

Acute Damage to the Sperm Quality and Spermatogenesis in Male Mice Exposed to Curcumin-Loaded Nanoparticles

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Background: Curcumin has shown many pharmacological activities in both preclinical and clinical studies. Many technologies have been developed and applied to improve the solubility and bioavailability of curcumin, especially the nanotechnology-based delivery systems. However, there has been evidence that certain nanoparticles have potential reproductive toxicity in practice.

Methods: Curcumin-poly (lactic-co-glycolic acid) (PLGA)-PEG nanoparticles (Cur-PLGA-NPs for short) were prepared. The Cur-PLGA-NPs were evaluated with its effect on the proliferation of mouse testicular cell lines in vitro and spermatogenesis in vivo, while PLGA-NPs were used as control. For animal experiments, male BALB/c mice were treated with 20 mg/kg of Cur-PLGA-NPs for continuous 10 days via tail vein injection.

Results: We found the curcumin nanoparticles suppressed the proliferation of testicular cell lines in vitro. Furthermore, a short-term intravenous delivery of curcumin-loaded nanoparticles could be harmful to the differentiation of spermatogonia, the elongation of spermatids, as well as the motility of mature sperms.

Conclusion: In the present study, we disclosed the acute damage on mouse spermatogenesis and sperm parameters by curcumin-loaded nanoparticles. Our results suggested that the reproductive toxicity of nanoformulated curcumin needs to be prudently evaluated before its application.

Keywords: nano-curcumin, reproductive toxicity, Sertoli cell, sperm motility, spermatogenesis

Introduction

Curcumin is extracted from the plant *Curcuma longa* and has been used as food additives and traditional medications for thousands of years in Asia.¹ In recent decades, curcumin has been proved to have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, anti-mutagen, and anti-carcinogenic effects.^{2,3} However, the clinical application of curcumin had long been hampered by its poor solubility in aqueous solvents, the low oral bioavailability and the rapid clearance in practice. Therefore, many technologies have been developed to overcome this limitation, including liposomes, phospholipid complexes, microemulsions, as well as polymeric micelles and nanoparticles.^{4,5} Specifically, the nanoformulated curcumin is promising for therapeutic applications. For example, the combination of nano-curcumin and chemotherapy is likely to increase the effectiveness of cancer treatment as well as reduce the adverse outcomes.⁶ Another case is, curcumin-loaded nanoparticles could successfully pass the blood-brain barrier, providing the neuroprotective effects in various central nervous system (CNS)-related diseases.⁷

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However, there have been contradictory scientific reports about the impacts of curcumin on reproductive systems, which partially dependent on the doses and cell/animal models used in different studies. In mouse and rat models, curcumin displayed a protective function on ovary and testis under exogenous stress or pathological conditions.⁸ In contrast, growing evidence supported that curcumin would adversely affect female and male reproduction. For instance, in vitro studies revealed that incubating murine sperms with curcumin would significantly suppress the sperm motility, capacitation, acrosomal reaction, as well as the in vitro fertilization outcomes.^{9,10} Similarly, when incubated with curcumin, the motility of human spermatozoa was decreased.^{10–12} In male mice and rats, curcumin exhibited the antifertility functions in vivo.^{13–15} Particularly, curcumin possessed the potential androgen-antagonizing property, as it could down-regulate the expression of human *androgen receptors*,¹⁶ *HSD17B3*,¹⁷ *HSD3B2* and *CYP11A1* genes,¹⁸ as well as the mouse *Cyp11a1* and *Star* genes.¹⁹

To summarize, the multifaceted bioactivities of curcumin contribute to its diverse effects on the reproductive systems. The potential reproductive toxicity of curcumin is also dependent on the dosage, preparations and delivery approaches. Along with the rise of the researches on nanoformulated curcumin, it is essential to analyze the reproductive safety of nano-curcumin carefully. In the present study, we prepared curcumin-loaded poly (lactic-co-glycolic acid) nanoparticles,²⁰ tried to evaluate its influence on testicular cell viability in vitro, as well as the short-term impact on spermatogenesis and sperm parameters in mouse model.

Materials and Methods

Curcumin-Loaded PLGA Nanoparticles Preparation

Curcumin was purchased from Sigma-Aldrich (St Louis, USA). Curcumin-Poly (lactic-co-glycolic acid) (PLGA)-PEG nanoparticles (Cur-PLGA-NPs for short) were prepared by double-emulsion method.²⁰ The identical concentration of free curcumin or PLGA nanoparticles (PLGA-NPs for short) was used as controls for in vitro and in vivo experiments.

Cell Culture and Proliferation Assay

Mouse Sertoli cell line TM4 and Leydig cell line TM3 were obtained from the Cell Bank of Chinese Academy of Sciences, Shanghai, China. TM4 and TM3 cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL

penicillin/streptomycin at 37°C in a humidified 5% CO₂ incubator. Spermatogonial stem cell line C18-4 were cultured in DMEM/F12, supplemented with 10% FBS, 2 mM L-glutamine and 50 U/mL penicillin/streptomycin in a humidified incubator at 34°C.²¹

Uncapsuled curcumin was diluted to 0.5 M in DMSO, stored at –20°C until use. Then, curcumin or PLGA-NPs or Cur-PLGA-NPs were diluted into the culture media to the final concentration indicated. After the treatment, the cell proliferation MTS assay was performed according to the manufacture's protocol (GENMED, GMS10043, China). The absorbance of each well was measured at 540 nm using Multiskan™ GO (Thermo Fisher Scientific, USA). The treatment was performed in triplicate and repeated three times.

