Article Type: Original Article

The Effects of L-carnitine against Cyclophosphamide-induced Injuries in Mouse Testis

Yuanchao Cao[1], Xinsheng Wang[2], Shenqian Li[2], Hongqiang Wang[2], Lei Yu[2] and Peitao Wang[2]

[1] Department of Urology, Affiliated Hospital of Qingdao University, Qingdao, Shandong 266001, China

[2] Department of Andrology, Affiliated Hospitalof Qingdao University, Qingdao ,Shandong, 266001, China

Author for correspondence: Peitao Wang, Department of Andrology, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao 266001, P.R. China (e-mail: PeitaoWang126@126.com).

(Received 6 April 2016; Accepted 22 August 2016)

Abstract: In order to explore the possibility of L-carnitine (LC) as a protector of male fertility in chemotherapy, we observed the damage of cyclophosphamide (CTX) to Sertoli cells and the protective effect of LC on the testis Sertoli cells from such damage in this study. Healthy adult male mice were divided into three groups. Chemotherapy group were

injected intraperitoneally with the CTX; Protective agent group were injected both LC and CTX; Control group mice were injected only with isochoric physiological saline; all once a day for 5 days. After 5 days, the mice were respectively killed at 24 hr after the last injection. The testis and epididymis were removed. Epididymis was for sperm analysis immediately, and immunohistochemistry, RT-PCR and Western blot for the assessments of occludin, GDNF and TGF-β3 mRNA and protein expression. The sperm analysis of epididymis showed that CTX can significantly decrease sperm count and motility; and administration of LC resulted in significant recovery of the sperm count and sperm motility. Compared with control group, the expressions of occludin and GDNF decreased and the expression of TGF-β3 increased significantly ($P<0.05$) in the CTX group. In the LC+CTX group, the expressions of occludin and GDNF were higher than those of the CTX group and similar to thos of the control group; the TGF-β3 expression was lower ($P<0.05$) than that of the CTX group and similar to that of the control group. The results of this study showed that CTX could damage the spermatogenesis and reduce the expression of occludin and GDNF, and increase the expression of TGF-β3 in testis of mouse, which indicates CTX's damage or efficacy to testis Sertoli cells. LC could protect the Sertoli cells of testis from these damages caused by CTX, and promote or protect the spermatogenesis. In conclusion, this study provides meaningful information about the

possible damage to male fertility by chemotherapeutics and potential of LC in the protection of male fertility during chemotherapy.

Keywords: cyclophosphamide(CTX), L-carnitine(LC), Sertoli cells, occludin, GDNF, TGF-β3

INTRODUCTION

Gonadal toxicity is a valued problem in chemotherapy of patients with various malignancies. With the development of diagnosis technology and treatment, the survival rate of many malignancies has increased rapidly in recent decades. A subsequent awkward situation that the survivors have to face is infertility or sub-fertility caused by chemotherapy[1]. This problem may lead to great physical and emotional pressure for these patients, and also greatly impact their family life thereafter.

CTX is a widely used chemotherapeutic alkylating agent. Its alkylating metabolite, phosphoramide mustard, is responsible for the therapeutic activity, and produces a wide range of adverse effects including testicular toxicity[2]. Animal studies have shown that it can lead to a decreased testicular weight, transitory oligospermia[3], decreased DNA synthesis in spermatogonia and protein synthesis in spermatids[4]. The mechanism by which CTX causes these damages is not well

explored. Some reports showed that CTX could disrupt the redox equilibrium of tissues, which suggests that the biochemical and physiological disturbances may result from oxidative stress[5]. Biological compounds with antioxidant properties may protect cells and tissues from deleterious effects of reactive oxygen species (ROS) and other free radicals generated during CTX exposure[6]. L-carnitine(LC), a vitamin substance occurring naturally, also known as Vitamin BT, plays an important role in energy metabolism of the human body. LC could eliminate free radicals, protecting sperm cells from oxidative damage[7].

Within the seminiferous epithelium of the testis, somatic Sertoli cells are known as the 'nurturing cells' by aiding germ cells in their development, proliferation and maturation during spermatogenesis. They separate the seminiferous epithelium into a basal and an adluminal compartment through the formation of the blood–testis barrier (BTB) that is located around the basal of the seminiferous tubule. To evaluate the functional state of Sertoli cells associated with spermatogenic damage during chemotherapy in this study, we examined the expressions of several proteins of Sertoli cells, including occludin, transforming growth factor-β3 (TGF-β3) and glial cell-derived neurotrophic factor (GDNF); Occludin is an integral plasma-membrane protein located at the tight junctions (TJs). Occludin is a series of bands between 62 and 82 kDa, depending upon the degree of phosphorylation. Together with the group of

proteins, it is the main component of the TJs. The regulation of occludin function is known to be greatly influenced by phosphorylation of Serine (Ser), Threonine (Thr), and Tyrosine (Tyr) residues in the cytoplasmic tail[8]. TGF-β3 is an important regulating factor of Sertoli cell TJ kinetics[9]. Supplementation with recombinant TGF-β3 may interfere with well-formed Sertoli cell TJ barrier *in vit*ro[10]. TGF-β3 is known to have multipotent functions controlling cell proliferation, differentiation and apoptosis, as well as cell–cell and cell–extracellular matrix interactions[11]. TGF-β3 apparently was up-regulated at the time of germ cell detachment from the epithelium and may have mediated its effects to perturb anchoring junctions in the testis via the Ras/MEK/ERK pathway[12]. GDNF was the first molecule known to mediate the self-renewal and differentiation of undifferentiated spermatogonia in a dose-dependent manner[13, 14]. Secreted by Sertoli cells after birth, it is an important growth factor that facilitates communication between Sertoli cells and spermatogonia. GDNF induces the proliferation of undifferentiated spermatogonia under *in vivo* and *in vitro* conditions[15] and stimulates DNA synthesis in spermatogonia[16]. Tadokoro and colleagues established that the expression of GDNF by Sertoli cells and the subsequent proliferation of SSCs were dependent on FSH[17] and also dependent on Fgf2, tumor necrosis factor alpha (Tnfα) and interleukin-1β (Il-1β) *in vitro*[18].

