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EXPERIMENTAL STUDY

Interventional effects of squid ink polysaccharides on cyclophosphamide-associated testicular damage in mice

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Abstract: Cyclophosphamide (CP) is a commonly used antitumour and immunosuppressive drug, but it is inevitable that the chemotherapeutic agent may cause long-term or permanent reproductive damage on young male patients through inducing oxidative stress in the testes. Squid ink polysaccharides (SIP), a newly found marine glycosaminoglycon have been proved to have antioxidant capabilities and chemotherapy-protective activities on model animals in our recent investigations. This study was conducted to assess whether or not SIP could protect male mice against gonadotoxicity during CP exposure. Sexually mature male Kunming mice were allocated to one of four groups. CP was abdominally administered at dose of 15 mg/kg body weight to two groups of mice for ten weeks, once a week, one group of mice received SIP at dose of 80 mg/kg body weight by gavage for ten weeks, once a day. The other two groups comprised a vehicle treated group and an SIP treated group. Toxicity of CP and protective activity of SIP on the testes were assessed by: sperm parameters, organ index, testicular antioxidant ability, activities of marker enzymes, sex hormone content, and histopathological features. Data showed CP-induced, serious negative changes on murine sperm parameters, organ index, testicular antioxidant ability, activities of marker enzymes, sexual hormone contents, and histopathological features which were all significantly impaired by SIP. This study found that SIP were demonstrated to offer protective effects against CP-induced toxicity on testes in mice *(Tab. 2, Fig. 3, Ref. 29)*. Text in PDF *www.elis.sk.*
Key words: chemoprotection, squid ink polysaccharides, cyclophosphamide, testis, mice

Introduction

It is well known that chemotherapy is still a critical remedy to malignant neoplasm, and that cyclophosphamide (CP) is a commonly used chemotherapeutic drug in treatment of cancer and immunosuppression for nephritic disorders and lupus erythematosus. It is also known that the antineoplastic drug decreases tumour patients' sperm counts during treatment (1). Although recovery from moderate doses of CP has been proved to be possible, a prolonged period of azoospermia has been found, moreover high doses of CP caused permanent azoospermia as reported elsewhere (2); this potentially results in infertility in young cancer patients. Background research showed that differentiating spermatogonia undergoing constant mitotic activity were sensitive to chemotherapeutic drugs (3), death of the testicular cells was responsible for depletion of the subsequent differentiating stages of germ cells and reductions in sperm counts.

Recovery of spermatogenesis depends on survival stem cells and an unaffected microenvironment exposed to CP, but if the chemotherapeutic agent kills enough spermatogonial stem cells, the recovery process would be more arduous. Data in model mice indicated that the interval needed for the recovery of fertility was directly related to the killing level of the spermatogonial stem cells, and that chemical agents could injury the somatic tissue, even though numerous spermatogonia survived the cytotoxic treatment in rat testes, recovery of the seminiferous epithelium was still impossible (4). From the aforementioned information, it is found that CP-induced damage to the male reproductive system is both inevitable and severe, and that reduction of the therapeutic effect of this chemotherapeutic drug on male tumour patients originates from clinical inadequate dosing of the drug due to its chemical toxicity.

To decrease negative effects of CP on normal testes and to increase the clinical dose of the agent for elevating its therapeutic effects in young male tumour patients, development and application of a cytoprotector against chemotherapy is both necessary and urgent. It has been presently reported that some natural compounds such as: carotenoids, astaxanthin, and lipoic acid can protect the testes from CP-induced oxidative stress damage through Nrf2/ARE signal pathway that is also employed by CP to produce gonadotoxicity (5-10), which implies that natural material may be a potential resource to be used when developing a cytoprotector against chemotherapy for the clinical treatment of cancer.

In recent years, squid ink polysaccharides (SIP), a natural marine bioactive substance derived from squid ink were proved to have chemotherapy-protective roles in our previous investigations. SIP is a type of glycosaminoglycon with a unique structure

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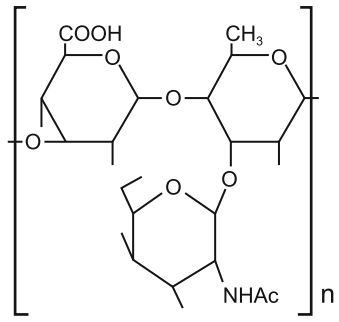

Fig.1. Structure of SIP.

