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Effects of Holothurian Spermatophore Extracts on Spermatogenesis of Reproductive System in Mice Injured by Cyclophosphamide*

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ABSTRACT Objective: To investigate the protective effects of holothurian spermatophore extracts on spermatogenesis of the mice reproductive system injured by cyclophosphamide (CP). **Methods:** Male Kunming mice were equally allotted to groups, and they were intraperitoneally injected with CP (28 mg/kg) once a day for 5 days to construct injured reproductive system and partial androgen deficiency model except that the control group were intraperitoneally injected with normal saline. Mice in treatment groups orally received extracts once a day for 4 weeks. Record the changes of body weight and sprit state. Quantity, motility rate and abnormal rate of sperm and enzyme activity of T-SOD were determined and compared with those of model group. **Results:** Compared with that in model group, though the dose of holothurian spermatophore extracts had no obvious difference ($P>0.05$), the quantity, sportive rate and abnormal rate of sperm and T-SOD enzyme activity were all significantly improved ($P<0.01$), even close to or higher than the normal level. **Conclusion:** Holothurian spermatophore extracts had a protective effect on the spermatogenic lesion of reproductive system in mice injured by CP.

Key words: Holothurian spermatophore; Extract; Cyclophosphamide; Spermatogenesis

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Introduction

In recent years, the role of oxygen-free radicals in the pathogenesis of various diseases is increasingly valued. The polyunsaturated fatty acids in sperm membrane lipids can react lipid peroxidation with radical, damage sperm membrane, reduce sperm motility and even lose[1-3]. Cyclophosphamide(CP) is a bifunctional cytotoxic alkylating agent. It is harm to genital system, especially the permatogenic function. Recent studies showed that the harmful mechanism of radical and the spermatogenic dysfunction caused by CP have a close relation[4,5].

As the well-know treasures of the sea, holothurian have high nutritional value. The ingredient of holothurian such as sea cucumber and sea cucumber glycosides has many pharmacologic effects. It has anti-tumor effect and anticlotting effects. It can enhance immunity and delaying senescence [6-9]. Holothurian spermatophore is the reproductive organ of holothurian and many studies showed that it has high nutritional value also. But it is always regarded as waste to discard so that the nutritional ingredient and active factor are out of use. Therefore, this paper takes the spermatogenic function of testis and the antioxidant indices as parameter to investigate the protective effects of holothurian spermatophore extracts on the reproductive system during use of CP.

1 Materials and methods

1.1 Materials

1.1.1 Experimental animals and raw materials 96 male Kunming mice, 10~12 weeks (25~35 g), have been bought in ShanDong LuKang Pharmaceutical Group Co.,Ltd., clean level, qualified number:SLXK lu 20080002. They have been fed water and food freely at room temperature. Holothurian spermatophores are supplied by Dalian Bangchui Island Seafood Co.,Ltd.

1.1.2 Experimental drugs and reagents Water extracts of holothurian spermatophore: weighed a certain amount of holothurian spermatophores, smashed them with potter-elvehjem tissue grinders. Mixed homogenate and distilled water at a mass ratio of 1:3, extracted the mix by ultrasonic wave for 25 min under 30 ℃; Then stirred at 30 ℃ water bath to extract 2 h, and 4000 r/min centrifuged 5 min, combined the supernatant. Precipitate and distilled water at a mass ratio of 1:2, repeated the above operation, mixed the supernatants and filtrated. Evaporated filtrate to 1/3 by rotary evaporator and lyophilized them into crude extracts.

Alcohol extracts of holothurian spermatophore: weighed a certain amount of lyophilized powders of holothurian spermatophores, mixed them and anhydrous ethanol at a mass ratio of 1:10, extracted the mix at 30 ℃ water bath for 3 h; Filtrated and collected filtrates. Residue and anhydrous ethanol at a mass ratio of 1:5, repeated the above operation, combined filtrate. Evaporated filtrate to 1/3 by rotary evaporator and got the extract by vacuum drying. The suspension were prepared with the different concentrations of extracts to use. 0.9% sodium chloride injection, from Cisen Pharm-

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aceutical Co.,Ltd., batch number: 1207215121; methyltestosterone piece, 5mg/piece, from Shanghai Sine Kangjie Pharmaceutical Co., Ltd., batch number: 110802; T-SOD test box, from Nanjing Jiancheng Bioengineering Institute, batch number: 20130603; cyclophosphamide for injection, 0.2g/bottle, from Jiangsu Hengrui Medicine Co., Ltd., batch number: 12031425.

1.1.3 Experimental instruments Electronic balance AR5120 (Ohaus Shanghai International Trade Co.,Ltd.); Electric-heated thermostatic water bath DK-98-1 (Tianjin Taisite Instrument Co.,Ltd.). Ultrasonic cleaners SK3200H (Shanghai Kudos Ultrasonic Instrument Co.,Ltd.). Freeze dryer (Beijing Boyikang Experimental Instrument Co.,Ltd.). Biological camera microscope B1 (Motic Industrial Group Co.,Ltd.). Low speed centrifuge LD4-2A (Beijing Lab Centrifuge Co.,Ltd.). Visible spectrophotometer 723N (Shanghai Precision and Scientific Instrument Co.,Ltd.).