Whereas the effect of LC on CTX-induced injuries in Sertoli cell of mouse testis has not been studied, this research was aimed to evaluate the effect of LC on mice semen parameters such as average number, motility, viability and mRNA, protein expressions of target protein.

MATERIALS AND METHODS

1. Animals and interventions

Sixty healthy adult (8-10 weeks) male Kunming mice were purchased from the Experiment Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 20130010), weighing 30-35g and fed with normal forage for 7 days. The mice were randomly divided into 3 groups: (1) control group, 20 mice; (2) CTX group, 20 mice (3) LC+CTX group, 20 mice. For the CTX group, the mice were injected intraperitoneally with CTX once a day for 5 days. For the LC+CTX group, the mice were injected intraperitoneally with LC and CTX once a day for 5 days, and LC injection was 30 min. earlier than CTX injection. In the control group, mice were injected intraperitoneally with an equivalent amount of normal saline.

CTX and LC were obtained from Sigma and dissolved in a 0.9% saline solution for intraperitoneal injection. The dosage of CTX and LC was calculated with the formula: $dB = dA \times (RB / RA) \times (WA / WB)^{1/3}$ [19]. dB: mouse amount ($mg \cdot kg^{-1}$), dA: human dosage ($mg \cdot kg^{-1}$), RA: body shape coefficient for the 100, RB: mouse shape factor, as 59; WA: adult weight, calculated according to 60 kg; WB: mouse body weight, calculated

according to 35 g. Human therapeutic dosage is 16mg·kg^{-1}. It was calculated that the mouse intraperitoneally dosage is 100mg·kg^{-1}. LC dosage is 100mg·kg^{-1}.

The animals were killed by cervical dislocation, and the testes and epididymis were dissected and processed at 24 hr after the last injection. The testes were immediately rinsed with ice-cold physiological saline, cleared of the adhering tissues and rinsed with ice-cold physiological saline. The testes were stored at -80°C until use.

2. Determination and analysis

2.1 Assessment of spermatozoon motility and viability : The caudal epididymis was dissected and placed in modified Hank's balanced salt solution (M-HBSS) for 10 min. at 37°C. The solution was then gently filtered through nylon gauze and centrifuged for 5 min. at 800 r/min. The deposit was re-suspended in 1 ml of M199 medium (Abcam Chemical, USA). 10 ul of spermatozoon suspension was uniformly smeared on to sheet glass, and the mean number of viable spermatozoa in at least four of the random squares was counted. The computer-assisted spermatozoon assay (CASA) with a spermatozoon motility analyzer (Weili Medical Treatment, China) was used to assess spermatozoon motility. Those with fast progressive motility (greater than 20 μm/sec.) were labelled as Level A, and those with modest motility (less than 20

μm/sec, greater than 5 μm/sec) were marked as Level B.

Spermatozoon viability was visualized by eosin and nigrosin staining. Briefly, the spermatozoon suspension was mixed with 1% eosin (0.01 g/ml) and 10% nigrosin (0.1 g/ml), and a smear of the mixture placed on a clean glass slide, which was allowed to air-dry. The slide was then observed by light microscopy. Those stained pink were interpreted as dead spermatozoa and those unstained live spermatozoa. The viability of spermatozoa was expressed as a percentage.

2.2 Immunohistochemistry staining: occludin(ab31721, Abcam), TGF-β3 (ab15537, Abcam), GDNF(ab18956, Abcam), SABC immunohistochemistry kit (SP-90001, ZSGB-BIO). Firstly, paraffin sections were dewaxed and incubated 3%H_2O_2 for 10 min., soaked by PBS for 5 min. Reagent A was dropped on the section to incubate at room temperature for 10 min., and washing with PBS for 5 min. × 3 times. Then, respectively, primary Occludin (1:1000), TGF-β3 (1:500), and GDNF (1:1000) were added to incubate at 37℃ for 1 hr, PBS washing 5 min. × 3 times. Secondly, reagent B was added to react at 37℃ for 15 min., PBS douching 5 min. × 3 times. Thirdly, reagent C was added to react at 37℃ for 10 min., PBS douching 5 min. × 3 times. Finally, the sections were coloured by DAB chromogenic reagent and re-stained by hematoxyllin. The positive cells could be seen under microscope with their cytopoasm or membrance stained brown.Negative control sections

were stained with 0.1mol/L PBS instead of without primary antibody and no colour appeared. Under optical microscope magnified 400 times, five non-overlapping visual fields in each section were randomly selected to observe under optical microscope of 400 times magnifications, and then the absorbance value of occludin, TGF-β3 and GDNF expression by Image-Pro Plus software were analysed. The occludin, TGF-β3 and GDNF expression intensity were presented by the positive cell absorbance value subtracting the background.

