Original Article

Preliminary research on abating rat testicle toxicity due to triptolide after oral polymer nanoparticle delivery

Mingxing Liu[1,2,*], Jing Dong[3], Yajiang Yang[3], Xiangliang Yang[2], Huibi Xu[3]

[1] *Department of Pharmacy, College of Bioengineering, Hubei University of Technology, Wuhan, China;*
[2] *College of Life Science & Technology, Huazhong University of Science & Technology, Wuhan, China;*
[3] *Department of Chemistry, Huazhong University of Science & Technology, Wuhan, China.*

ABSTRACT: This study was undertaken to investigate the effect of triptolide (TP) on male rats after oral polymer nanoparticle delivery (TP-loaded poly(D,L-lactic acid) nanoparticles, TP-PLA-NPs). Free TP and TP-PLA-NPs were administered orally at doses of 0.2 and 0.6 mg/kg for 15 days and rats were sacrified at end of the dosage period. All rat testes were weighed, fructose content and activity of acid phosphatase (ACP) were assayed, and testis tissues were observed histopathologically. Testis weight, testis index, ACP activity, and fructose content of the treated animals were lower than those of the control group. Moreover, ACP activity and fructose content were markedly decreased for free TP of 0.6 mg/kg in comparison to the same dose of TP-PLA-NPs. Obvious pathological changes were not observed for a dose of 0.2 mg/kg free TP and TP-PLA-NPs at the end of 15 days. At a dose of 0.6 mg/kg, however, free TP caused more serious damage to the testes than TP-PLA-NPs. These results revealed that TP-PLA-NPs might decrease the testis toxicity of TP because TP was slowly released from polymer nanoparticles. Further research on this mechanism of abated toxicity is in progress.

Keywords: Triptolide, Testis, Polymer nanoparticle delivery, Poly(D,L-lactic acid)

1. Introduction

Triptolide (TP, Figure 1), a purified diterpenoid triepoxide compound that is extracted from the Chinese medicinal plant *Tripterygium wilfordii* Hook.f. (TWHf), has several forms of pharmacological activity including an immunosuppressive effect (*1*,*2*), anti-cancer activity (*3*), and anti-inflammatory activity (*4*). However, the clinical use of TP is known to present several practical disadvantages mainly due to its lower water solubility and severe toxicity. The incidence of adverse drug reactions (ADRs) due to TP is higher than due to other drugs in China. The organic systems affected by ADRs of TP relate to the digestive, urogenital, and circulatory systems as well as bone marrow.

In recent years, the effect of TWHf or TP on the testis tissue of male rats or men has been extensively noted (*5-8*). Oral administration of TP at a dosage of 0.1 mg/kg daily for 70 days left male rats completely infertile, with sperm motility reduced to zero, and cauda epididymal sperm content decreased to 68% (*6*). TP was reported to impair the cauda epididymal sperm ultrastructure with minimal abnormalities in the fine structural cytology of the testes (*9*). Daily oral doses of 20-30 mg extract of TWHf for 2 months caused a sharp decline in the epididymal sperm number and motility for Chinese men; interestingly, though, fertility returned to normal between 1 and 2 months after the cessation of administration (*10*). Therefore, these results suggest that TWHf or TP might be developed as a promising male contraceptive.

Earlier immunological studies have indicated that TP does not have an immunosuppressive effect at dose levels inducing infertility; however, higher doses (5 to

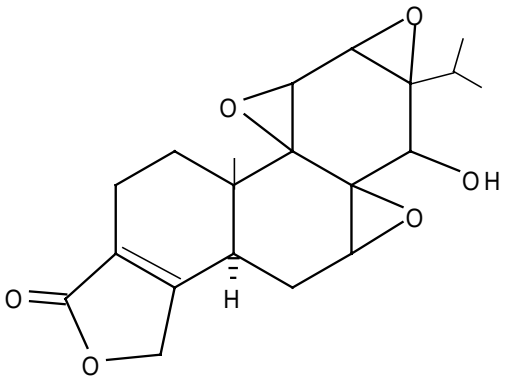

Figure 1. Chemical structure of triptolide.

