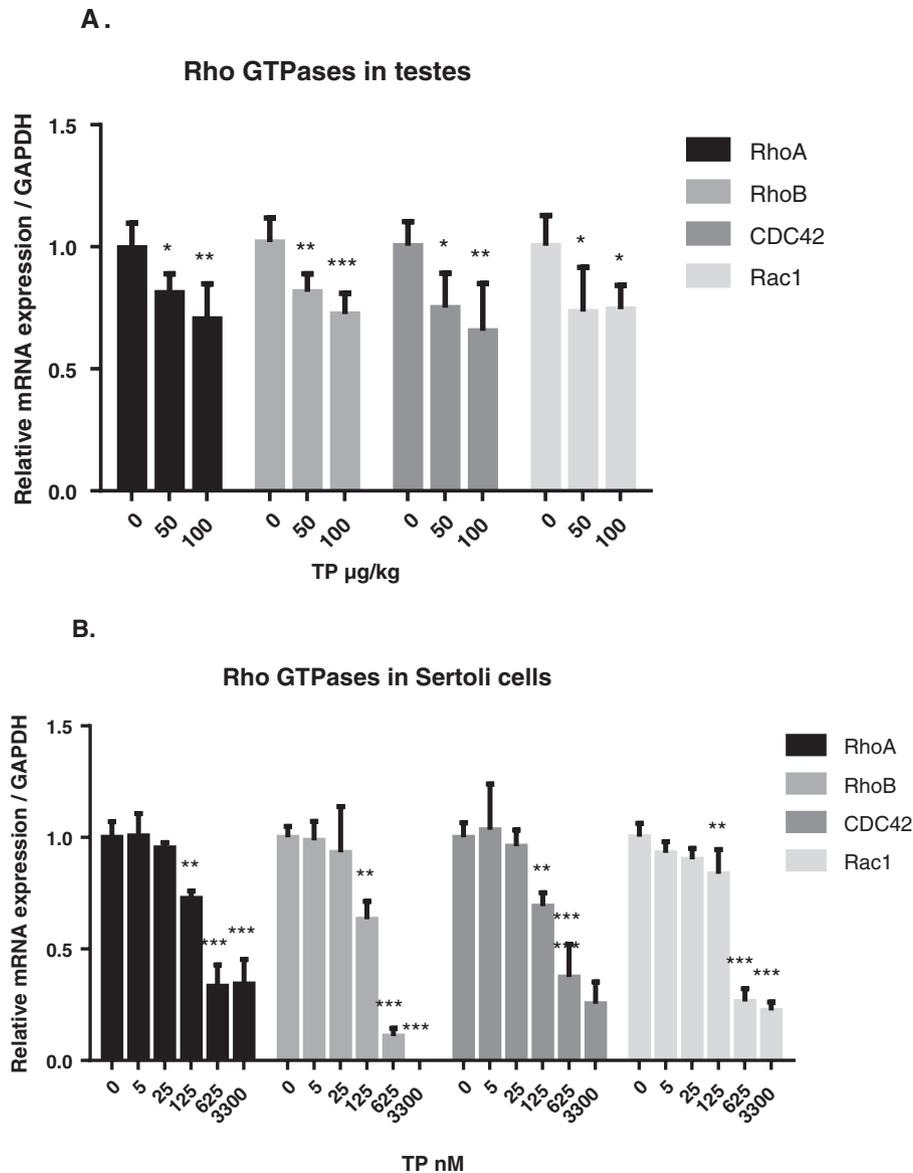


Fig. 4. Real-time PCR analysis of mRNA levels of RhoA, RhoB, Cdc42 and Rac1. A. Animals were treated with different concentrations of TP (0, 50, 100 µg/kg) for 28 days. B. Sertoli cells were treated with different concentrations of TP (0, 5, 25, 125, 625, 3300 nM) for 24 h. Statistical significance relative to vehicle control, *P < 0.05, **P < 0.01, ***P < 0.001.

Amplification conditions were as follows: 95 °C initial denaturation for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative expression levels of the target genes were calculated based on 2- $\Delta\Delta$ Ct according to the manufacturer's specifications by using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as the reference housekeeping gene (Chang et al., 2007; Zinzow-Kramer et al., 2014).

2.9. Western blotting

Testes and whole-cell lysates were prepared using cell lysis buffer supplemented with protease-phosphatase inhibitor cocktail (Vazyme, Nanjing, China). Protein concentrations were measured using the BCA (Bicinchoninic acid) assay and protein (50 μ g) was resolved by polyacrylamide gel electrophoresis.