–[3GlcAβ1-4(GalNAcα1-3)-Fucα1]$_n$– (Fig. 1) (11, 12) and it has been discovered that the bioactive polysaccharides could increase antioxidant capabilities of some tissues including the liver, heart, lungs, and kidneys of model animals exposed to CP (13), which implies that SIP protects normal tissues of model animals from damage induced by the chemotherapeutic drug. Especially, our latest preliminary findings revealed intervention of SIP on CP-induced spermatogenetic damage in mice, the investigated parameters including sperm density, survival rate, abnormality rate, as well as superoxide dismutase activity in testis indicated that SIP could protect the testes from chemotherapeutic damage caused by CP (14, 15) and that the marine polysaccharides could be potentially developed as a cytoprotector. However, the decidedly protective effects and the possible interventional mechanisms of SIP on the spermatogenesis injured by CP are not still known. We further assessed the interventional effects of SIP on CP associated testicular toxicity in model mice with: testis index, epididymal sperm characteristics, testicular antioxidant ability, activities of marker enzymes, sex hormone contents, and pathological features of the testes, which would be helpful when determining the protective mechanisms of SIP during the intervention process.

Materials and methods

Preparation of SIP

Live squids purchased from a local aquatic products market were sacrificed to harvest fresh ink sacs that were then stored at –28 °C for future use. According to our previous methods (15), ink collected from sacs thawed at 4 °C and was suspended with pH 6.7 PBS, and was then ground and ultrasonically treated. The resultant ink solution was stored at 4 °C for 24 h and was then centrifuged at 14000 g for 1 h at 4 °C. The supernatant was subjected to enzymolysis with 1 % papain in PBS (pH 6.7) at 60 °C for 24 h, and was then mixed with a 1/4 volume liquid mixture of chloroform and n-butanol (v/v, 4/1) followed by stirring for 30 min on a magnetic stirrer plate. After centrifugation at 5000 g for 15 min, the supernatant was re-digested with papain, the digestion process was performed twice. SIP in the resulting supernatant was precipitated with four volumes of absolute alcohol, and was subjected to freeze-drying in a vacuum. Solid powder SIP was stored at 4 °C before use.

Animal experimental scheme

Sexually mature male Kunming mice purchased from the Experimental Animal Centre of Guangdong Medical College were adaptively domesticated for one week under the following constant experimental conditions: a relative humidity of 55 ± 5 %, a temperature of 22 ± 2 °C, a quasi-diurnal cycle of 12 h light and 12 darkness, and free feeding and drinking. They were then allocated to one of four equal sized test groups: the control group (administered orally with normal saline and injected abdominally with normal saline), a CP-treated group (administered orally with normal saline and injected abdominally with CP in normal saline), an SIP-treated group (administered orally with SIP and injected abdominally with normal saline), and a co-treated group (administered orally with SIP and injected abdominally with CP in normal saline): each group contained three replicates of ten animals. The SIP dose was 80 mg/kg body weight, once a day for a continuous ten week period, and the CP dose was 15 mg/kg body weight, once a week (again for a continuous ten week period).

Sperm analysis

The epididymis was cut and incubated in 1 ml of normal saline at 37 °C for 5 min. A blood cell counting plate was used to analyse the total, and live, sperm counts which were used to derive the survival ratio of sperm. One drop of sperm suspension was smeared on a slide and was coloured with 2 % eosin. Abnormal sperm were distinguished from 200 sperm and were used to calculate their abnormality ratio.

Biochemical analysis of the testes

A 10 % testicular homogenate was prepared with normal saline. Detection kits purchased from Nanjing Jiancheng Bioengineering Institute of China were used to determine some enzyme activities, such as those of: γ-glutamyltransferase (γ-GT), alkaline phosphatase (ALP), acid phosphate (ACP), lactate dehydrogenase (LDH), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST), and to determine the: glutathione (GSH), nitric oxide (NO), malonyldialdehyde (MDA), and intratesticular testosterone (ITT) contents.

Analysis of serum sex hormones

Mouse serum was used to determine levels of testosterone (T), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estrogen (E) with enzyme-linked immunosorbent assay kits purchased from Nanjing Jiancheng Bioengineering Institute of China.