**Correspondence to:* Dr. Mingxing Liu, Department of Pharmacy, College of Bioengineering, Hubei University of Technology, Wuhan 430068, China;
e-mail: lmxing@mail.hbut.edu.cn

12 times higher than its antifertility doses) can produce immunosuppressive effects (*11,12*). However, higher dosages of TP might induce more severe toxicity in animals or man. The question is how to retain or improve the pharmacological activity of TP while decreasing its toxicity on the testes.

Over the last few decades, various drug delivery systems (DDS) such as liposomes, microemulsion, and polymer micro/nanoparticles have shown great promise in controlled release and targeted drug delivery in order to decrease or avoid a drug's side effects (*13-15*). Fortunately, novel types of delivery systems for TP were widely developed in China. Loaded-TP solid lipid nanoparticles efficiently penetrated the skin, had desirable anti-inflammatory activity, and significantly decreased hepatotoxicity in comparison to control groups (*16,17*). Microemulsions containing TP significantly increased the in vitro permeation rate of TP through mouse skin and reduced skin irritation when compared to a TP solution (*18*).

Poly(D,L-lactic acid) (PLA) has been universally used as a microsphere/nanoparticles carrier in DDS due to its desirable biocompatible and biodegradable properties; in particular, it was approved by the US Food and Drug Administration for clinical use. In previous studies, TP-loaded PLA nanoparticles (TP-PLA-NPs) were successfully prepared and characterized (*19*). Animal experiments indicated that TP-PLA-NPs significantly inhibited adjuvant-induced arthritis and had a desirable anti-inflammatory effect with long-time administration (*4*). The main objective of the present study was to investigate if TP-PLA-NPs would decrease the testis tissue toxicity caused by TP in male rats after oral administration.

2. Materials and Methods

2.1. *Materials*

TP (purity > 99%) was supplied by Fujian Chinese Medical Research Institute (China). PLA (MW, 10,000 Da) was obtained from Shangdong Medical Treatment and Instrument Institute (China). Poloxamer 188 was purchased from Jiqi Medicine Co., Ltd., Shenyang Pharmaceutical University (China). Methanol was of HPLC grade and other reagents were of analytical grade.

2.2. *Preparation of TP-PLA-NPs and characterization*

TP-PLA-NPs were prepared according to the method previously described (*4*). Briefly, an amount of PLA/TP (20:1, w/w) was co-dissolved in a mixture of acetone/ethanol (9:6, v/v). The solution obtained was then added dropwise to 40 mL of poloxamer 188 solution (1%, w/v) with continuous gentle stirring at ambient temperature until complete evaporation of the organic solvent. The obtained suspension was then used for animal experiments after adjustment of the drug concentration (calculated according to TP). The produced nanoparticles were collected by ultracentrifugation (12,000 rpm, 3 h, 4°C) using a GL 20 centrifuge (Xiangxi, China) and freeze-dried (LGJ 0.5, Beijing, China) to obtain white, powdered nanoparticles.

The mean particle size, size distribution, and polydispersity index of nanoparticles were assessed by dynamic light scattering with a particle size analyzer (Zeta plus, Brookhaven). The shape and surface morphology of nanoparticles were observed with a transmission electron microscope (JEM-2000 CX-II, JEOL).

The amount of drug incorporated into nanoparticles was determined by HPLC (Agilent 1100) using a reverse phase Lichrospher ODS column (5 μm, 250 × 4.6 mm i.d.). A release experiment for release of TP from nanoparticles was performed using a dialysis bag technique. Analysis of drug content and the release experiment are described in detail elsewhere (*19*).