Electron Microscopy Analysis

TM4 cells were cultured with 50 µM PLGA-NPs or Cur-PLGA-NPs for 48 hrs. For electron microscopy analysis, the cells were collected and fixed with glutaraldehyde, postfixed in reduced osmium, dehydrated, and embedded as previously described.²² The sections were examined under CM-120 transmission electron microscope (PHILIPS, Eindhoven, Netherlands) at 80 kV.

Design of Animal Experiment

All experiments were conducted following the Guide for the Care and Use of Laboratory Animals of National Institutes of Health. This study was approved by the animal ethics committee of Shanghai Jiao Tong University School of Medicine (DAS-A-2016-038). Male BALB/c mice were purchased from the Shanghai Laboratory Animal Center and hosted in the Animal Center of Shanghai Jiao Tong University School of Medicine under the standardized SPF conditions.

Six-week mice were randomly divided into two groups (n=6 per group) as follows: Group 1, PLGA-NPs; Group 2, Cur-PLGA-NPs. For Group 2, mice were injected 20 mg/kg body weight (~55 mM/kg b.w.)^{8,13} of Cur-PLGA-NPs in saline via tail vein. For Group 1, mice were injected 20 mg/kg body weight of PLGA-NPs. The injections were executed for continuous 10 days.

After the treatment, blood was obtained from the retro-orbital venous plexus and used for testosterone measurement assay (n≥4 per group). Then, mice were sacrificed, the weight of testes, epididymis and seminal vesicles were recorded, as well as the body weight (n≥4 per group). Sperms were collected immediately and subjected to the

computer-assisted sperm analysis (CASA) ($n=5$ per group). At the same time, testes were fixed in Bouin's solution for subsequent haematoxylin-eosin (H-E) staining, TdT-mediated dUTP Nick-End Labeling (TUNEL) and immunohistochemistry assays.

Testosterone Measurements

After in vivo treatment to the mice, the testosterone levels in serum were measured using the immunoassay kit (R&D Systems, Minneapolis, USA) according to the manufacturer's protocols.

CASA Analysis

The cauda epididymis was dissected from an individual mouse and then incubated in Tyrode's Solution (Sigma-Aldrich, St Louis, USA) at 37°C for 15 min to release the sperms. The supernatant was collected, sperm counts and motility were evaluated using the computer-assisted sperm analysis (CASA) system (Hamilton Thorne, Beverly, USA).

Histopathological Examination

Five-micrometer-thick serial paraffin sections were prepared and H-E staining was executed by standard protocols. The images were captured under ECLIPSE E600 microscope (Nikon, Tokyo, Japan) and analyzed by Image-Pro Plus 6.0. The relative volume (%) of lumen was evaluated according to the previous publications.^{23,24} The staging of seminiferous tubules was determined,²⁵ 200 tubules from each group were counted and the percentage of every spermatogenic stage was quantified.

TUNEL Assay

The paraffin sections were incubated in buffered citrate (pH 6.0) for 15 mins at 105°C. TUNEL analysis was performed

using in situ Cell Death Detection Kit (Roche, Indianapolis, USA) according to the manufacturer's instructions. Tubules from every group were counted and the percentage of the TUNEL positive tubules was quantified, as well as the average number of apoptotic cells per tubule.

Immunohistochemistry

For antigen retrieval, the paraffin sections were incubated in buffered citrate (pH 6.0) for 15 mins at 105°C and then soaked in methanol containing 0.3% H₂O₂ to neutralize endogenous peroxidase activity. For primary antibody incubation, the sections were incubated with 1:50 rabbit anti-Pan-Acetylated Histone4 antibody (Abcam, ab10807, Cambridge, USA) or normal rabbit IgG (negative control) at 4°C overnight. On the following day, the signals were visualized using a Histostain-Plus Kit, DAB (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis data are expressed as mean \pm SEM or as proportions. For continuous variables, the differences between groups were analyzed by one-way ANOVA, followed by Student's *t*-test. For categorical variables, Pearson's χ^2 test was used to analyze the correlation between variables. $P<0.05$ was considered to be statistically significant.

Results

The Cytotoxicity of Cur-PLGA-NPs on Testicular Cell Lines

In the previous study, we have proved that curcumin affected the growth of spermatogonial stem cell line