2.3 RT-PCR: Trizol extraction kit was purchased from Invitrogen Co. Ltd. Firstly, 1ml Trizol solution was added into 100 mg testis tissue, standing 5 min. at 4℃, and then centrifuged at 12000r/min for 15 min. to separate the supernatant into sterile EP tube. Secondly, 0.2ml chloroform was added, mixed for 15 sec. at room temperature (22℃) and then centrifuged at 12000r/min for 15 min. to separate the colourless aqueous phase into another sterile EP tube. Thirdly, 0.5 ml isopropanol was added, mixed gently and centrifuged at 12000r/min for 15 min. to discard the supernatant carefully, and then 75% pre-cooled ethanol 1ml was added to wash RNA precipitation and centrifuged (4℃) at 7500r/min for 5 min. Then, the supernatant was carefully discarded to dry about 20 min. in a cupboard (RNA precipitate becomes transparent). Finally, 0.1% DEPC·H2O 100μl was added to dissolve RNA for 10 min. at 57℃ in a water bath. Micro-spectrophotometer (K5500, Beijing Kaiao Tech. Co.,

Ltd) was used to detect RNA abundance. Primers were synthesized by Shanghai Yingjiekai & Co.Ltd.

Semi-quantitative PCR was conducted according to Takara DRR014A PrimeScript™ RT-PCR kit. Primer sequences for Occludin, GDNF, TGF-β3 and β-actin amplification were: Occludin forward primer 5′-TAT GAT GAA CAG CCC CCC AAT-3′, reverse primer 5′-TCA GGC ACC AGA GGT GTT GAC-3′; GDNF forward primer 5′-TTG CAG CGG TTC CTG TGA A-3′, reverse primer 5′-CAT GCC TGG CCT ACT TTG TCA-3′; TGF-β3 forward primer 5′-AAC ACC CTG AAC CCA GAG GC-3′, reverse primer 5′-TCA CCA CCA TGT TGG ACA GCT-3′; β-actin forward primer 5′-GAG ACC TTC AAC ACC CCA GC-3′, reverse primer 5′-ATG TCA CGC ACG ATT TCC C-3′. The reactions were initiated at 95℃ for 5 min., followed by 94℃ for 30 sec., 58℃ (for occludin) or 60℃ (for GDNF) or 61℃ (for TGF-β3) or 55℃ (for β-actin) for 30 sec. and 72℃ for 30 sec., 35 cycles, then followed by 72℃ for 10 min. Electrophoresis: PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The absorbance (A) of interest gene occludin , TGF-β3 , GDNF, and internal reference β-actin were imaged by Vilber Lourmat gel imaging system and analysed by Quantity One software. The occludin, TGF-β3 , GDNF mRNA expression level was presented as a ratio of samples A/β-actin A value. The experiments were repeated at least three times, and results are

presented as mean ± standard deviation ($\bar{x}$±s).

2.4 Western blot: testis tissue was obtained and grinded in homogenizer with RIPA lysis buffer (P0013B, Beyotime Co., Ltd.) on ice, and centrifuged at 12000r/min for 15 min. to separate the supernatant. The concentration of the proteins was determined by enhanced BCA protein assay kit (P0010, Beyotime Co., Ltd.). According to total protein 50 μg, interest protein were separated by 10% SDS-PAGE electrophoresis, transferred to a PVDF membrane and sealed for 1 hr by 5% evaporated skimmed milk. Then, occludin was added (rabbit anti-mouse primary antibody, ab31721, 1:500), TGF-β3(ab15537, 1:500), GDNF(ab18956, 1:1000), β-actin(mouse anti-mouse primary antibody, TA-09, 1:1000), overnight at 4℃, TBST washing 10 min × 3 times. Peroxidase labelling goat anti-rabbit secondary antibodies (Abcam, ZB-2301,1:8000) and horseradish enzyme marker goat anti-mouse lgG (ZB-2305, 1:10000) were added to incubate at 37℃ for 1 hr. The membrane was taken out and washed with TBST for 10min×3times, TBS for 5min×2times. Then,plus A, B liquid developer (1:1) was added and imaged by Vilber Fusion FX7 imaging system. Quantity One software was used to analysis the absorbance value (B) of the interest protein occludin (59kD), TGF-β3(50kD), GDNF(24kD) and internal reference (43kD), and calculate the relative content of occludin (occludin *B*/β-actin B value), TGF-β3 (TGF-β3 *B*/β-actin B value) and GDNF (GDNF B/β-actin B

value).The experiment was repeated 3 times and result is presented as mean ± standard deviation ($\bar{x}$ ±s).

3. Statistical analysis

According to SPSS 20.0 software analysis, data were expressed as mean±standard deviation (x ±s) that multi-groups were compared using analysis of variance (ANOVA) .

RESULTS

1. Sperm anaylsis of epididymis

1.1 Sperm count. The results showed a significant difference between average number of sperms in the CTX group compared to the control group and LC+CTX group ($p<0.05$) (table 1).

1.2 Sperm viability. Results of alive sperms using eosin-nigrosin staining indicated a significant decrease of sperm viability in the CTX group compared with the control group and the LC+CTX group ($p<0.05$) (table 1).