2.3. *Animals and treatment*

Healthy male Wistar rats (n = 30) weighing 152-171 g were obtained from the Laboratory Animal Center, Tongji Medical College, Huazhong University of Science & Technology (Wuhan, China). The animals were housed in stainless steel cages in the animal room. The room temperature and the relative humidity were controlled at 23 ± 2°C and 50 ± 10%, respectively. All animals were allowed free access to drinking water and feed throughout the study.

Animals were randomly divided into five groups with six per group. The control group was given only saline every day. TP-PLA-NPs groups were given a 0.05% CMC (sodium carboxymethyl cellulose) suspension of TP-PLA-NPs (0.2 and 0.6 mg(TP)/kg), respectively, by gavage. Free TP groups were given a 0.05% CMC suspension of free TP (0.2 and 0.6 mg(TP)/kg), respectively, by gavage. All animal experiments followed a protocol approved by the Institutional Animal Care and Use Committee of this Center.

2.4. *Organ weight and preparation of testis serum*

All rats were weighed on the 15th day and immediately sacrificed under ether anesthesia. Testes were quickly removed, cleared of fat and connective tissue, washed with normal saline (0.9% sodium chloride) and weighed after removing water on their surface. One gram of testes was placed in 10 mL phosphate buffered saline (1 mM, pH 7.0) and homogenized by 2 min of sonication (Scientz-IID, Ningbo, China) under low temperature conditions (4°C). The suspension obtained was

centrifuged for 10 min at 7,000 rpm and the supernatant was stored at -20°C before biochemical analysis.

2.5. *Enzyme assay and fructose content*

Acid phosphatase (ACP) activity was assayed using *p*-nitrophenyl phosphate as a substrate at pH 4.8 (*20*). One unit of enzyme is defined as μmoles of *p*-nitrophenol released per min and per gram protein at 37°C.

Fructose levels from the seminal vesicles were quantified colorimetrically by employing a resorcinol reagent (*21*).

2.6. *Histopathological analysis*

The excised testes were fixed in 10% buffered formalin (0.01 mM, pH 7.4) and dehydrated in aqueous alcohol (25%, 50%, 70%, and 90% EtOH). The testes were embedded in paraffin blocks and sectioned perpendicular to the longest axis of the testis at a thickness of 5-8 μm. The sections were mounted onto regular glass slides and stained with hematoxylin and eosin prior to observation by light microscopy (Olympus, BH-2, Japan).

2.7. *Statistical analysis*

Data were expressed as mean ± standard deviation (S.D.) and analyzed using the Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

3. Results and Discussion

TP is a potent anti-inflammatory and immunosuppressive agent. However, thousands of adverse events related to TWHF or TP are reported in China. The patients usually experience nausea, vomit, dyspnoea, hepatomegaly, duodenal ulcer, and gastrointestinal bleeding in the digestive system if taking the drug for a long time. Some organic systems including the gastrointestinal, urogenital, cardiovascular, and circulatory systems as well as bone marrow and skin can also be affected by these systems' toxic reactions to TP (*2*).

TP is reported to not be immunosuppressive at dose levels inducing infertility (*11*), though doses about 5 to 12 times higher than infertility doses can have immunosuppressive effects (*12*), TP also has potent toxicity associated with the renal, cardiac, hematopoietic, and reproductive systems. In recent years, several advanced dosage forms have been developed in order to effectively improve the immunosuppressive effect of TP and decrease its toxicity (*4,17,18*). The current study mainly investigated the effect of TP-PLA-NPs on the testes of male rats after continuous oral administration at different dosages for 15 days.

3.1. *Characterization of nanoparticles*

TP-loaded PLA nanoparticles showed a narrow size distribution, low polydispersity index of 0.088, and mean particle size of about 150 nm. Moreover, nanoparticles appeared to be a fine spherical shape with smooth surfaces and without any aggregation or adhesion. Drug encapsulation efficiency into nanoparticles was 85.7 ± 4.3% (n = 3). The *in vitro* release profiles of TP from nanoparticles showed a biphasic release phenomenon, namely an initial burst release and then a slow release. The initial burst release resulted in 11.45% of the cumulative amount of TP being released from nanoparticles at the first sampling time of 0.5 h. In contrast, about 22.35% of the cumulative amount of TP was released from nanoparticles within 48 h during the constant slow release process. The above results are shown for reference (*4*).