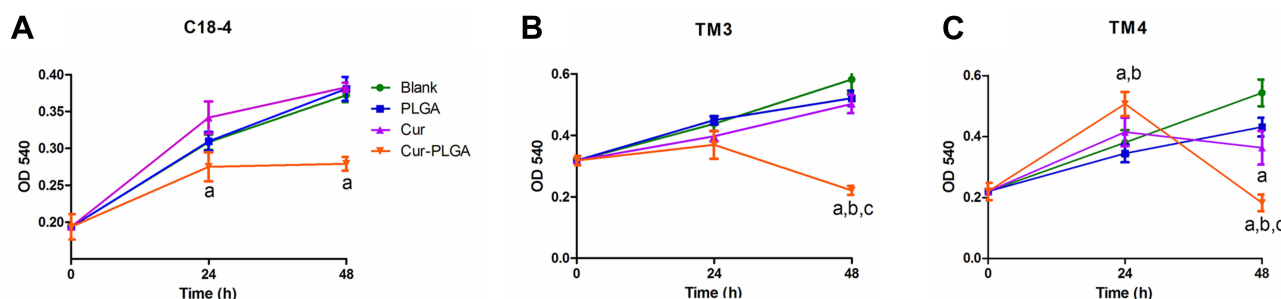


Figure 1 Effects on proliferation of testicular cell lines by Cur-PLGA-NPs treatment. Blank, untreated; PLGA, 50 μ M PLGA-NPs treatment; Cur, 50 μ M curcumin treatment; Cur-PLGA, 50 μ M Cur-PLGA-NPs treatment. (A) Effects on proliferation of C18-4 spermatogonia cell line by 50 μ M curcumin or Cur-PLGA-NPs treatment for continuous 48 h. (B) Effects on proliferation of TM3 Leydig cell line by 50 μ M curcumin or Cur-PLGA-NPs treatment for continuous 48 h. (C) Effects on proliferation of TM4 Sertoli cell line by 50 μ M curcumin or Cur-PLGA-NPs treatment for continuous 48 h. a, $P<0.05$ when compared to Blank control. b, $P<0.05$ when compared to PLGA-NPs vehicle control. c, $P<0.05$ when compared to uncapsulated Curcumin group.

C18-4 in a dose-dependent manner. C18-4 cells treated with 50 μM curcumin entered into the apoptotic process after 48 h.²⁶ In the present study, we prepared curcumin-PLGA nanoparticles (Supplemental Figure 1), considering the biosafety of PLGA nanoparticles has been verified convincingly.^{27,28} The average size and surface charge (zeta-potential) of the Cur-PLGA-NPs were 208.0 nm and 0.161 ± 3.45 mV, respectively. Firstly, we evaluated the cytotoxicity of Curcumin-PLGA nanoparticles to testicular cell lines, including C18-4, Leydig cell line TM3 and Sertoli cell line TM4. As shown in Figure 1, the given treatment of PLGA-NPs had no significant effect on the growth of cells. However, comparing to blank control, PLGA-NPs and curcumin, 50 μM Cur-PLGA-NPs treatment exhibited the obvious negative impact by 48 h incubation ($P < 0.05$) (Figure 1A–C). These results confirmed that nano-curcumin exhibited stronger biological activity than naked curcumin.

Among the cell lines we tested, TM4 seemed more sensitive to the curcumin/Cur-PLGA-NPs treatment. Interestingly, the proliferation of TM4 cells treated with Cur-PLGA-NPs was promoted by 24 h, but declined rapidly by 48 h (Figure 1C). The electron microscope observation revealed that, in the 50 μM PLGA-NPs group, there were continuous cell junctions formed between the TM4 cells, highlighted by the anchoring junctions (Figure 2A, arrowhead). In contrast, after the 50 μM Cur-PLGA-NPs treatment for 48 h, the anchoring junctions were disappeared and the TM4 cells were detached (Figure 2B). Furthermore, a considerable amount of the TM4 cells had gone into the program of the apoptosis, showing the characteristics of nuclei fragmentation and chromatin condensation, as well as the vacuolus and degenerated organelles in the cytoplasm (Figure 2C and D).

These results implied that Sertoli cells would probably be damaged by Cur-NPs delivery in vivo, which could be a risk factor to the blood-testis barrier (BTB) and the spermatogenesis. In order to elucidate this question, we applied a moderate treatment of 20 mg/kg body weight^{8,13} Cur-PLGA-NPs in the following tests, by tail intravenous injection for continuous 10 days, with the PLGA-NPs used as control.

A Short-Term Treatment of Cur-PLGA-NPs Reduced the Sperm Motility in vivo

As shown in Figure 3, after 10-day in vivo treatment, the weight of sex organs, the level of testosterone and the quality