Histopathological analysis of the testes

Samples were prepared into paraffin sections by routine methods, each section was photographed and its histopathological features analysed.

Statistical analysis

Experimental data were analysed by ANOVA using JMP 7.0 statistical software. Results were expressed as means and standard errors. Differences were distinguished by Duncan's multiple range test. Significance was considered at $p < 0.05$ or $p < 0.01$.

Tab. 1. Body weight, testis weight, organ index, and epididymal sperm characteristics in mice.

Groups	Body weight (g)	Testis weight (mg)	Testis index (wt., ‰)	Sperm count (10^6/mL)	Survival ratio (%)	Abnormal ratio (%)
Control group	43.16 ± 2.45^{Aab}	282 ± 30^{Aa}	6.71 ± 0.61^{Aa}	1.29 ± 0.23^{Aa}	72.61 ± 4.21^{Aa}	20.80 ± 4.05^{Aa}
CP-treated group	36.49 ± 2.77^{Bc}	213 ± 25^{Bb}	5.76 ± 0.79^{Bb}	0.90 ± 0.26^{Bb}	50.97 ± 8.04^{Bb}	36.17 ± 3.76^{Bb}
SIP-treated group	44.10 ± 1.45^{Aa}	292 ± 46^{Aa}	6.38 ± 1.11^{ABab}	1.26 ± 0.20^{Aa}	73.66 ± 7.08^{Aa}	22.75 ± 5.73^{Aac}
Co-treated group	41.61 ± 3.27^{Ab}	258 ± 39^{Aa}	6.28 ± 0.67^{ABab}	1.07 ± 0.16^{ABab}	68.98 ± 6.95^{Aa}	25.80 ± 4.10^{Ac}

Tab. 2. Biochemical parameters in mice testes.

Parameters	Control group	CP-treated group	SIP-treated group	Co-treated group
γ-GT (U/g protein)	7.81 ± 2.62^{Bb}	13.36 ± 2.64^{Aa}	7.01 ± 3.65^{Bb}	8.85 ± 3.02^{Bb}
AKP (U/mg protein)	0.25 ± 0.020^{Cc}	0.33 ± 0.035^{Aa}	0.27 ± 0.017^{BCb}	0.30 ± 0.039^{ABb}
ACP (U/mg protein)	0.69 ± 0.11^{ABb}	0.83 ± 0.13^{Aa}	0.65 ± 0.10^{Bb}	0.69 ± 0.10^{ABb}
LDH (U/mg protein)	10.35 ± 1.86^{Aa}	8.43 ± 1.08^{Bb}	9.92 ± 1.00^{Aa}	9.84 ± 1.61^{Aa}
MDA (nmol/mg protein)	1.21 ± 0.09^{Bb}	1.48 ± 0.06^{Aa}	1.29 ± 0.12^{Bb}	1.27 ± 0.10^{Bb}
GSH (mg/g protein)	1.07 ± 0.11^{Aa}	0.67 ± 0.24^{Bb}	1.08 ± 0.18^{Aa}	1.00 ± 0.26^{Aa}
GPX (U/mg protein)	6.46 ± 0.67^{Aa}	3.38 ± 0.64^{Bb}	5.95 ± 1.71^{Aa}	6.36 ± 1.36^{Aa}
GST (U/mg protein)	59.47 ± 5.45^{Aa}	45.86 ± 5.70^{Bb}	56.38 ± 3.93^{Aa}	55.75 ± 6.87^{Aa}
SOD (U/mg protein)	4.74 ± 0.33^{ABa}	4.43 ± 0.23^{Bb}	4.70 ± 0.21^{ABa}	4.90 ± 0.31^{Aa}
CAT (U/mg protein)	1.33 ± 0.20^{ABab}	0.96 ± 0.10^{Cc}	1.38 ± 0.13^{Aa}	1.17 ± 0.14^{Bb}
NO (μmol/mg protein)	0.61 ± 0.38^{Bb}	1.32 ± 0.44^{Aa}	0.56 ± 0.34^{Bb}	0.96 ± 0.30^{ABa}