1.3 Sperm motility. The results for mean percentage of motile sperms in the studied groups indicated a significant decrease of this parameter in the CTX group compared with the control and LC+CTX groups ($p<0.05$). Compared with the control group, CTX significantly decreased the Level A and Level B sperm ($P<0.05$) in the CTX group. However, LC can efficiently protect sperm degradation ($P<0.05$) in the LC+CTX group when compared with the CTX group (table 2).

2. Immunohistochemical staining

Seminiferous tubules of mouse testes were severely damaged in the CTX group. There was a progressive loss of occludin and GDNF, while a gain of TGF-β3, immunostaining from its normal intercellular location at the basolateral site of Sertoli cells. (The dyes of occludin and GDNF became light-coloured or absent, while the dye of TGF-β3 became darkened). Immunoreactivity of occludin and GDNF became stronger and more concentrated; TGF-β3 became weaker in the LC+CTX groups.(fig. 1)

3.RT-PCR and Western blot

RT-PCR and Western blot were performed to determine the effects of CTX and LC on occludin, GDNF and TGF-β3 in mouse testes. After being normalized according to the β-actin signal, CTX significantly decreased the expressions of occludin, GDNF mRNA and protein, increased the expressions of TGF-β3 mRNA and protein in the CTX group as compared with the control group (*P<0.05*). LC significantly improved the occludin, GDNF mRNA and protein expressions levels, decreased signifcantly the TGF-β3 mRNA and protein expressions levels in the LC+CTX group when compared with the CTX group. (*P<0.05*; fig. 2 and 3)

DISCUSSION

Chemotherapy for cancer treatment may result in a temporary or long-term gonadal damage resulting in sub-fertility or infertility. Chemotherapeutics kill or destroy the body cells by two main ways: one is to disrupt the normal DNA synthesis of dividing cells undergoing meiosis and mitosis by their metabolites[20]; another is to generate reactive oxygen species to improve the apoptosis or death of the cells[21]. Studies have shown that CTX can cause a noticeable increase in the apoptotic rate in the testis[22]. Our preliminary data showed that CTX significantly decreased sperm count and motility. Reduction of sperm count was mainly attributed to the beginning of testicular tissue damage.

Carnitines are widely distributed in nature and their potential health benefits have been popularized. It has been found that only the L-isomer was found bioactive and could accelerate lipid metabolism. LC acts as a shuttle of the activated long-chain fatty acids into the mitochondria, where beta-oxidation of the long-chain fatty acids takes place. LC exhibits a wide range of biological activities including anti-inflammatory, neuroprotective and antiapoptotic. LC has been found to have an antiperoxidative effect on several tissues[23]. Pre-treatment with LC attenuates germinal cells damages in the ischaemia-reperfusion rats and prevents a further increase in MDA (malondialdehyde) levels[24].

LC is synthesized from the essential amino acids lysine and

methionine. LC has an antioxidant activity and has been shown to be effective in diseases characterized by increased oxidative stress. LC scavenges superoxide anion radical and hydrogen peroxide. LC stimulates directly the gene and protein expression of endothelial nitric oxide synthase (eNOS) that is known as antioxidant and anti-inflammatory [25].

Studies have identified that LC could protect the spermatogenic process and decrease germ cells apoptosis in the testis[22]. In our study, administration of LC resulted in significant enhancement in sperm count as well as motility. Therefore, we could suggest that LC might have up-regulated the antioxidant status, which in turn degraded CTX-induced damage in testicular tissue and up-regulated the CTX-reduced sperm motility.

In testis, germ cells undergo various stages of division circle and will be easily destroyed on account of its DNA damage. Other somatic cells, mainly including Sertoli cells and Leydig cells, do not divide further and are stable and relatively resistant to many environmental factors, including chemotherapeutics, so they cannot be affected by the DNA synthesis disturbance during chemotherapy. However, more studies showed that these somatic cells were also damaged during chemotherapy[2]. For example, in an animal experiment with CTX intervention, Sertoli cells underwent morphological and functional

disruptions resulting in damage of testis blood barrier and abnormal expression of functional proteins[26]. Further investigation showed that these effects may have resulted from the oxidation stress generated by chemotherapeutics[27], and antioxidants could alleviate or avoid this damage[28]. The exact mechanism has not been completely explained.

In the present study, using immunohistochemistry, RT-PCR and western blotting, we observed the cytotoxicity of CTX on Sertoli cells of mouse testis by the expression of occludin, GDNF and TGF-β3. They are all secreted by Sertoli cells, as extrinsic signals from the surrounding micro-environment play an important role in spermatogenesis. Occludin is the main component of TJs and it plays a role in regulating TJs properties[29]. TGF-β3 might be involved in down-regulating TJs associated proteins occludin, leading to cell junction reduction[30]. It has previously been reported that changes in TGF-β3 expression relate to various disease states[31] and in injury repair processes[32]. Studies in *in vivo* and *in vitro* models have shown that $Cdcl_2$-induced TJs damages were related to increases in TGF-β3 mRNA and protein levels in testicles[33]. GDNF, which is secreted by Sertoli cells, was implicated in spermatogonial self-renewal because GDNF+/- mice show gradual spermatogonial depletion[34]. The differentiation and self-renewal of spermatogonial stem cells(SSCs) can be stimulated and inhibited, respectively, by the reduction of GDNF expression. Stimulated

differentiation and decreased self-renewal of SSCs could bring about stem cell pool depletion and impair the spermatogenesis. The results showed that the expressions of occludin and GDNF decreased, and the expression of TGF-β3 increased significantly (*P<0.05*) in the CTX group compared with that of the control group.