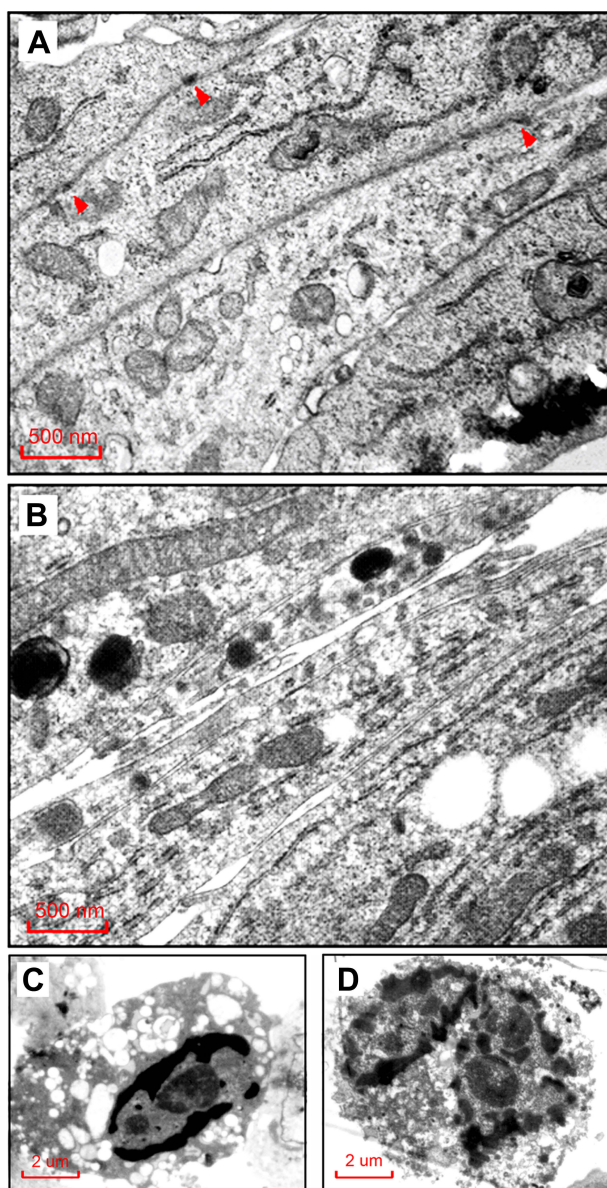


Figure 2 Morphologic changes of TM4 cells by Cur-PLGA-NPs treatment under transmission electron microscopy. (A) Control group treated by 50 μM PLGA-NPs, arrowhead indicating the anchoring junction structure. (B) Experimental group treated by 50 μM Cur-PLGA-NPs, no anchoring junctions were found. Bar = 500 nm. (C–D) TM4 cells in experimental group showing typical signs of apoptosis, including chromatin condensation, nuclei fragmentation and degenerated organelles. Bar = 2 μm .

of semen were measured in Cur-PLGA-NPs delivered male mice. Comparing to the PLGA-NPs controls, the gross weight of sex organs was unchanged (Figure 3A), as well as the serum concentration of testosterone (Figure 3B), which infer the normal function of Leydig cells. However, there was a slight inclination of the decreased sperm concentration in the Cur-PLGA-NPs treated group (Figure 3C). Meanwhile, the CASA analysis disclosed the depressed total motility of sperms after the short-term Cur-PLGA-NPs

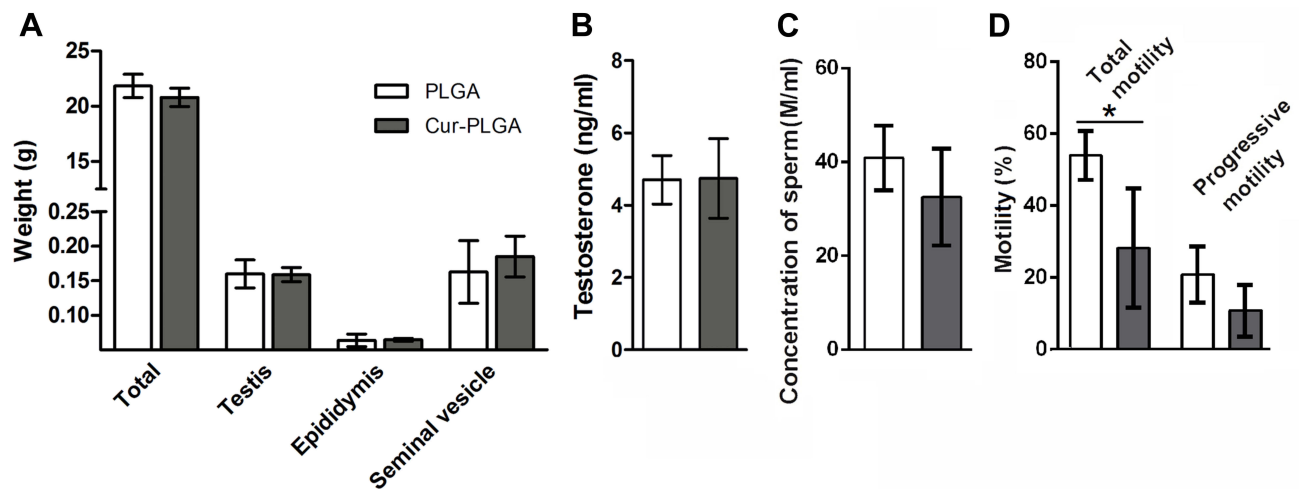


Figure 3 Impaired sperm motility by Cur-PLGA-NPs treatment in vivo. **(A)** Weight of body, testis, epididymis and seminal vesicle. **(B)** Serum testosterone level. **(C)** Sperm concentration and **(D)** Sperm motility. * $P < 0.05$.

treatment ($P < 0.05$), and the relatively lower progressive motility (Figure 3D).

Cur-PLGA-NPs Disturbed the Progress of Spermatogenesis

Consistently, the pathological analysis of testes provided interesting evidence. Although the average weight of the testis was not different between the two groups, the lumen of the seminiferous tubules was visibly enlarged in the Cur-PLGA-NPs treated ones (Figure 4A–C, Figure 6B). The relative volume of the tubular lumen rose from 12.78% in the control group to 17.11% in the Cur-PLGA-NPs treated group ($P < 0.01$) (Figure 4B). This phenotype was caused by the general loss of cell layers in the spermatogenic epithelia, which could be found throughout all the existing stages of the spermatogenesis (Figure 4C).