Briefly, after electrotransfer to PVDF (polyvinylidene difluoride) membranes, nonspecific binding sites were blocked with 5% nonfat

milk in TBST for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C, washed with TBST at room temperature, and then incubated with appropriate HRP-conjugated secondary antibody for 1 h. Immune complexes were visualized using enhanced chemiluminescence. Consistent loading and transfer were confirmed by probing the same membrane with anti-GAPDH.

According to manufacturer's instructions, the dilution of β -Actin and GAPDH are 1:1000, and the dilution of RhoA, RhoB, Cdc42, Rac1/2/3 and p-Rac1/Cdc42 are 1:500.

2.10. Statistical analyses

Statistical analyses were performed with GraphPad Prism 6.0 software (GraphPad, USA). The results are expressed as the mean \pm SD of individual values from three independent experiments. Data were compared by one-way ANOVA followed by a Dunnett's Multiple Comparison

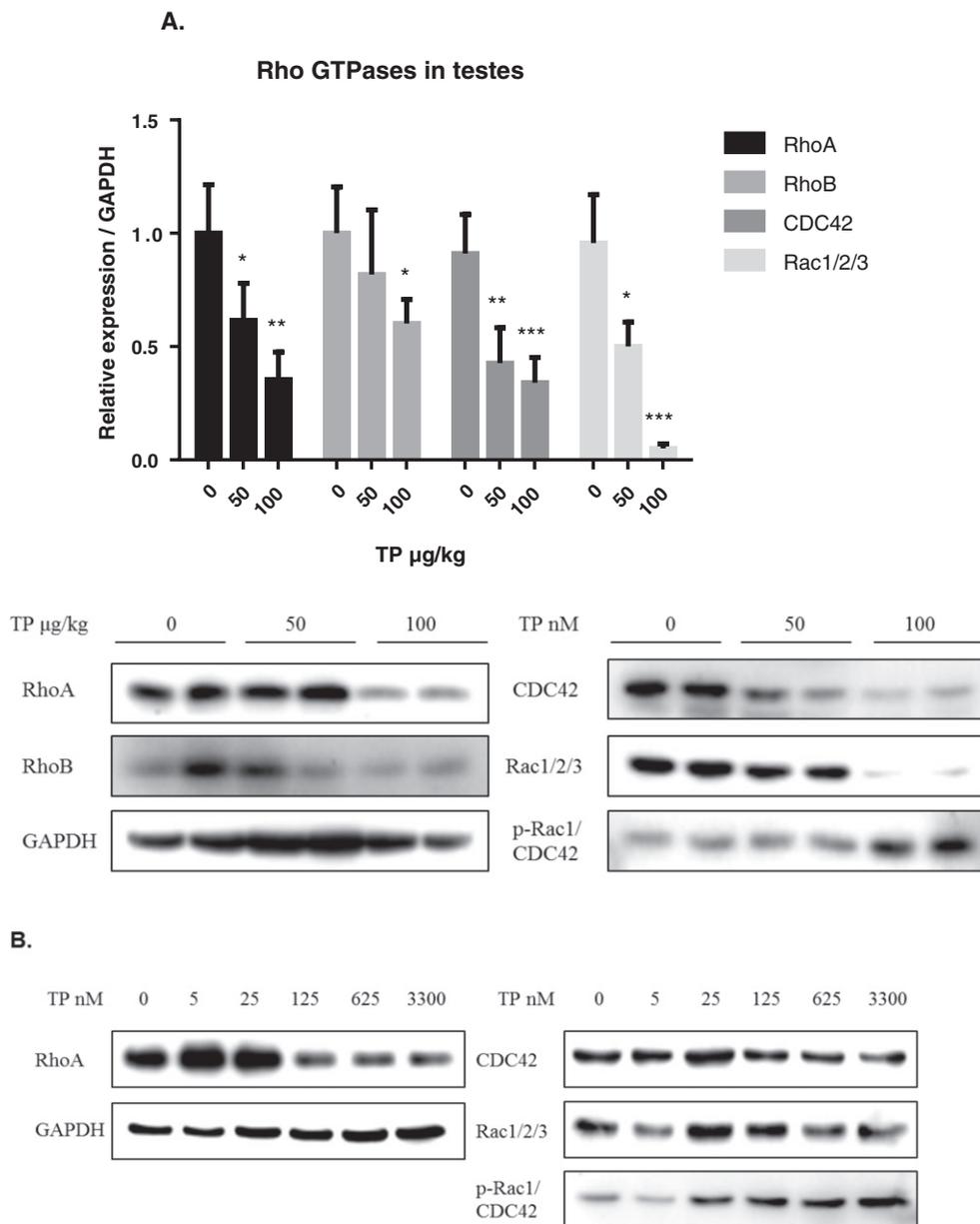


Fig. 5. Western bolt analysis of protein levels of Rho GTPases families. A. Animals were treated with different concentrations of TP (0, 50, 100 μ g/kg) for 28 days. B. Sertoli cells were treated with different concentrations of TP (0, 5, 25, 125, 625, 3300 nM) for 24 h. Statistical significance relative to vehicle control, data from a representative experiment are presented. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Test. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Antifertility effects of TP in animals and cytotoxicity on Sertoli cells