1.2 Methods

1.2.1 The preparation of animal model and grouping,dosing 96 male Kunming mice were equally allotted to 8 groups, and were intraperitoneally injected with CP (28 mg/kg) once a day for 5 days to construct injured genital system and partial androgen deficiency model except that the control group were intraperitoneally injected with normal saline. Meanwhile they orally received different extracts such as water extracts of holothurian spermatophore (300,600 mg/kg), alcohol extracts of holothurian spermatophore (300,600 mg/kg), free-dried power of holothurian spermatophore (600 mg/kg). Mice in positive group orally received methyltestosterone, 1.5 mg/kg. Mice in control and model group orally received the same volume of distilled water once a day for 4 weeks.

1.2.2 The collection of sperm and analysis Killed mice by cervical dislocation, stripped epididymis and weighed them by analytical balance. Mixed them and 0.5 ml normal saline and then cut into pieces, incubated them under water bath of 37 ℃ for 5 min to make the sperms come out of epididymis and got the suspension of sperm. Took 25 μL suspension of sperm in the hemocytometer, counted quantity and sportive rate of sperm. After that, took 10 μL suspension of sperm in slide, stained with eosin, observed and counted abnormal rate of sperm by microscope.

1.2.3 The preparation of tissue homogenate and the determination of enzyme activity of T-SOD Took out one testicle, mixed it and 0.9% normal saline at the weight volume ratio of 1:9, homogenized them under ice-water bath to make the homogenate of 10%, centrifuged them at 2500～3000 r/min for 10 min, sucked out the supernatant. Determined the content of protein in homogenate by coomassie brilliant blue and the enzyme activity of T-SOD was deteted by hydroxylamine method.

Table 1 Effects of holothurian spermatophore extracts on sperm concentration,motility rate and abnormal rate of mice(n=10,mean± sd)

Groups	Sperm concentration /$1\times10^7\cdot ml^{-1}$	Sperm motility rate/%	Sperm abnormal rate/%
Control group	4.4170± 0.4167	47.50± 4.37	30.33± 1.63
Model group	2.1330± 0.5086°	28.33± 6.31°	36.67± 0.82°
Low dose of water extract group	5.1000± 0.3889°	51.17± 3.19	33.17± 1.47°
High dose of water extract group	4.4500± 0.1871	49.17± 4.36	32.50± 1.05°
Low dose of alcohol extract group	4.6330± 0.3077	52.83± 1.47●	32.33± 1.03°
High dose of alcohol extract group	4.6170± 0.5076	47.83± 4.79	31.83± 1.17●
Free-dried power group	5.2170± 0.4021°	52.50± 5.54	32.00± 1.41●
Positive drug group	5.0500± 0.2168°	58.00± 2.83°	31.17± 0.75

Note: ● P<0.05,° P<0.01, compared with control group; P<0.01,compared with model group.

1.2.4 Statistical processing The data were expressed in ± s, and analyzed by one-way analysis of variance with SPSS17.0.

2 Results

2.1 The sperm concentration of mice

Table 1 showed that compared with that in the control group, the sperm concentration of mice in model group decreased significantly (P<0.01). The sperm concentration in treatment groups were significantly higher than that in model group (P<0.01), though there was no significant difference between high and low dose (P>0.05), and they were close to or higher than the normal level.

2.2 The sperm motility rate of mice

Table 1 showed that compared with that the control group, the sperm motility rate decreased significantly (P<0.01). Compared with that in the model group, holothurian spermatophore extracts could significantly enhance the sperm motility rate (P<0.01).

2.3 The sperm abnormal rate of mice

Compared with that in the control group, the sperm abnormal rate increased significantly (P<0.01). Holothurian spermatophore extracts could alleviate the toxic action of CP and reduce the sperm abnormal rate significantly (P<0.01).

2.4 The determination of the concentration of protein in testis

Table 2 showed that compared with that in control group, the concentration of protein in testis decreased significantly after the mice in model group treated with CP(P<0.01). Compared with that in the model group, the concentration of protein in treatment group increased significantly (P<0.01).

Table 2 Effects of holothurian spermatophore extracts on anti-oxidative capacity of testis in the mice(n=10,mean± sd)

Groups	10% concentration of protei(g/L)	T-SOD(U/mgprot)
Control group	6.55± 0.92	46.38± 6.31
Model group	5.35± 0.23°	38.60± 4.73°
Low dose of water extract group	6.26± 0.66▲	45.22± 2.67▲
High dose of water extract group	6.49± 0.72	60.03± 6.10°
Low dose of alcohol extract group	6.41± 0.38	51.68± 3.84●
High dose of alcohol extract group	6.55± 0.54	53.99± 4.65°
Free-dried power group	7.34± 0.25●	51.27± 2.83
Positive drug group	7.12± 0.62	46.55± 2.07

Note:● P<0.05,° P<0.01,compared with control group;▲ P<0.05, P<0.01,compared with model group.