Another phenomenon was the abnormal proportion of given stages during the spermatogenesis process. The staging of seminiferous tubules was determined according to the previous publications,²⁵ especially based on the characteristics of the developing spermatids. To quantify the percentage of each spermatogenic stage, at least 200 seminiferous tubules from 3 different mice were counted in each group. Surprisingly, the proportion of Stage IV–V and Stage VIII–IX were severely affected by the Cur-PLGA-NPs treatment, since the tubules at Stage IV–V were reduced, but the ones at Stage VIII–IX were increased (Figure 5A).

In fact, some of the tubule sections from the Cur-PLGA-NPs group could not be categorized into any spermatogenic stages, on account of the misarranged cell type combination

(Figure 5B, I–II). Additionally, the vacuole in the basal compartment (arrowhead) implied the faulted BTB structure, and the multinucleated giant cells (arrow) were indications of the cellular apoptosis (Figure 5B, III–IV).²⁹

In that way, we then performed the TUNEL analysis on the testicular tissues. As shown in Figure 6, compared to the testicular tissue from the PLGA-NPs group (Figure 6A), apoptotic spermatogenic cells were remarkably accumulated in the Cur-PLGA-NPs group ($P < 0.01$) (Figure 6B and E), especially the apoptotic spermatogonia (arrow) and early spermatocytes (Figure 6C and D). Taken together, a short-term in vivo treatment of Cur-PLGA-NPs would cause the acute injury to the testis.

Histone Acetylation Was Downregulated in Spermatogenic Cells After Cur-PLGA-NPs Treatment

One of the well-known properties of curcumin is the inhibition activity on kinds of histone acetylase (HATs).^{30,31} Herein, we investigated the histone acetylation expression after in vivo treatment of Cur-PLGA-NPs. In PLGA-NPs group, the signal of acetylated histone 4 (ACh4) was detected in the intermediate (arrowhead) and Type B (arrow) spermatogonia, as well as in the daughter leptotene spermatocytes (asterisk) (Figure 7, upper panel). In contrast, the ACh4 signal was diminished in the spermatogonia from the Cur-PLGA-NPs group (Figure 7, lower panel). Particularly, the progress of spermatogonia differentiation seemed blocked in some tubules from the Cur-PLGA-NPs group (Stage VI', VIII'). Furthermore, the