Cauda epididymal fluid was used in computer-aided semen analysis (CASA). A significant decrease (Fig. 1A) in sperm density, percent sperm motility and percent progressive sperms was found in TP treated groups compared to control group. TP treatment induced sperm loss and vitality decrease.

Cell viability of Sertoli cells exposed to TP was assessed by the CCK8 assay. Compared with 0 nM group, exposure to TP at a concentration of 625 nM and 3300 nM resulted in significant reduced (Fig. 1B) cell viability ($31.48 \pm 5.66\%$ and $36.54 \pm 4.32\%$). No significant differences were observed following the exposure to lower concentrations (5 nM, 25 nM, 125 nM).

3.2. TP disturbs Sertoli-germ cells adherence junctions

To illustrate the effect of TP on Sertoli-germ cells adherence junctions, we use Sertoli-germ cells co-culture model for cell adherence assay. Our data showed that TP (>25 nM) induced germ cell loss (Fig. 2A) in the co-culture system. Moreover, both live and dead cells increased in the supernatant, the ratio of live cells (in total cells) was increased with high dose of TP (Fig. 2B). This evidence indicates that not cytotoxicity of TP but the cell-cell junction disruption is the major cause of sperm cell loss.

Testin mRNA level, which is tightly coupled to the integrity of specialized junctions between testicular cells, has been detected by qRT-PCR. As a result, testin mRNA level in both testes and Sertoli cells had been increased by TP treatment (Fig. 2C and D). Which associated with cell adherence assay confirmed that TP disturbs Sertoli-germ cells adherence junctions.

3.3. TP induced actin suppression

F-actin expression has been detected in order to evaluate the effects of TP. As described before, Sertoli cells were treated for 24 h. Analysis of actin cytoskeletal rearrangement was performed by immunofluorescence staining with FITC Phalloidin (0.1 μM). The nuclei were stained with hoechst 34,580 (Fig. 3-1).

With the increasing concentration of TP, F-actin organization has been disrupted. Under 125 nM TP, cell number remained while some F-actin fiber bundles began to rupture. Exposure to 625 nM and 3300 nM TP, cell number decreased obviously and actin cytoskeleton distinctly disassembled.

Since TP induced depolymerization of F-actin filaments, we detected β -actin expression in both mRNA and protein levels. Unexpectedly, we found that TP can significantly decrease β -actin expression (Fig. 3-2) both *in vivo* and *in vitro*. In animals, the suppression of β -actin agreed with the result of CASA. Impairment was observed in 50 $\mu\text{g}/\text{kg}$ group and was severe in 100 $\mu\text{g}/\text{kg}$ group. In Sertoli cells, 125 nM TP had no cytotoxicity on cells but significantly inhibited β -actin expression indicating that the disruption of actin organization was prior to cytotoxic effect.

3.4. TP decreases Rho GTPases expression both *in vivo* and *in vitro*

Rho GTPases families are crucial regulators in actin dynamics. Studies by qRT-PCR revealed that both testes and Sertoli cells express Rho GTPases family members (RhoA, RhoB, Cdc42 and Rac1) and TP treatment significantly decreased these genes (Fig. 4).

To verify the above observation that TP inhibited Rho GTPases expression, protein levels of Rho GTPases have been examined. The results of western blot proved that TP suppressed RhoA, RhoB protein levels, activated Cdc42/Rac1 phosphorylation while reducing Cdc42 and Rac1 protein levels in testes. And a similar performance on RhoA and Cdc42/Rac1 activation was observed in Sertoli cells (Fig. 5).

In gene analysis, all Rho GTPases genes have been down-regulated both *in vivo* and *in vitro*. But for proteins, triptolide induced a mild reduction on Cdc42 and Rac1 proteins in Sertoli cells. This change of Cdc42 and Rac1 is not as remarkable as that in testis.

In a long-term toxicity study, first action of TP appeared earlier and manifested mainly in epididymal sperm, then directly on germ cells. In animal model, it is apparent that not only Sertoli cells but also germ cells contributed to the significant decrease in protein levels. Considering the different duration of treatment and changes derived from sperm cells, the different performance of TP is reasonable.