Results

Testis index and sperm parameters of mice

CP coincidently caused a serious reduction of body weight, testis weight, and testis index, although SIP successfully reversed the negative effects of CP on body and testis weights, the organ index of testis could not benefit from the marine agent and was still close to that in CP-injured mice (Tab. 1). In addition, three parameters of sperm characteristics listed in Table 1 include sperm count, survival rate, and abnormality rate. It was clear that CP markedly decreased sperm count and survival rate, while sharply increasing the abnormality ratio of epididymal sperm in mice, and that like body weight and testis weight, the induced toxic damage of CP on sperm was also alleviated by SIP. It is necessary to see that, however, although SIP succeeded in its protective effects to reduce the chemical toxicity of CP on the testis, the marine active material failed to change the six parameters of natural mice in our control group and the SIP-treatment group, and in reverting the data to those of natural mice, there was no obvious difference between the control group and the co-treatment group.

Biochemical indicators in mice testes

Biochemical indicators can properly reflect functional state of tissue/organ, so this paper investigated some important parameters to show whether SIP could relieve gonadotoxicity of CP in male mice, the data were presented in Table 2. In this paper, we examined eleven parameters and found that CP significantly changed all of the designed parameters ($p < 0.01$) except for SOD ($p < 0.05$), which indicated that severe injury occurring in the testes was caused by CP. SIP remodelled activities of the four detected marker enzymes of metabolism: γ-GT and LDH ($p < 0.01$), as well as AKP and ACP ($p < 0.05$) were each changed by the antitumour drug. In addition, other correlative indicators of antioxidant ability were mostly obviously rescued by SIP except NO.

Sexual hormones levels in sera and testes

Testosterone and oestrogen produced in testes regulate development and maturation thereof as well as spermatogenesis. Production and secretion of the two hormones are regulated by two upstream sexual hormones: follicle-stimulating hormone and luteinizing hormone produced in anterior pituitary gland. Our measured data (Fig. 2) suggested that although CP obviously decreased ITT content, the reduction was not clearly suppressed by SIP. However, the same result did not appear in serum, where the content of circulating T which was decreased by CP was elevated to the same extent by SIP. Similarly, elevation of the CP-induced circulating LH content was inhibited by SIP. The other two hormone levels in the serum, FSH and E, exhibited similar trends during the stimulating processes of either CP or SIP, the former promoted levels of the two hormones, the latter impaired their promotion.

Histopathological characteristics of the testes

As shown in Fig. 3, in the control, and SIP-treated, groups, the thick seminiferous epithelium contained different stages of the spermatogenic cells in well-stratified sequences including rapidly growing spermatogonia, spermatocytes, and sperm cells. The Leydig cells were well-developed in the spaces among closely arranged seminiferous tubules. However, in CP-treated mice, the seminiferous epithelium was either thin or incomplete, various spermatogenic cells in the thinner cellular layers were either disarranged or lost, and the cells were undergoing depletion, the cavity of the seminiferous tubule was larger than that in the control, and SIP-treated, groups. The Leydig cells were either reduced in number or lost. Seminiferous tubules were not closely arranged. Meanwhile, co-treated mice showed little difference from control mice, but compared with CP-treated mice, some distinct changes were visible in the testes, such as the regular arrangement and increased quantities of various stages of the spermatogenic cells, and a decreased volume of cavity in the seminiferous tubule.