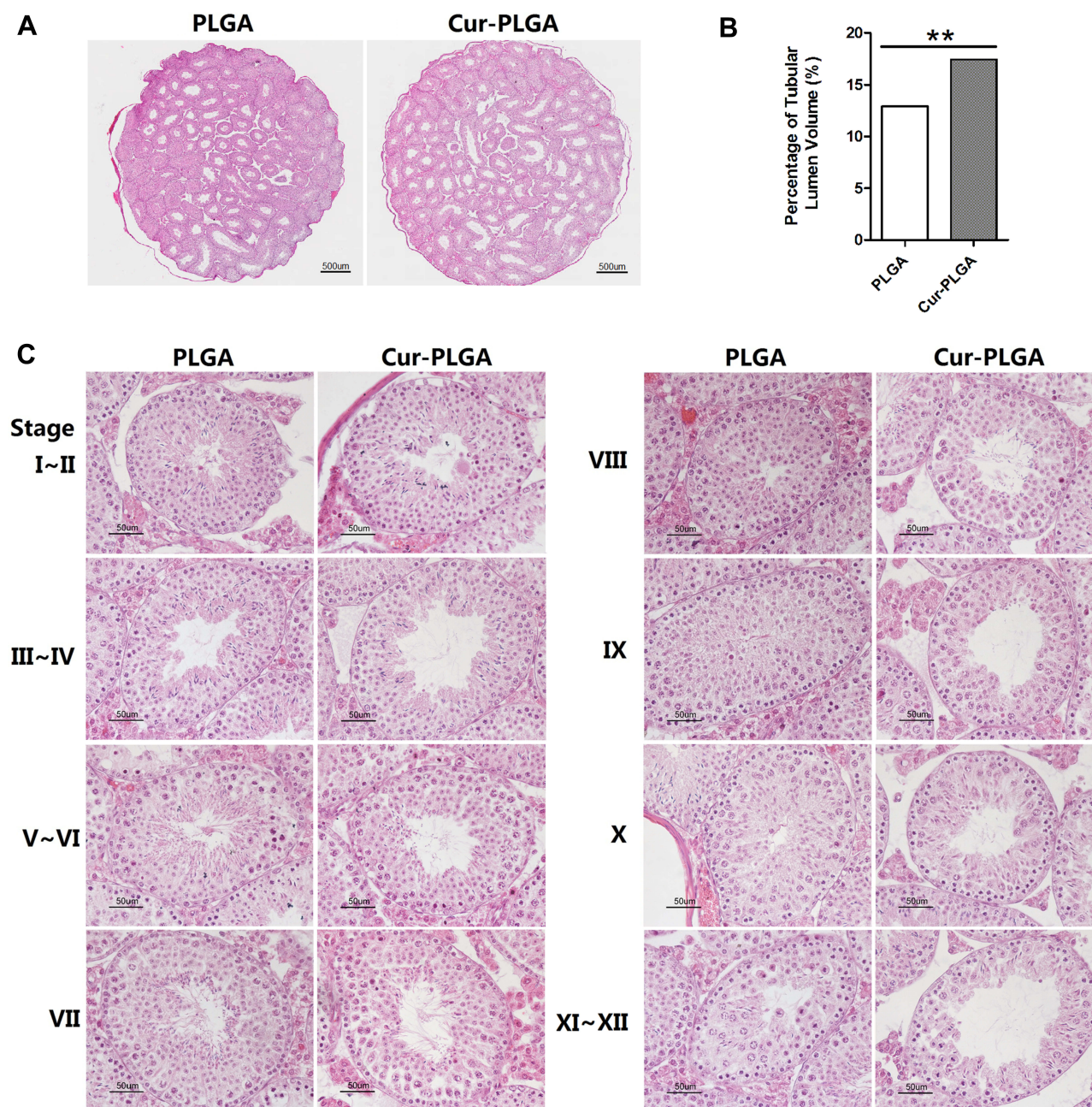


Figure 4 Disrupted structure of seminiferous epithelia by Cur-PLGA-NPs treatment. **(A)** Representative macroscopic images of testicular sections. Bar = 500 μ m. **(B)** Relative volume (%) of tubular lumen. ** P < 0.01. **(C)** Morphologic changes in different stages during spermatogenesis. Bar = 50 μ m.

typical hyperacetylated signal in elongating spermatids was dramatically weakened (Stage X', XI'), which would impede the maturation steps and contribute to the lower concentration/quality of sperms.

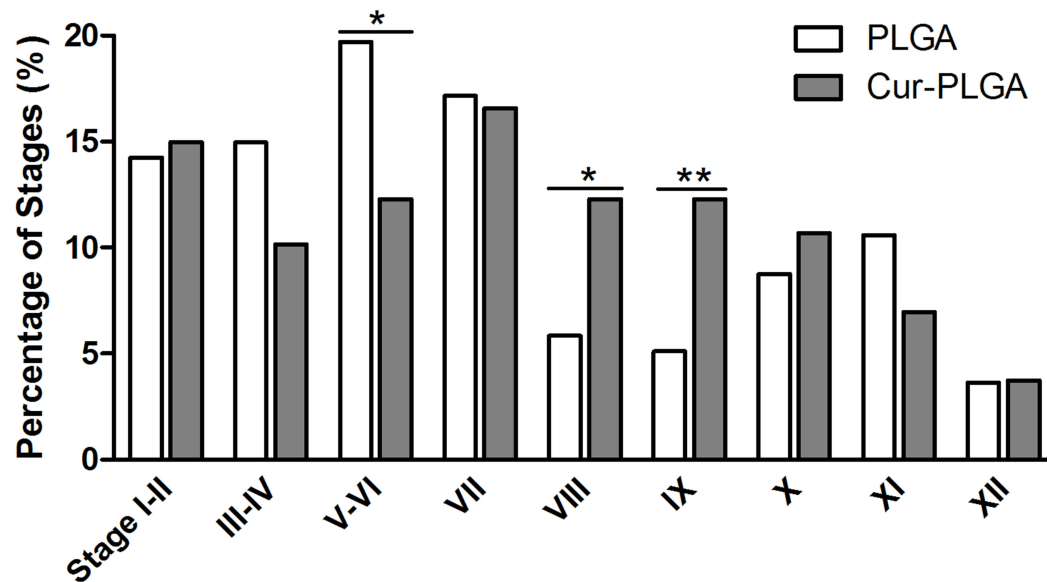
Discussion

Blood–testis barrier is mainly constituted by the tight junctions between the adjacent Sertoli cells, which is crucial to the homeostasis in the testis and the process of spermatogenesis.³² Noteworthy, there have been increasing evidence on

nanoparticles penetrating the blood–testis barrier successfully and demonstrating the potential adverse effects on the reproductive system.^{33–36} It put forward the emergent question that whether the nanoformulated agents might induce the unintended reproductive dysfunction.

Curcumin is a prominent biomolecule in the field of drug development. The nano-based drug delivery system has effectively optimized the aqueous solubility and bioavailability of curcumin. Ahmed-Farid OAH reported that, 5 mg/kg b.w. curcumin nano-emulsion showed significant

A



B

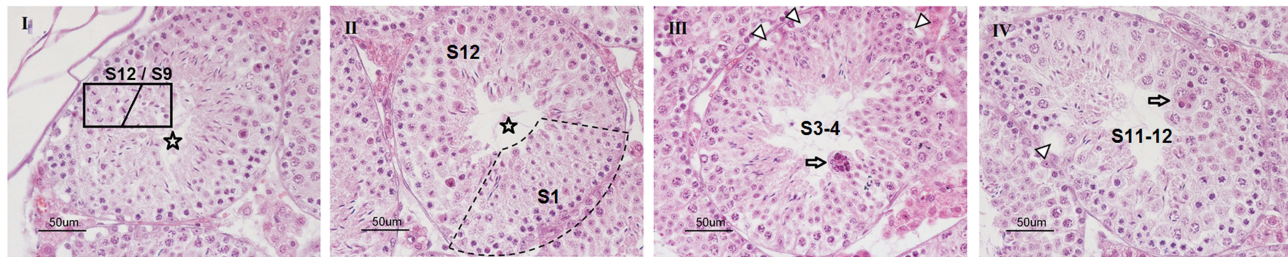


Figure 5 Disturbed spermatogenic progress by Cur-PLGA-NPs treatment. **(A)** Analysis of the proportion of spermatogenic stages. * $P < 0.05$, ** $P < 0.01$. **(B)** Disorder in the seminiferous epithelia. Asterisk, tubules can not be classified; arrow, multinucleated giant cell; arrowhead, vacuole. Bar = 50 μ m.