3.5. Triptolide inhibits gene expression of Rocks

To further expand and illustrate the potential regulating pathway, Rocks gene expression has been detected, which demonstrated that TP induced Rock1 and Rock2 inhibition in mRNA level (Fig. 6). This down-regulation can be a downstream effector of Rho GTPases inhibition, result in actin suppression.

4. Discussion

Triptolide has been used to treat inflammatory and autoimmune diseases (Li et al., 2014). In preclinical research, TP revealed an anticancer effect against various cancer cells (Chugh et al., 2012; Xiong et al., 2016). The pro-drug of TP (Minnelide) is being tested in a phase 1 clinic trail. However, it has been proved in numerous studies that TP inhibits

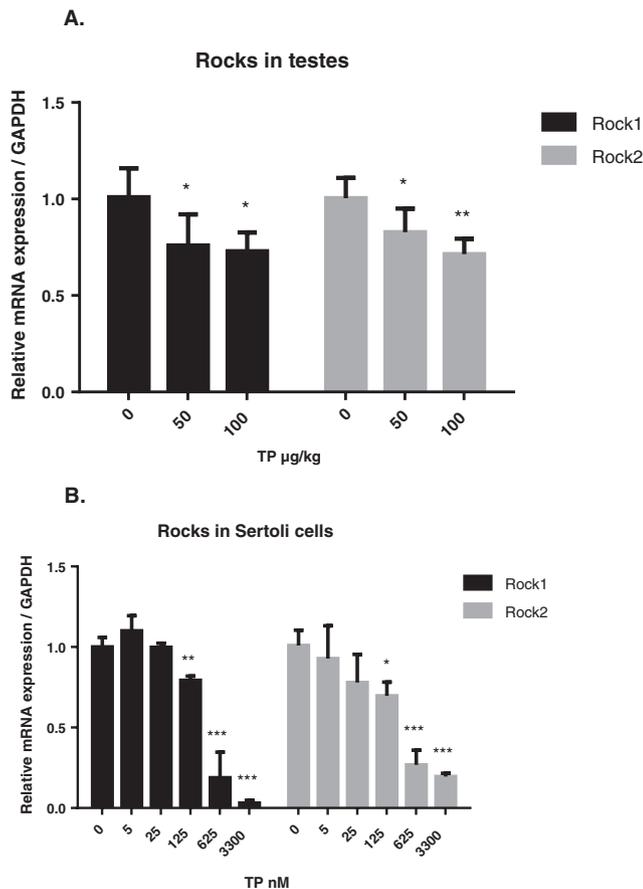


Fig. 6. Real-time PCR analysis of mRNA levels of Rock1 and Rock2, A. in testes and B. in Sertoli cells. Statistical significance relative to vehicle control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

productive functions, which restricts its application. Research on the antifertility effects will lead us to further knowledge of TP's functional mechanism as well as guidance in clinical use. In this study, we demonstrate that inhibition of Rho GTPases and disruption of Sertoli-germ cells AJ are involved in the antifertility effects of TP.

In the processes of spermatogenesis, developing germ cells have to move across the seminiferous epithelium, which must be supported by actin-based AJ such as ES. As previously described, mRNA level of testin in the testis reflects the integrity of inter-testicular cell junctions correlates with the disruption of Sertoli-germ cell junctions but not the inter-Sertoli tight junction (Xia et al., 2005). As a protein found predominantly at ES (Grima et al., 1995), testin expression can be induced whenever ES function was disrupted, leading to aspermatogenesis (Cheng et al., 2001). More studies on gene profiling have identified testin as one of the most induced genes (Xia et al., 2007). Furthermore, testin is reported strongly binds to actin (Mruk and Cheng, 2011b). The increasing level of testin and the inhibition in actin dynamics indicate that TP authentically induced male fertility impairment not only in sperm cell loss but also in the disruption of actin-based AJ. Germ cells would exfoliate at inappropriate time, resulting in sperm deformity and the reduction in sperm cell number and motility. In this context, AJ disruption might be one of the pivotal actions in TP-induced antifertility effect.

To elucidate the regulation of actin dynamics, we have investigated the effects of TP on Rho GTPases. Rho GTPases, such as Rho, Cdc42 and Rac1, are known to regulate many cellular processes including cell movement and cell adhesion (Lui et al., 2003a).