In the present study, we treated the mice with LC while they received CTX and compared the expression of occludin, GDNF and TGF-β3 between the different groups. The results show that the expression of these proteins in the LC+CTX group was obviously different from that of the CTX group. In the LC+CTX group, there was significantly high expression of occludin and GDNF and lower expression of TGF-β3, compared with that of the CTX group (*P<0.05*). Similar results were obtained in immunohistochemistry, western blot and RT-PCR with these three proteins in all groups.

Studies have identified that the testis Sertoli cell may be a possible testicular target of carnitine activity[35], which demonstrates a direct action of LC on the metabolism of Sertoli cells. LC influences fat and carbohydrate metabolism of rat Sertoli cells in primary culture by increasing fatty acid oxidation, glucose uptake and lactate/pyruvate secretion. Previous studies have also shown that LC and its derivatives protect against radiation damage by inhibiting radiation-induced increasing nitric oxide and malondialdehyde levels and by modulating

radiation-induced changes in the antioxidant defence mechanisms in rat tissues[36,37].

From the results of this study, we could draw a conclusion that CTX might impair spermatogenesis by disrupting protein expression of Sertoli cells. The expression of occludin and GDNF was suppressed, and the expression of TGF-β3 was enhanced. LC could resist the damage of CTX to Sertoli cells by alleviating the decrease of occludin and GDNF and the increase of TGF-β3. Occludin, GDNF and TGF-β3 are important proteins or factors for stable or normal micro-environment of seminiferous tubule in testis. These results suggest that LC might play a protective function against the toxic effects of CTX, and benefits the protection or recovery of fertility during and after chemotherapy of tumours.

COMPETING INTERESTS The authors declare that they have no competing interests.

ACKNOWLEDGEMENT This study was supported in part by Young Foundation(Scheme No.1347) of The Affiliated Hospital of Qingdao University and prospective protection study of reproductive function on High-risk male infertility crowd of Qingdao people's livelihood project (Scheme No. 13-1-3-22-nsh).

REFERENCE

1. Marcon L, Hales BF, Robaire B. Reversibility of the effects of subchronic exposure to the cancer chemotherapeutics bleomycin, etoposide, and cisplatin on spermatogenesis, fertility, and progeny outcome in the male rat. Journal of andrology. 2008;29:408-17.

2. Drumond AL, Weng CC, Wang G, Chiarini-Garcia H, Eras-Garcia L, Meistrich ML. Effects of multiple doses of cyclophosphamide on mouse testes: Accessing the germ cells lost, and the functional damage of stem cells. Reproductive Toxicology. 2011;32:395-406.

3. Jalali AS, Hasanzadeh S, Malekinejad H. Crataegus monogyna aqueous extract ameliorates cyclophosphamide-induced toxicity in rat testis: stereological evidences. Acta medica Iranica. 2012;50:1-8.

4. Bakhtiary Z, Shahrooz R, Ahmadi A, Zarei L. Evaluation of antioxidant effects of crocin on sperm quality in cyclophosphamide treated adult mice. Veterinary Research Forum : an International Quarterly Journal. 2014;5:213-8.

5. Das UB, Mallick M, Debnath JM, Ghosh D. Protective effect of ascorbic acid on cyclophosphamide-induced testicular gametogenic and androgenic disorders in male rats. Asian journal of andrology. 2002;4:201-7.

6. Tang MS, Wang HT, Hu Y, Chen WS, Akao M, Feng Z, et al. Acrolein induced DNA damage, mutagenicity and effect on DNA repair. Molecular nutrition & food research. 2011;55:1291-300.

7. Abd-Allah AR, Helal GK, Al-Yahya AA, Aleisa AM, Al-Rejaie SS, Al-Bakheet SA. Pro-inflammatory and oxidative stress pathways which compromise sperm motility and survival may be altered by L-carnitine. Oxidative medicine and cellular longevity. 2009;2:73-81.

8. Rao R. Occludin phosphorylation in regulation of epithelial tight junctions. Annals of the New York Academy of Sciences. 2009;1165:62-8.

9. Lui WY, Lee WM, Cheng CY. Transforming growth factor-beta3 perturbs the inter-Sertoli tight junction permeability barrier in vitro possibly mediated via its effects on occludin, zonula occludens-1, and claudin-11. Endocrinology. 2001;142:1865-77.

10. Lui WY, Lee WM, Cheng CY. TGF-betas: their role in testicular function and Sertoli cell tight junction dynamics. International journal of andrology. 2003;26:147-60.

11. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. Current opinion in genetics & development. 2002;12:22-9.

12. Xia W, Cheng CY. TGF-beta3 regulates anchoring junction dynamics in the seminiferous epithelium of the rat testis via the Ras/ERK signaling pathway: An in vivo study. Developmental biology. 2005;280:321-43.

13. Ebata KT, Yeh JR, Zhang X, Nagano MC. Soluble growth factors stimulate spermatogonial stem cell divisions that maintain a stem cell pool and produce progenitors in vitro. Experimental cell research. 2011;317:1319-29.

14. Kubota H, Wu X, Goodyear SM, Avarbock MR, Brinster RL. Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2011;25:2604-14.

15. Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science (New York, NY). 2000;287:1489-93.

16. Viglietto G, Dolci S, Bruni P, Baldassarre G, Chiariotti L, Melillo RM, et al. Glial cell line-derived neutrotrophic factor and neurturin can act as paracrine growth factors stimulating DNA synthesis of Ret-expressing spermatogonia. International journal of oncology. 2000;16:689-94.