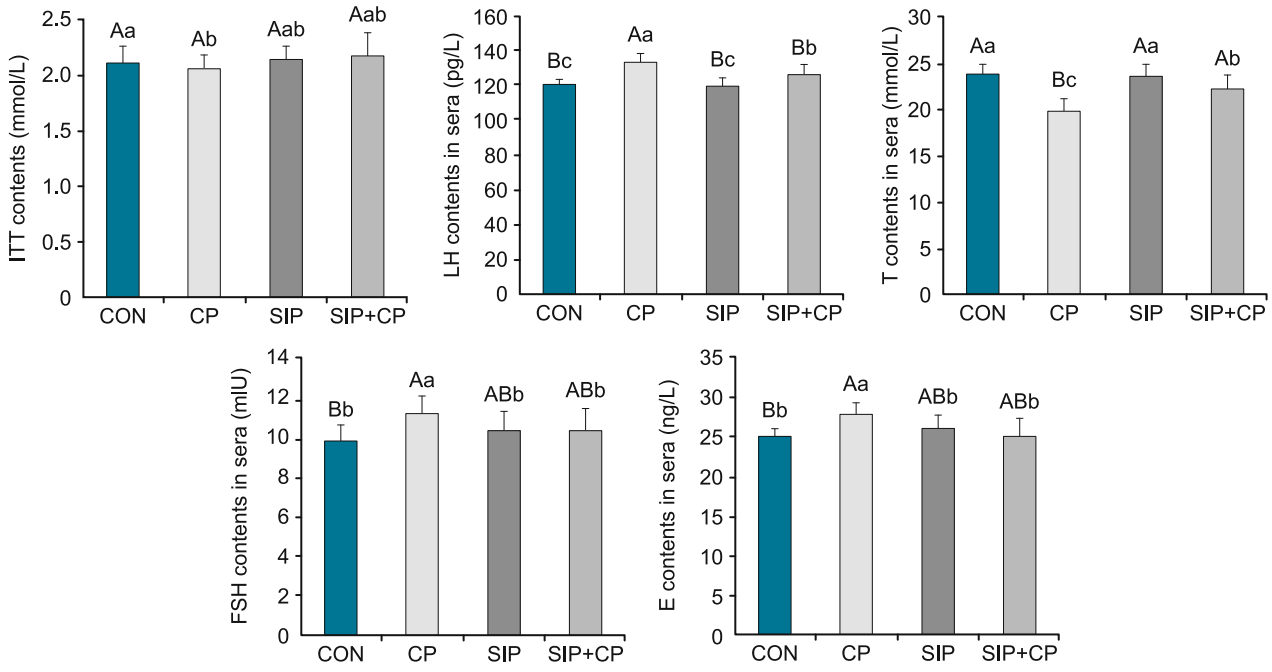

Fig. 2. Sexual hormone contents in mice sera and testes. CON: Control group; CP: CP-treated group; SIP: SIP-treated group; SIP+CP: co-treated group

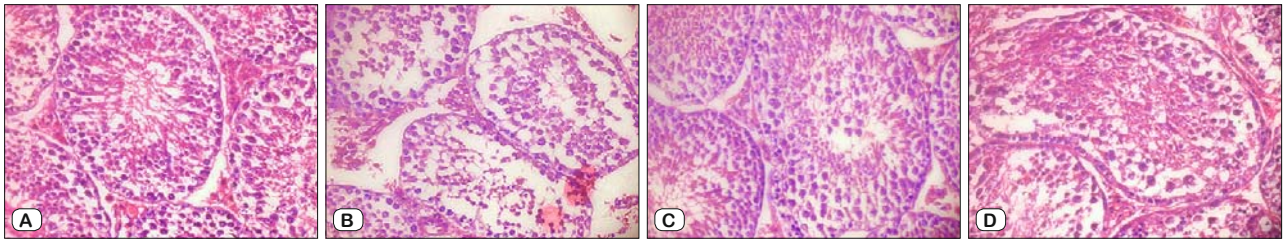

Fig. 3. Photomicrographs of murine testicular paraffin sections stained with haematoxylin and eosin (400 ×). A: control mice, B: CP-treated mice, C: SIP-treated mice, D: co-treated mice.

Discussion

Cyclophosphamide, a biofunctional alkylating agent, is an inactive precursor that is metabolized to acrolein and phosphoramide mustard by the hepatic cytochrome P450 system, the two metabolic products exert anti-cancer effects in childhood and adult malignancies, as well as immunosuppression (1). Nowadays, chemotherapeutic damage of CP on testis is universally acknowledged. Male reproductive toxicity of CP was firstly attributable to its leading spermatogonial stem cells to apoptosis, but the following investigation discovered that after CP exposure, a large number of stem cells still survived, which implied that the actual reasons for spermatogenic impairment were loss of spermatogonial stem cells and loss of their proliferation and differentiation ability (16). In fact spermatogenesis is regulated by a combination of factors, such as Sertoli cells and hormones, that can be rendered dysfunctional by CP. CP-induced damage to spermatogenesis is most directly reflected by sperm count, survival ratio, and abnormality ratio. Previous studies reported that long-term low-dose CP exposure should result in reductions in body weight, testis weight, and testis index in humans or animals (17), and reduction of sperm count and quality (18), which would affect growth of offspring and increase the percentage of deformities therein. Testis weight is connected with quantities of different differentiational spermatids (19). We found results here that were consistent with those in our previous work which indicated that CP exposure resulted in decreases in sperm count and survival ratio, an increased sperm abnormality ratio, as well as significant reductions in body weight, testis weight and index in CP-treated mice, which also agreed with current data (3, 4, 9, 20).