3.2. *Body and testis weight, testis index, and biochemical parameters of testes serum*

As shown in Table 1, the results revealed that there were no changes in body weight between each group for 15 days. However, testis weight and the testis index were reduced by administration of different dosage forms and decreased significantly ($P < 0.05$) for free TP of 0.6 mg/kg in comparison to the control group.

Monitoring of body weight, testis weight, and testis index provides an index of the general health of animals and such information may also be important for the

Table 1. Effect of different dosage forms on the body weight and testis weights of rats and testis index (gavage daily for 15 days)

Groups	Body weight before experiments (g)[a]	Body weight after experiments (g)[a]	Testis weight (g)[a]	Testis index (g/g, %)[a,b]
Control (n = 6)	164.6 ± 3.4	207.1 ± 3.3	3.44 ± 0.17	1.67 ± 0.2
Free TP (mg/kg)				
0.2 (n = 6)	164.2 ± 5.1	201.6 ± 5.2	2.51 ± 0.13	1.25 ± 0.2
0.6 (n = 5)	164.7 ± 3.6	198.3 ± 3.9	1.81 ± 0.26[c]	0.91 ± 0.13[c]
TP-PLA-NPs (mg/kg)				
0.2 (n = 6)	164.8 ± 4.6	203.1 ± 2.7	2.38 ± 0.18	1.18 ± 0.14
0.6 (n = 6)	164.2 ± 4.3	202.3 ± 4.1	2.22 ± 0.12	1.1 ± 0.13

[a] Values are mean ± S.D.
[b] Testis index: the percent weight of the testis to body weight after 15 days.
[c] Significant difference from control ($P < 0.05$).

interpretation of reproductive health (*22*). The results obtained revealed that TP might be responsibility for the rats' health. This decrease after administration is understandable, though, because TP might greatly affect the digestive system, causing symptoms such as nausea (*2*). Furthermore, testis weight and testis index of free TP decreased less with free TP of 0.6 mg/kg than with the same dose of TP-PLA-NPs. The explanation might be that TP enveloped into polymer nanoparticles was slowly released (*4*).

As shown in Table 2, ACP activity and fructose content of the treated animals decreased significantly ($P < 0.05$) for free TP of 0.2 mg/kg and TP-PLA-NPs of 0.6 mg/kg and very significantly ($P < 0.01$) for free TP of 0.6 mg/kg in comparison to the control group. Moreover, they decreased markedly ($P < 0.05$) for free TP of 0.6 mg/kg when compared with the same dose of TP-PLA-NPs.

Fructose content has been used as a parameter to evaluate the function of sexual glands because fructose in the seminal plasma serves to induce the glycolytic metabolism of spermatozoa (*23*). The enrichment of ACP in human sperm has been proven to restrain the activity of neutrophils and NK cells and provide better immunosuppression to avoid the spermatozoa excluded in the vagina of females (*24*). The fructose content and ACP activity of the treated animals decreased significantly for free TP in comparison to the control group. The explanation might be that TP hampered the glycolytic metabolism of spermatozoa and resulted in abnormal sperm function, ultimately giving rise to complete male sterility. Moreover, fructose content and ACP activity decreased markedly for free TP of 0.6 mg/kg when compared with the same dose of TP-PLA-NPs. The results might be because TP was slowly released from polymer nanoparticles matrix and polymer nanoparticles helped to prevent the testes from being harmed by TP. These changes in parameters were well supported by histopathological observations.