17. Tadokoro Y, Yomogida K, Ohta H, Tohda A, Nishimune Y. Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mechanisms of development. 2002;113:29-39.

18. Simon L, Ekman GC, Tyagi G, Hess RA, Murphy KM, Cooke PS. Common and distinct factors regulate expression of mRNA for ETV5 and GDNF, Sertoli cell proteins essential for spermatogonial stem cell maintenance. Experimental cell research. 2007;313:3090-9.
19. Brock N, Pohl J, Stekar J, Scheef W. Studies on the urotoxicity of oxazaphosphorine cytostatics and its prevention--III. Profile of action of sodium 2-mercaptoethane sulfonate (mesna). European journal of cancer & clinical oncology. 1982;18:1377-87.
20. Kehe K, Balszuweit F, Steinritz D, Thiermann H. Molecular toxicology of sulfur mustard-induced cutaneous inflammation and blistering. Toxicology. 2009;263:12-9.
21. Makin G, Hickman JA. Apoptosis and cancer chemotherapy. Cell and tissue research. 2000;301:143-52.
22. Zhu B, Zheng YF, Zhang YY, Cao YS, Zhang L, Li XG, et al. Protective effect of L-carnitine in cyclophosphamide-induced germ cell apoptosis. Journal of Zhejiang University Science B; 2015 Sep. 2015. p. 780-7.
23. Pignatelli P, Lenti L, Sanguigni V, Frati G, Simeoni I, Gazzaniga PP, et al. Carnitine inhibits arachidonic acid turnover, platelet function, and oxidative stress. American journal of physiology Heart and circulatory physiology. 2003;284:H41-8.
24. Idrovo JP, Yang WL, Matsuda A, Nicastro J, Coppa GF, Wang P. Post-treatment with the combination of 5-aminoimidazole-4-carboxyamide ribonucleoside and carnitine improves renal function after ischemia/reperfusion injury. Shock (Augusta, Ga). 2012;37:39-46.
25. Miguel-Carrasco JL, Monserrat MT, Mate A, Vazquez CM. Comparative effects of captopril and l-carnitine on blood pressure and antioxidant enzyme gene expression in the heart of spontaneously hypertensive rats. European journal of pharmacology. 2010;632:65-72.
26. Aghaie S, Nikzad H, Mahabadi JA, Taghizadeh M, Azami-Tameh A, Taherian A, et al. Protective effect of combined pumpkin seed and ginger extracts on sperm characteristics, biochemical parameters and epididymal histology in adult male rats treated with cyclophosphamide. Anatomical science international. 2015.
27. Ghosh D, Das UB, Misro M. Protective role of alpha-tocopherol-succinate (provitamin-E) in cyclophosphamide induced testicular gametogenic and steroidogenic disorders: a correlative approach to oxidative stress. Free radical research. 2002;36:1209-18.
28. Mythili Y, Sudharsan PT, Selvakumar E, Varalakshmi P. Protective effect of DL-alpha-lipoic acid on cyclophosphamide induced oxidative cardiac injury. Chemico-biological interactions. 2004;151:13-9.
29. Sundstrom JM, Tash BR, Murakami T, Flanagan JM, Bewley MC, Stanley BA, et al. Identification and analysis of occludin phosphosites: a combined mass spectrometry and bioinformatics approach. Journal of proteome research. 2009;8:808-17.
30. Liu Y, Li X. Molecular basis of cryptorchidism-induced infertility. Science China Life sciences. 2010;53:1274-83.
31. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. The New England journal of medicine. 2000;342:1350-8.
32. Grande JP. Role of transforming growth factor-beta in tissue injury and repair. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY). 1997;214:27-40.
33. Xia W, Mruk DD, Lee WM, Cheng CY. Differential interactions between transforming growth factor-beta3/TbetaR1, TAB1, and CD2AP disrupt blood-testis barrier and Sertoli-germ cell adhesion. The Journal of biological chemistry. 2006;281:16799-813.
34. Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science (New York, NY). 2000;287:1489-93.

35. Shang XJ, Wang XL, Huang YF. [Carnitines and male reproduction]. Zhonghua nan ke xue = National journal of andrology. 2006;12:726-9.
36. Topcu-Tarladacalisir Y, Kanter M, Uzal MC. Role of L-carnitine in the prevention of seminiferous tubules damage induced by gamma radiation: a light and electron microscopic study. Archives of toxicology. 2009;83:735-46.
37. Dokmeci D, Inan M, Basaran UN, Yalcin O, Aydogdu N, Turan FN, et al. Protective effect of L-carnitine on testicular ischaemia-reperfusion injury in rats. Cell biochemistry and function. 2007;25:611-8.

tables and figures

Table 1 Spermatozoon count and viability rate

Group	density ($\times 10^6$ ml^{-1})	Viability rate (%)
Control	64.622±17.345	38.763±4.857
CTX	32.279±11.012 #	23.468±4.324#*
LC+CTX	43.435±11.446 #	35.597±5.856