RhoA and RhoB, 83% identical to each other (Jeffrey Kroon et al., 2013), can regulate actin polymerization and Sertoli-germ cell adherens junction dynamics (Siu et al., 2011). Cdc42 and Rac1 are responsible for regulating the IQGAP (White et al., 2009) function (an effector protein involved in regulating E-cadherin activity), resulting in weakening adhesion (Lui et al., 2005). And their function is specifically between Sertoli-germ cells, but not Sertoli-Sertoli cells junction (Xia et al., 2005). The observation that TP disrupted F-actin filaments polymerization and induced ES structure disruption corresponds with functional changes of Rho GTPases inhibition.

As a member of cell adhesion molecules, β 1-integrin is reported to be present at ES site (Mulholland et al., 2001) in testis and it is also reported as the upstream regulator of RhoA (Siu et al., 2011). The cadherin/catenin is reported one of the protein complexes mediating AJ dynamics in testis (Lee et al., 2005). Many studies have demonstrated that Rho GTPases are required for cadherin-dependent cell-cell adhesion (Menke and Giehl, 2012; Shiratsuchi et al., 2012) and actin organization (Buul and Timmerman, 2016). Inhibition of Rho GTPases might be relevant to abnormal expression and/or dysfunction of adhesion molecules. At present experiments, we also observed a significant decrease in β 1-integrin and β -catenin gene expression (not published). Still, we need further work to demonstrate the role of adhesion molecules.

An interesting result is that TP significantly induced phosphorylation of Cdc42/Rac1 both *in vivo* and *in vitro*. It has been reported that Cdc42 played an opposing roles in regulating MMP-2 (matrix metalloproteinase 2) activation (Ispanovic et al., 2008). Other studies on the hierarchy of Rho GTPases signaling showed that Cdc42 activation induced Rac1 activation and then decreased RhoA activity (Tatin et al., 2006). Our observation of Cdc42 and Rac1 activation is in agreement with this signaling cascade.

In the past decades, numerous candidate downstream targets for Rho GTPases have been identified (Hanna and El-Sibai, 2013), the most important downstream molecules are Rho associated kinases, such as Rock1 and Rock2. Rock is an important target protein and affects actin regulators, such as MLC (myosin light chain), LIM kinases and adducin (Guan et al., 2013). Our findings on Rock suggest that Rho-Rock could be the potential regulating pathway of actin dynamics.

As the major component of microfilaments, β -actin was unexpectedly down-regulated by a non-toxicity dose of TP, suggesting that TP might have an inhibitive effect on cytoskeletal proteins. Vimentin, an important molecule for adhering spermatogenic cells to the seminiferous epithelium (Kumar et al., 2015), did not show a consistent tendency in our experiments. We also detected tubulin, the core protein of microtubules, gene expression indicates that TP inhibited tubulin only at cytotoxic doses. Therefore, we can deduce that the effects of TP on cytoskeletal proteins are mainly through regulating actin dynamics, which is regulated by Rho-Rock pathway and correlated to actin-based AJ.

Noteworthy, many studies highlight the metabolic cooperation between Sertoli cells and germ cells since these events are essential for the normal occurrence of spermatogenesis (Alves et al., 2015). Sertoli cells maintain a high glycolytic flux in order to sustain the energetic needs of the developing germ cells. In the future study, considering the development of metabolomics research on TP (Ma et al., 2015), the metabolic cooperation of Sertoli-germ cells could be a potential originator of TP-induced antifertility effect.

5. Conclusion

To the best of our knowledge, this is the first report demonstrating a potential toxicant mechanism that disruption of Sertoli-germ cells AJ is the critical process in TP-induced antifertility effect. On the other hand, we found that TP induces Rho GTPases inhibition in Sertoli cells prior to its cytotoxicity, suggesting that TP could be a potential male contraceptive. Furthermore, Rho-Rock pathway might be involved in the regulation of its antifertility effect.

Conflict of interest statement

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted "Triptolide disrupts the actin-based Sertoli-germ cells adherens junctions by inhibiting Rho GTPases expression".

Transparency document

The [Transparency document](#) associated with this article can be found, in the online version.

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References

- Alves, M.G., Dias, T.R., Silva, B.M., Oliveira, P.F., 2015. Metabolic cooperation in testis as a pharmacological target: from disease to contraception. *Curr. Mol. Pharmacol.* 7, 83–95.
- Aravindan, G.R., Pineau, C., Bardin, C.W., Cheng, C.Y., 1996. Ability of trypsin in mimicking germ cell factors that affect sertoli cell secretory function. *J. Cell. Physiol.* 168, 123–133.
- Buul, J.v., Timmerman, I., 2016. Small rho GTPase-mediated actin dynamics at endothelial adherens junctions. *Small GTPases* 7, 21–31.
- Cai, L., Kato, T., Chen, M., Wang, H., Sekine, E.-i., Izumi, S.-i., Kato, Y., 2012. Accumulated HSV1-TK proteins interfere with spermatogenesis through a disruption of the integrity of sertoli-germ cell junctions. *J. Reprod. Dev.* 58, 544–551.