2. 5 The determination of T-SOD enzyme activity

The content of T-SOD of testis in the mice decreased significantly after treated with CP for 5d, while the holothurian spermatophore extract and power reversed the decline of T-SOD and the content of T-SOD began to rise again. They were close to or higher than the normal level. Compared with that in the model group, there was a significant difference (P<0.05).

3 Discussion

The ability of body to clear the free radical will decrease as people get old. It can break the oxidation-antioxidation balance so that the oxidation will be increased and the anti-oxidative capacity will be decreased. Excessive free radicals generate peroxide fat. Accordingly, the body will be damaged[10,11]. As the clinically common anticancer drugs and immunosuppressive agents, a variety of basic and clinical studies show that CP can cause spermatogenic dysfunction[12-14]. Researches show the mechanisms of spermatogenesis of testis injured by CP are the following ways: (1) CP can damage genetic materials in germ cells and induce apoptosis of spermatogenic cells. In the acute phase, CP mainly induce the apoptosis of spermatogonia and spermatocytes that are in active phase of mitosis. The mechanism of inducing apoptosis may be that activating the induction of regulating apoptosis in germ cells such as c-Kit and p53 [15]. (2) The proliferation and differentiation of self-renewing in type A spermatogonial cells are disturbed. Study find that the toxic effects of CP can downregulate the expression of stem cell factor (SCFm) in rat testis,thus decreasing the proliferation index [16]. From histological observation, the researchers also find that there is a single remnant type A spermatogonial cell in the basal and atrophied adjacent Sertoli cells. And their limited self-renewal capacity makes it difficult to return to normal spermatogenesis level. (3) CP can damage the microenvironment of spermatogenesis, and its mechanism include decrease of seminiferous tubule cell layers, structural disorder,morphological change of Sertoli cells, germ cells shedding and spermatogenesis. (4) The activity of 3β-HSD and 17β-HSD in testis are inhibited by the reproductive toxicity of CP, thereby reducing the levels of serum testosterone and the number of germ cells[17,18].

The mice which reproductive system injured by CP orally received holothurian spermatophore extraction. The effects of holothurian spermatophore extraction on sperm concentration, sperm motility rate, abnormal rate and T-SOD enzyme activity were observed. The protective effects of holothurian spermatophore extraction on spermatogenesis of reproductive system injured by CP were investigated. Testis is vital organ that it can promote spermatogenesis and testosterone secretion. Seminiferous tubules are the part of spermatogenesis. And the most direct manifestation of oxidative damage caused by CP on testis is damaging the spermatogenesis of testis. The study found that the quantity and motility rate of sperm of mice in model group decreased and the abnormal rate increased [19-20]. However, holothurian spermatophore extraction can relieve the toxic effect of CP, enhance the quantity of sperm effectively, decrease the abnormal rate, have a certain extent protective effect on reproductive function of mice. In addition, the T-SOD activity of testis of mice in model group decreased significantly, suggesting that CP can cause the increasing of oxygen free radicals in testis spermatogenic cells, consume more T-SOD and hardly clear the excess free radicals. Thereby it aggravate the degree of lipid peroxidation, break the oxidation-antioxidation balance,injure germ cells and metabolic disorders, and then damage the reproductive system. Compared with model group, the T-SOD activity of testis of mice in treatment groups increased significantly, indicating that holothurian spermatophore extraction can improve the T-SOD activity of testis, increase the capacity of antioxidation and scavenging free radicals, effectively inhibit the damage of oxidation-antioxidation system on testis of mice caused by CP.

In summary, holothurian spermatophore extraction can alleviate the reproductive toxicity on Kunming mice of CP, have a certain extent protective effect on reproductive function. It provides theoretical basis in order to further the development and utilization of holothurian,but its specific mechanism also need further study.

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海参精囊提取物对环磷酰胺诱导生殖系统受损小鼠生精功能的影响 *

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摘要 目的:本文旨在探讨海参精囊提取物对环磷酰胺诱导的生殖系统受损小鼠生精功能的保护作用。**方法:**昆明小白鼠随机分组,除正常组用生理盐水外其他各组均腹腔注射环磷酰胺(CP,28 mg/kg),每天 1 次,连续 5 天,复制生殖系统受损、雄激素部分缺乏模型;同时治疗组每天灌胃不同剂量的药物 1 次,连续 4 周,记录小鼠的体重并观察其精神状态。计算精子总数、运动率、畸形率及睾丸组织中 T-SOD 酶活力,并与模型组进行比较。**结果:**相较于模型组小鼠,虽海参精囊提取物高低剂量间无明显差异(P>0.05),但治疗组小鼠的精子总数、运动率、畸形率和 T-SOD 酶活力均有明显的改善(P<0.01),甚至接近或高于正常水平。**结论:**海参精囊提取物对环磷酰胺导致的生殖系统受损小鼠的生精功能具有一定的保护作用。

关键词:海参精囊;提取;环磷酰胺;生精功能

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