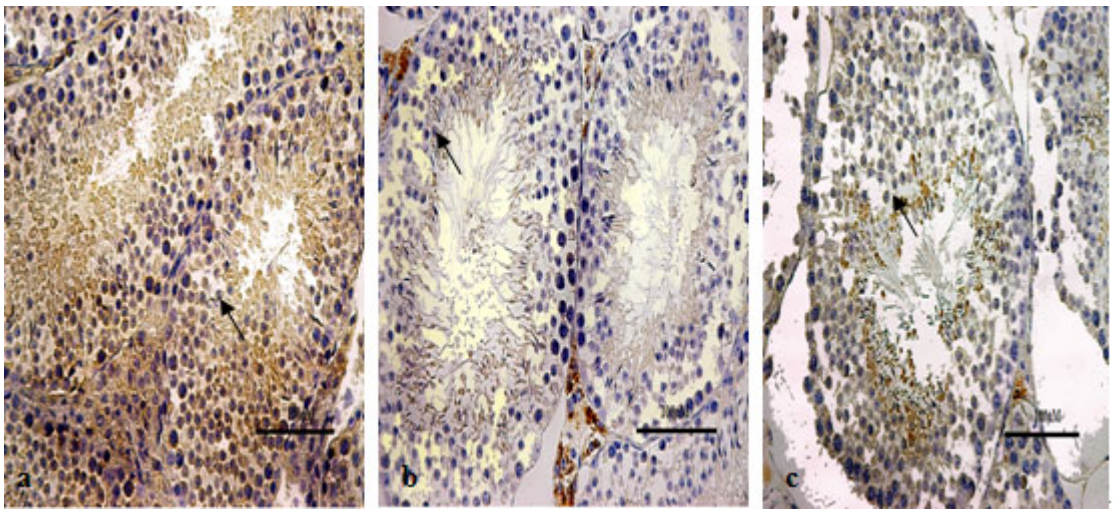
Data are expressed as mean±SD (n=10). # indicates significant decrease in the CTX or the LC+CTX group compared with the control group, * indicates significant decrease in the CTX group compared with that of the LC+CTX group ($p < 0.05$).

Table 2 Spermatozoon motility

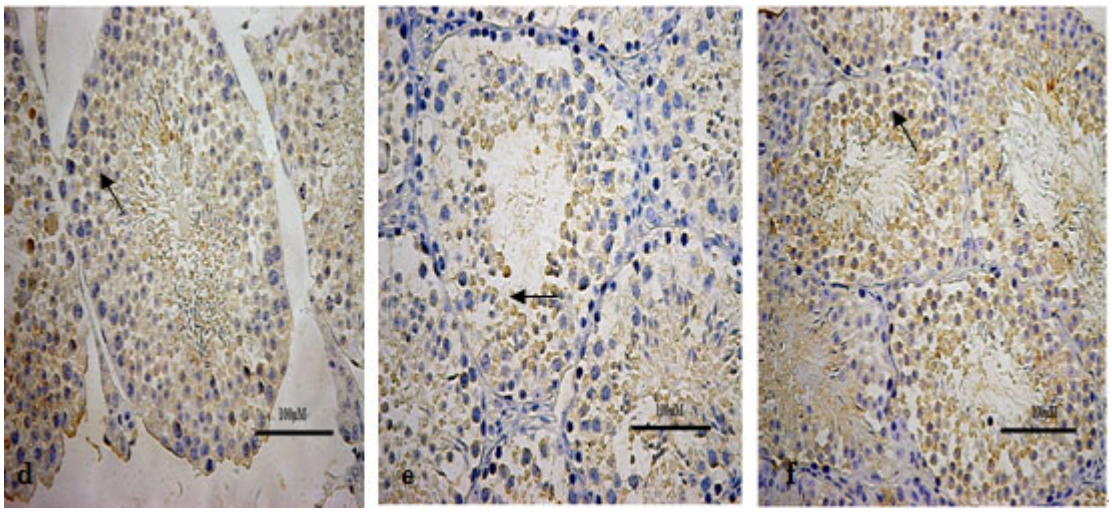
Group	Level (A%)	Level B (%)
Control	3.274±0.476	9.390±2.642
CTX	1.102±0.625 # *	3.218±1.126 # *
LC+CTX	2.315±0.676	8.481±4.367

Data are expressed as mean±SD (*n*=10). # indicates significant decrease in the CTX or the LC+CTX group compared with the control group, * indicates significant decrease in the CTX group compared with that of the LC+CTX group ($p < 0.05$).