Recent studies have shown that long-term administration of TP (over a period of 70 days or more) can result in male rat infertility due to glutathione depletion and a decrease in microsomal epoxide hydrolase activity (*7*). Moreover, glutathione conjugation and microsomal epoxide hydrolase can detoxify the reactive metabolites present in the chemical constituents of triptolide, such as glycosides, diterpenoids, and epoxide (*11*,*25*,*26*). Glutathione transferases also play an important role in sperm motility and epididymal sperm counts (*27*,*28*), which should be further investigated in subsequent research.

Table 2. Biochemical parameters of testis serum of treated and control rats (gavage daily for 15 days)

Groups	ACP (U/g protein)[a,b]	Fructose (mg/g of testis)[a]
Control ($n = 6$)	350 ± 12	0.32 ± 0.03
Free TP (mg/kg)		
0.2 ($n = 6$)	150 ± 11[c]	0.19 ± 0.02[c]
0.6 ($n = 5$)	80 ± 13[d,e]	0.09 ± 0.01[d,e]
TP-PLA-NPs (mg/kg)		
0.2 ($n = 6$)	190 ± 12	0.23 ± 0.02
0.6 ($n = 6$)	170 ± 14[c]	0.2 ± 0.02[c]

[a] Values are mean ± S.D.
[b] Enzyme activity in Table 2 is expressed in U/g protein where one unit is defined as μmoles of *p*-nitrophenol released per min and per gram protein under specified assay conditions.
[c] Significant difference from control ($P < 0.05$).
[d] Very significant difference from control ($P < 0.01$).
[e] Significant difference from the same dose of free TP-PLA-NPs ($P < 0.05$).

3.3. *Histopathology*

Seminiferous tubules, spermatogenic cells, and Sertoli cells appeared to be normal in the control rats. Multi-nucleation was seen in some tubules. Scattered Leydig cells and blood vessels were found in the interstitial connective tissue between the tubules. Meanwhile, the seminiferous tubules appeared uniform in size and shape (Figure 2A).

As shown in Figure 2, histopathological changes in rat testes occurred as a result of oral administration of different dosage forms after 15 days. Obvious pathological changes were not observed in seminiferous tubules and interstitial tissue at a dose of 0.2 mg/kg free TP and TP-PLA-NPs at the end of 15 days (Figure 2B and 2C). However, spermatogenic cells in some seminiferous tubules of testes decreased slightly in comparison to normal testis tissue.

At a dose of 0.6 mg/kg TP-PLA-NPs, the testes showed slight shrinkage of tubules particularly in the central region, while the number of spermatogenic cells was reduced in some seminiferous tubules. Some tubules appeared to have moderate degeneration (Figure 2D). At a dose of 0.6 mg/kg free TP, fewer spermatid and secondary spermatocytes were observed in some of the seminiferous tubules. Some tubules appeared to have complete degeneration. Meanwhile, necrosis was observed in some seminiferous tubules and edema was observed in interstitial tissue. Large multinuclear cells were found in the lumen of some testes (Figure 2E). During the experiment, one rat died in the group given 0.6 mg/kg free TP.

TP affected the normal configuration of testis tissue; at a high dose of free TP, TP severely harmed the testes, causing fewer spermatid and secondary spermatocytes, degeneration, necrosis, and large multinuclear cells (Figure 2E). Interestingly, histopathological observation revealed that TP-PLA-NPs greatly deceased the damage to rat testes by TP when compared to the same dose of free TP (Figure 2D and 2E).

In conclusion, the results obtained indicated that TP-PLA-NPs can decrease the testis toxicity caused by TP because TP is slowly released from the polymer nanoparticle matrix. Further work is required to investigate the mechanism of decreased toxicity and tissue distribution of TP-PLA-NPs in male rats.