amelioration effects against the disrupted reproductive performance in rats induced by protein-deficient diet.³⁷ In a randomized clinical trial, Alizadeh F reported that daily uptake of 80 mg curcumin nanomicelle for 10 weeks would improve the semen parameters in infertile men.³⁸ On another hand, there have been studies focusing on the possible reproductive toxicity of nanoformulated curcumin. Using a rat model, Moshari S discovered that, a 48-day oral administration of 30 mg/kg b.w. of nanomicelle curcumin could induce DNA fragmentation in testis, accompanied by the suppression of the p53 and PCNA-related apoptotic program.¹⁴ Later, this group further testified that a 48-day nanomicelle curcumin treatment would negatively affect the spermatogenesis, sperm parameters and in vitro fertilization potential in rats.¹⁵ In our results, the curcumin-loaded nanoparticles demonstrated a more profound biological effect than the uncapsuled curcumin in vitro (Figures 1 and 2). We then delivered the dosage of 20 mg/kg b.w. curcumin-PLGA nanoparticles to male

mice via intravenous injection for continuous 10 days that the administration via blood circulation could evade the issue of oral bioavailability and enhance the drug utilization. Strikingly, our results suggested that (Figures 3–6), the Cur-PLGA-NPs treatment, even for a short time, might endanger the blood-testis barrier function, be harmful to the spermatogenesis and sperm motility, ultimately the male fertility.

Generally, the spermatogenic cycle cost 35 days in mice, which spreading asynchronously along the seminiferous tubules.³⁹ In our study, we observed the halted development of existing spermatogonia and elongating spermatids in Cur-PLGA-NPs group (Figure 5A, Figure 7). One appropriate interpretation is, the Cur-PLGA-NPs treatment probably caused the retarded transition of Stage II to VI and Stage VIII to X in the ongoing spermatogenic cycles. It leads to the reasonable assumption that, an accumulated, long-term treatment of nano-curcumin could eventually deplete the spermatogenic cells in the seminiferous tubules.