- Cao, J., Tan, M.H., Yang, P., Li, W.L., Xia, J., Du, H., Tang, W.B., Wang, H., Chen, X.W., Xiao, H.Q., 2008. Effects of adjuvant chemotherapy on bone marrow mesenchymal stem cells of colorectal cancer patients. *Cancer Lett.* 263, 197–203.
- Chang, S.L., Jin, B., Youn, P., Park, C., Park, J.D., Ryu, D.Y., 2007. Arsenic-induced toxicity and the protective role of ascorbic acid in mouse testis. *Toxicol. Appl. Pharmacol.* 218, 196–203.
- Cheng, C.Y., Silvestrini, B., Grima, J., Mo, M., Zhu, L., Johansson, E., Saso, L., Leone, M., Palmery, M., Mruk, D., 2001. Two new male contraceptives exert their effects by depleting germ cells prematurely from the testis. *Biol. Reprod.* 65.
- Chugh, R., Sangwan, V., Patil, S.P., Dudeja, V., Dawra, R.K., Banerjee, S., Schumacher, R.J., Blazar, B.R., Georg, G.I., Vickers, S.M., Saluja, A.K., 2012. A preclinical evaluation of Minnelide as a therapeutic agent against pancreatic cancer. *Sci. Transl. Med.* 4, 156ra139.
- Gao, Y., Lui, W., Lee, W.M., Cheng, C.Y., 2016. Polarity protein crumbs homolog-3 (CRB3) regulates ectoplasmic specialization dynamics through its action on F-actin organization in Sertoli cells. *Sci. Rep.* 6, 28589.
- Grima, J., Zhu, L., Zong, S.D., Catterall, J.F., Bardin, C.W., Cheng, C.Y., 1995. Rat testin is a newly identified component of the junctional complexes in various tissues whose mRNA is predominantly expressed in the testis and ovary. *Biol. Reprod.* 52, 340–355.
- Grima, J., Zhu, L., Cheng, C.Y., 1997. Testin is tightly associated with testicular cell membrane upon its secretion by sertoli cells. *J. Biol. Chem.* 272, 6499–6509.
- Grima, J., Wong, C.C.S., Zhu, L., Zong, S., Cheng, C.Y., 1998. Testin secreted by sertoli cells is associated with the cell surface, and its expression correlates with the disruption of sertoli-germ cell junctions but not the inter-sertoli tight junction. *J. Biol. Chem.* 273, 21040–21053.
- Guan, R., Xu, X., Chen, M., Hu, H., Ge, H., Wen, S., Zhou, S., Pi, R., 2013. Advances in the studies of roles of Rho/Rho-kinase in diseases and the development of its inhibitors. *Eur. J. Med. Chem.* 70, 613–622.
- Hanna, S., El-Sibai, M., 2013. Signaling networks of Rho GTPases in cell motility. *Cell. Signal.* 25, 1955–1961.
- Hikim, A.P.S., Lue, Y., Wang, C., Reutrakul, V., Sangsawan, R., Swerdloff, R.S., 2000. Posttesticular antifertility action of triptolide in the male rat evidence for severe impairment of cauda epididymal sperm ultrastructure. *J. Androl.* 21, 431–437.
- Ho, L., Chang, W., Chen, A., Chao, P., Lai, J., 2013. Differential immunomodulatory effects by Tripterium wilfordii Hook f-derived refined extract PG27 and its purified component PG490 (triptolide) in human peripheral blood T cells. *J. Transl. Med.* 11 (Published online).
- Hu, Y., Wang, R., Xiang, Z., Qian, W., Han, X., Li, D., 2014. Antagonistic effects of a mixture of low-dose nonylphenol and di-n-butyl phthalate (monobutyl phthalate) on the Sertoli cells and serum reproductive hormones in prepubertal male rats in vitro and in vivo. *PLoS One* 9, e93425.
- Ispanovic, E., Serio, D., Haas, T.L., 2008. Cdc42 and RhoA have opposing roles in regulating membrane type 1-matrix metalloproteinase localization and matrix metalloproteinase-2 activation. *Am. J. Phys. Cell Physiol.* 295, C600–C610.
- Johnson, S.M., Wang, X., Evers, B.M., 2011. Triptolide inhibits proliferation and migration of colon cancer cells by inhibition of cell cycle regulators and cytokine receptors. *J. Surg. Res.* 168, 197–205.