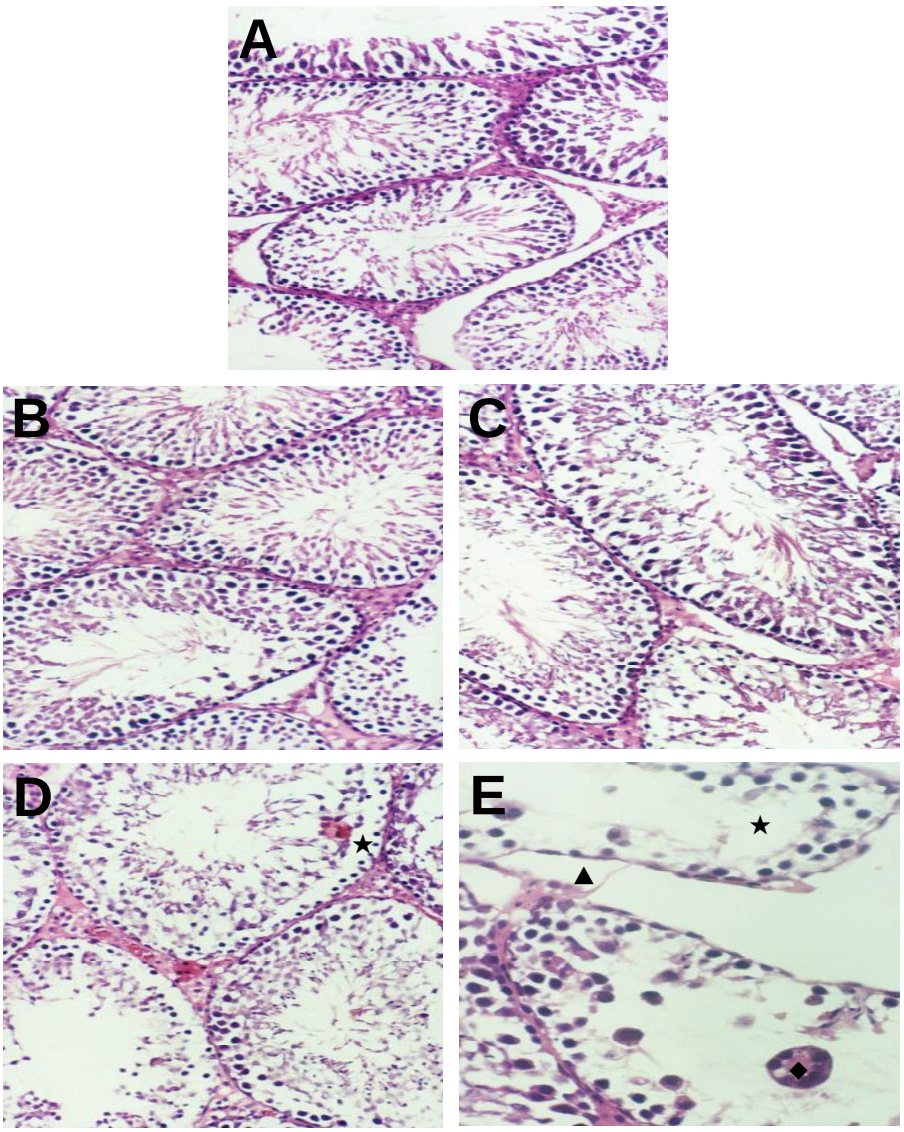

Figure 2. Histopathological changes in the testicle tissue after administration of triptolide and its nanoparticles (gavage daily for 15 days). (A) Normal pattern for control rats. (B and C) Only a slight decrease in spermatogenic cells at a dose of TP-PLA-NPs and TP (0.2 mg/kg). (D) Moderate degeneration, slight necrosis (★), and a decrease in spermatogenic cells at a dose of 0.6 mg/kg TP-PLA-NPs. (E) Severe pathological changes with complete degeneration, necrosis (★) in seminiferous tubules, edema (▲) in interstitial tissue, and appearance of large multinuclear cells (◆) at a dose of 0.6 mg/kg free TP. Magnifications: (A) ×200, (B) ×200, (C) ×200, (D) ×200, (E) ×400.

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