The antineoplastic effect of CP is associated with phosphoramide mustard; acrolein is linked to side-effects (21). The toxic mechanism by which CP was induced in testis may be the disruption of redox balance leading to oxidant stress. Acrolein is documented as having caused inactivation of microsomal enzymes which results in increased reactive oxygen species generation and lipid peroxidation (22). We can conclude that CP treatment gives rise to intra-testicular oxidative stress.

Our current research indicated that CP caused intensification of lipid peroxidation, an increase in MDA content, and reduction of the activities of SOD, CAT, and GPX, and diminution of GSH content and GST activity, which suggested that CP destroyed the redox balance in testis and influenced development and maturation of the male reproductive organ.

It was reported that CP elevated NO levels in testicular tissues indirectly resulted in infertility (10). So, it must be a reason, in this work, that explained why CP-upregulated NO content originated possibly from an increase in the activity of nitric oxide synthetase-injured animal testes: this was directly reflected in their apparent characteristics, such as: testis index, sperm parameters, and histopathological changes.

Presently some marker enzymes: LDH, AKP, ACP, and γ-GT are accepted as functional indicators during spermatogenesis. Modification of their activities may change the spermatogenetic process, and injure the functions of germ cells, Leydig cells, and Sertoli cells. LDH, correlated to the seminiferous epithelium, is in mitochondrial membranes of mature or maturing testicular spermatogenic cells and plays critical roles in the proton transfer process across mitochondrial membranes. Peroxidation of mitochondrial membranes induced by CP negatively affects activity of LDH, spermatogenesis, and maturation of testis (8). Gamma-GT is connected with maturation and proliferation of Sertoli cells, CP-induced increases in its activity means damaged Sertoli cells and disturbed spermatogenesis. Sertoli cell secreted stem cell factors are important to regulate proliferation and differentiation of spermatogonial cells (23), however, CY blocks proliferation and differentiation of spermatogonial cells (24, 25) through down-regulating the expression of stem cell factors in Sertoli cells. AKP and ACP are non-specific phosphatases released from injured degenerating cells and germ cells: increased activities of the two enzymes result in damage to seminiferous epithelium cells and original embryonic cells, a decrease in spermatogenetic time, and damage of reproductive function (26).

A metabolite of CP, acrolein, mainly targeted Sertoli cells in testis, consequently CP could induce sex hormone disorders in male mice (3). Our work revealed that T contents in serum and testis decreased and levels of FSH, LH, and E in serum increased. LH promotes Leydig cells to produce T, and acts with FSH to keep the seminiferous tubule's higher content of T that promotes development and maturation of spermatogenic cells. Decreased synthesis and secretion of T from the CP-destroyed Leydig cells caused a reduction of T content in serum (27) which resulted in a feedback stimulation of the anterior pituitary gland inducing the release of FSH and LH, thus increasing the contents of these two hormones in serum. Moreover, the increase of FSH level and the decrease of T level impaired their effects on Leydig cells and as a result inhibited expression of the 3β-hydroxysteroid dehydrogenase gene (a key enzyme inhibiting production of T) and promoted expression of the cytochrome P450 aromatase gene (P450arom), which converted T to E and thus increased the E content in serum (28). The P450arom was localised within Leydig cells in mice (29). Both Sertoli cells and Leydig cells were found to express P450arom whose expression gradually increased in Leydig cells and decreased in Sertoli cells during the course of rat maturation.

Based on our previous findings and on data in this paper, we observed effective protection and attenuation of SIP on testicular damage in mice administered with CP. The positive efficiency of SIP was not only found in apparent indicators, sperm parameters, testis index, and histopathological characteristics, which were all nearly restored to normalcy by SIP, but also discovered in levels of sexual hormones and activities of some marker enzymes. Meanwhile, antioxidant ability previously destroyed by CP was significantly restored in mice administered with SIP. Increasing evidence to support the idea that the toxicity of CP can be weakened by some natural materials (5, 6, 8, 9) including SIP, further indicates that these compounds are potential cytoprotectors that could be applied in clinical therapy for cancer.

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