- Kroon, J., Tol, S., Amstel, S.V., Elias, J.A., Fernandez-Borja, M., 2013. The small GTPase RhoB regulates TNF α signaling in endothelial cells. *PLoS One* 8, e75031.
- Kumar, N., Srivastava, S., Roy, P., 2015. Impact of low molecular weight phthalates in inducing reproductive malfunctions in male mice: special emphasis on sertoli cell functions. *Gen. Comp. Endocrinol.* 215, 36–50.
- Lee, N.P., Mruk, D.D., Wong, C.H., Cheng, C.Y., 2005. Regulation of sertoli-germ cell adherens junction dynamics in the testis via the nitric oxide synthase (NOS)/cGMP/protein kinase G (PRKG)/beta-catenin (CATNB) signaling pathway: an in vitro and in vivo study. *Biol. Reprod.* 73, 458–471.
- Li, Y., Han, X., 2012. Microcystin-LR causes cytotoxicity effects in rat testicular Sertoli cells. *Environ. Toxicol. Pharmacol.* 33, 318–326.
- Li, X., Jiang, Z., Zhang, L., 2014. Triptolide: progress on research in pharmacodynamics and toxicology. *J. Ethnopharmacol.* 155, 67–79.
- Li, N., Mruk, D.D., Lee, W.M., Wong, C.K.C., Cheng, C.Y., 2016. Is toxicant-induced Sertoli cell injury in vitro a useful model to study molecular mechanisms in spermatogenesis? *Semin. Cell Dev. Biol.*
- Lie, P., Mruk, D.D., Lee, W.M., Cheng, C.Y., 2010. Cytoskeletal dynamics and spermatogenesis. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 365, 1581–1592.
- Liu, Q., 2011. Triptolide and its expanding multiple pharmacological functions. *Int. Immunopharmacol.* 11, 377–383.
- Liu, L., Li, G., Li, Q., Jin, Z., Zhang, L., Zhou, J., Hu, X., Zhou, T., Chen, J., Gao, N., 2013. Triptolide induces apoptosis in human leukemia cells through caspase-3-mediated ROCK1 activation and MLC phosphorylation. *Cell Death Dis.* 4, e941.
- Lue, Y., S.H., A.P., Wang, C., Leung, A., Baravarian, S., Reutrakul, V., Sangsawan, R., Chaichana, S., Swerdloff, R.S., 1998. Triptolide a potential male contraceptive. *J. Androl.* 19, 479–486.
- Lui, W.Y., Lee, W.M., Cheng, C.Y., 2003a. Rho GTPases and spermatogenesis. *Biochim. Biophys. Acta (BBA) - Mol. Cell Res.* 1593, 121–129.
- Lui, W.Y., Lee, W.M., Cheng, C.Y., 2003b. Sertoli-germ cell adherens junction dynamics in the testis are regulated by RhoB GTPase via the ROCK/LIMK signaling pathway. *Biol. Reprod.* 68, 2189–2206.
- Lui, W.Y., Mruk, D.D., Cheng, C.Y., 2005. Interactions among IQGAP1, Cdc42, and the cadherin-catenin protein complex regulate Sertoli-germ cell adherens junction dynamics in the testis. *J. Cell. Physiol.* 202, 49–66.
- Ma, B., Qi, H., Li, J., Xu, H., Chi, B., Zhu, J., Yu, L., An, G., Zhang, Q., 2015. Triptolide disrupts fatty acids and peroxisome proliferator-activated receptor (PPAR) levels in male mice testes followed by testicular injury: a GC-MS based metabolomics study. *Toxicology* 336, 84–95.
- Menke, A., Giehl, K., 2012. Regulation of adherens junctions by Rho GTPases and p120-catenin. *Arch. Biochem. Biophys.* 524, 48–55.
- Mruk, D.D., Cheng, C.Y., 2011a. An in vitro system to study Sertoli cell blood-testis barrier dynamics. *Methods Mol. Biol.* 763, 237–252.
- Mruk, D.D., Cheng, C.Y., 2011b. Testin and actin are key molecular targets of adjuvin, an anti-spermatogenic agent, in the testis. *Spermatogenesis* 1, 137–146.
- Mulholland, D.J., Dedhar, S., Vogl, A.W., 2001. Rat seminiferous epithelium contains a unique junction (Ectoplasmic specialization) with signaling properties both of cellcell and cellmatrix junctions. *Biol. Reprod.* 64, 396–407.
- Murali, A., Rajalingam, K., 2014. Small Rho GTPases in the control of cell shape and motility. *Cell. Mol. Life Sci.* CMLS 71, 1703–1721.