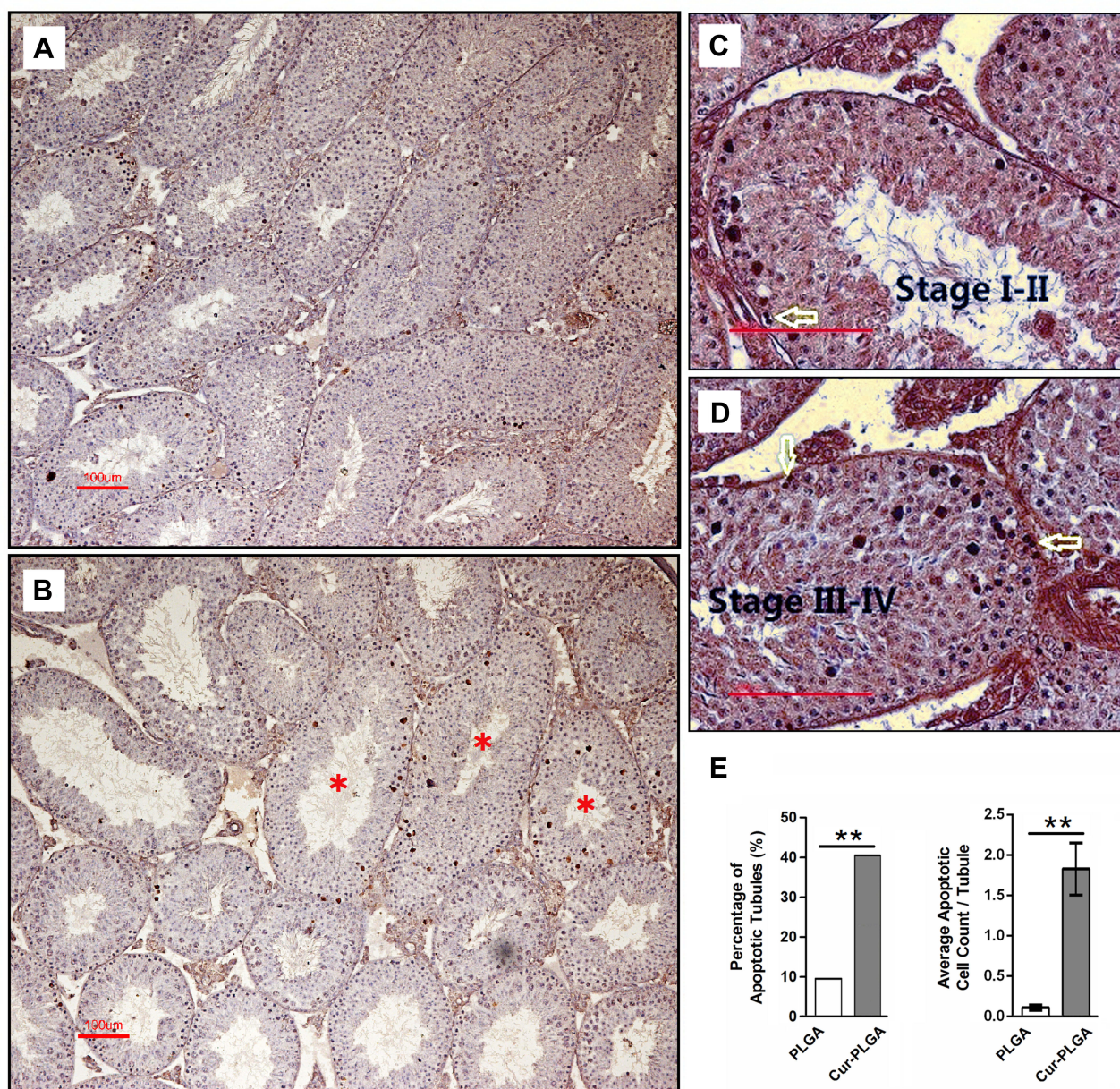


Figure 6 Induced apoptosis of spermatogenic cells by Cur-PLGA-NPs treatment. (A) Control group treated by 50 μ M PLGA-NPs. (B–D) Experimental group treated by 50 μ M Cur-PLGA-NPs. Asterisk, the representative affected tubules; arrow, spermatogonia. Bar = 100 μ m. (E). Quantitative measurement of apoptotic events. ** P < 0.01.

Mechanically, we detected the suppression of the histone 4 acetylation after Cur-PLGA-NPs treatment (Figure 7, lower panel), which is accordant to the reported activity of curcumin as HAT inhibitor.^{30,31} During spermatogenesis, the acetylation of histones is mainly happening in the spermatogonia under differentiating, as well as in the elongating spermatids. Importantly, the global hyperacetylation of histone 3 and 4 was found in mouse, rat and human elongating spermatids, critical to the later histone replacement and chromatin condensation.^{40,41} In Cur-PLGA-NPs group, the AcH4

signal was decreased in elongating spermatids, which implies the failure of spermatid maturation. However, considering the complexity of curcumin bioactivities, the mechanism of damage induced by curcumin-loaded nanoparticles in vivo should be cautiously analyzed.

In conclusion, our results revealed that curcumin-loaded nanoparticles might cause an acute injury to the testicular functions in male mice. There are extended questions as, whether the reproductive toxic effect of nano-curcumin is transient and reversible, and how to reduce the damage caused.

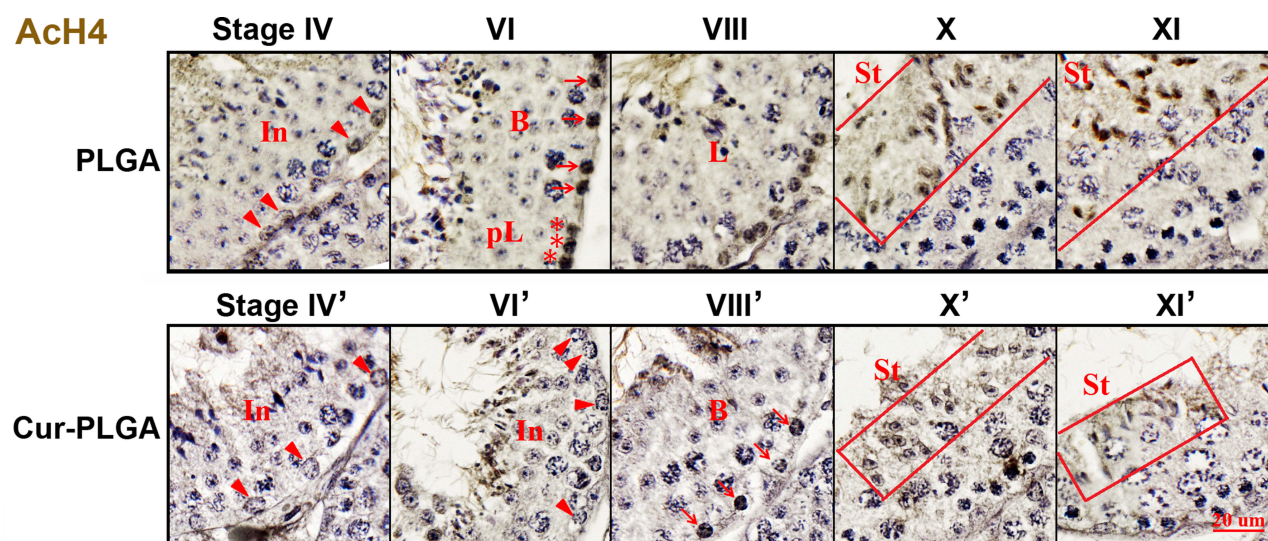


Figure 7 Inhibition on acetylated histone 4 expression by Cur-PLGA-NPs treatment. In (arrowhead), intermediate spermatogonia; B (arrow), B type spermatogonia; pL (asterisk), preleptotene spermatocytes; L, leptotene spermatocytes; St, elongating spermatids. Bars=20 μ m.

It is also interesting and meaningful to evaluate the influences of nano-curcumin on female fertility in vivo. To sum up, the reproductive toxicity of nanoformulated curcumin needs to be evaluated thoroughly before its therapeutic applications.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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