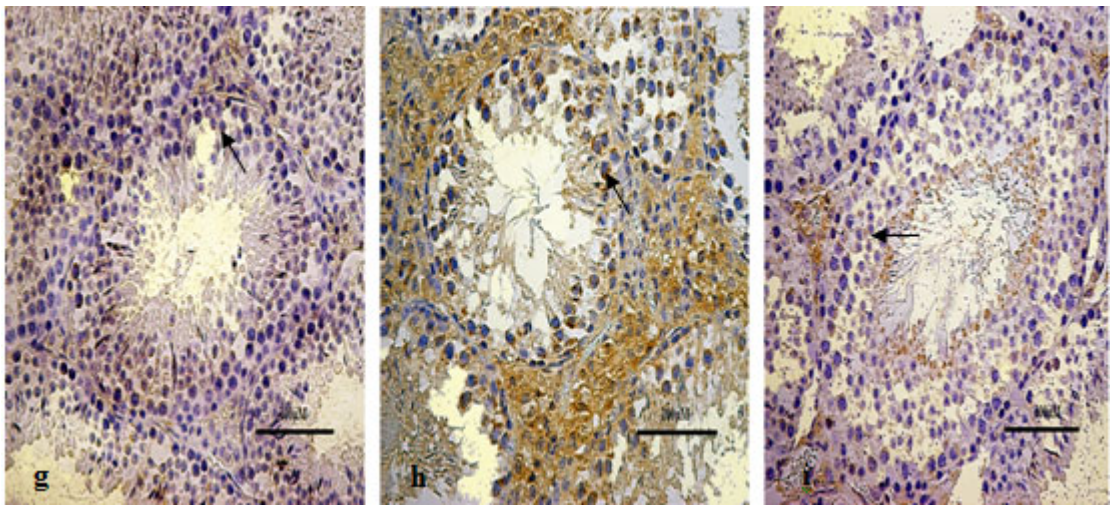
Figure 1 Immunohistochemistry



Occludin：Compared with the control groups(a), the expression of occludin decreased in the CTX groups(b) and LC+CTX groups(c).The expression in the LC+CTX groups was obviously more than that of the CTX group. Seminiferous tubule cross-sections of testis of mouse(×400). Scale bars = 100um.

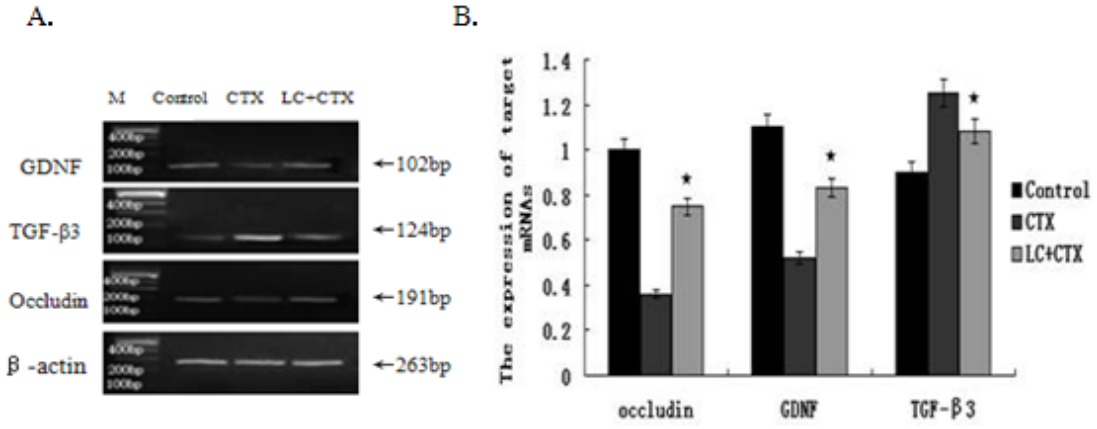


GDNF: Compared with the control groups(d), the expression of GDNF decreased in the CTX groups(e) and LC+CTX groups(f).The expression in the LC+CTX groups was obviously more than that in the CTX groups. Seminiferous tubule cross-sections of testis of mouse(×400). Scale bars = 100um.



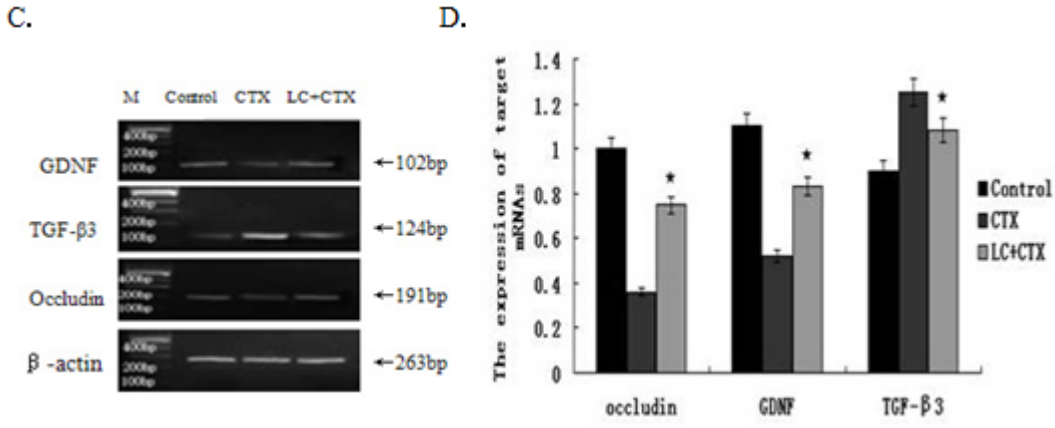
TGF-β3: Compared with the control groups(g), the expression of TGF-β3 increased in the CTX groups(h) and LC+CTX groups(i).The expression in the LC+CTX groups was obviously less than that in the CTX groups. Seminiferous tubule cross-sections of testis of mouse(×400). Scale bars = 100um.

Figure 2 RT-PCR



Semiquantitative RT-PCR analysis of the effect of CTX and LC on testicular TJs protein occludin,GDNF and TGF-β3 mRNA expression. (A) RT-PCR result; (B)Semiquantitative analysis of the RT-PCR products. Relative amounts of RT-PCR products of occludin,GDNF and TGF-β3 were calculated by dividing by the internal controls, β actin. The values are means ±S.D. (n=10).

Figure 3 Western blot



Expressions of occludin, GDNF and TGF-β3 protein in the testes determined by western blot analysis (C) and their respective quantitative analysis presented as bar graphs (D). Relative amounts of western blot products of occludin, GDNF and TGF-β3 were calculated by dividing by the internal controls, β-actin. The values are means ±S.D. (n=10).