- Ni, B., Jiang, Z., Huang, X., Xu, F., Zhang, R., Zhang, Z., Tian, Y., Wang, T., Zhu, T., Liu, J., Zhang, L., 2008. Male reproductive toxicity and toxicokinetics of triptolide in rats. *Arzneimittelforschung* 58, 673–680.
- Reis, M.M.S., Moreira, A.C., Sousa, M., Mathur, P.P., Oliveira, P.F., Alves, M.G., 2015. Sertoli cell as a model in male reproductive toxicology: advantages and disadvantages. *J. Appl. Toxicol.* JAT 35, 870–883.
- Shiratsuchi, H., Shimizu, O., Saito, T., Mashimo, T., Yonehara, Y., 2012. Immunohistological study of small Rho GTPases and beta-catenin during regeneration of the rat submandibular gland. *J. Mol. Histol.* 43, 751–759.
- Singla, N., Challana, S., 2014. Reproductive toxicity of triptolide in male house rat, *Rattus rattus*. *TheScientificWorldJOURNAL* 2014, 879405.
- Siu, M.K., Wong, C.H., Lee, W.M., Cheng, C.Y., 2005. Sertoli-germ cell anchoring junction dynamics in the testis are regulated by an interplay of lipid and protein kinases. *J. Biol. Chem.* 280, 25029–25047.
- Siu, M.K.Y., Wong, C., Lee, W.M., Cheng, C.Y., 2010. Sertoli-germ cell junctions in the testis: a review of recent data. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 365, 1593–1605.
- Siu, M.K.Y., Wong, C.H., Xia, W., Mruk, D.D., Lee, W.M., Cheng, C.Y., 2011. The beta1-integrin-p-FAK-p130Cas-DOCK180-RhoA-vinculin is a novel regulatory protein complex at the apical ectoplasmic specialization in adult rat testes. *Spermatogenesis* 1, 73–86.
- Tang, E.I., Lee, W.M., Cheng, C.Y., 2016. Coordination of actin- and microtubule-based cytoskeletons support transport of spermatids and residual bodies/phagosomes during spermatogenesis in the testis of the male rat. *Endocrinology*, en20151962.
- Tatin, F., Varon, C., Génot, E., Moreau, V., 2006. A signalling cascade involving PKC, Src and Cdc42 regulates podosome assembly in cultured endothelial cells in response to phorbol ester. *J. Cell Sci.* 119, 769–781.
- Vogl, A.W., Vaid, K.S., Guttman, J.A., 2008. The Sertoli cell cytoskeleton molecular mechanisms in spermatogenesis. *Adv. Exp. Med. Biol.* 636, 186–211.
- White, C.D., Brown, M.D., Sacks, D.B., 2009. IQGAPs in cancer: a family of scaffold proteins underlying tumorigenesis. *FEBS Lett.* 583, 1817–1824.
- Xia, W., Wong, C.H., Lee, N.P.Y., Lee, W.M., Cheng, C.Y., 2005. Disruption of Sertoli-germ cell adhesion function in the seminiferous epithelium of the rat testis can be limited to adherens junctions without affecting the blood-testis barrier integrity. *J. Cell. Physiol.* 205, 141–157.
- Xia, W., Mruk, D.D., Lee, W.M., Cheng, C.Y., 2007. Unraveling the molecular targets pertinent to junction restructuring events during spermatogenesis using the adjuvin-induced germ cell depletion model. *J. Endocrinol.* 192, 563–583.
- Xiong, W., Wang, H., Wu, H., Chen, Y., Han, D., 2009. Apoptotic spermatogenic cells can be energy sources for sertoli cells. *Reproduction* 137, 469–479.
- Xiong, J., Su, T., Qu, Z., Yang, Q., Wang, Y., Li, J., Zhou, S., 2016. Triptolide Has Anticancer and Chemosensitization Effects by down-Regulating Akt Activation through the MDM2/REST Pathway in Human Breast Cancer Oncotarget Epub ahead of print.
- Ying, W., Huang, L.Q., Tang, X.C., Zhang, H.Y., 2010. Retrospect and prospect of active principles from Chinese herbs in the treatment of dementia. *Acta Pharmacol. Sin.* 31, 649–664.
- Zinzow-Kramer, W.M., Horton, B.M., Maney, D.L., 2014. Evaluation of reference genes for quantitative real-time PCR in the brain, pituitary, and gonads of songbirds. *Horm. Behav.* 66, 267–275.