Squid ink polysaccharide prevents chemotherapy induced injury in the testes of reproducing mice

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Abstract: The present study was conducted to investigate the preventive effects of squid ink polysaccharides (SIP) on the damage of sperm and reproduction induced by cyclophosphamide that is most commonly used for treating clinically cancers. Male Kunming mice exposed to cyclophosphamide were administered with SIP and were sacrificed to determine sperm parameters, testicular antioxidant ability and reproductive capacity. Data indicated that cyclophosphamide caused obvious changes in mice such as significant reduction (P<0.01) of glutathione reductase activity (GR), vitamin C (Vc) content and total antioxidant capacity (T-AOC) in the testes, as well as elevation (P<0.01) of abnormal rates of sperm and fetus, and a decrease in the total fetal count and average fetal count (P<0.01), were totally alleviated by SIP. From these findings it can be concluded that SIP decreases chemotherapeutic damage to sperm and reproduction in mice induced by cyclophosphamide.

Keywords: Squid ink polysaccharide (SIP), cyclophosphamide, sperm, reproduction, mice.

INTRODUCTION

Cyclophosphamide (CP) is a well-known anti-neoplastic drug and immune-suppressive agent that has been extensively used in the treatment of various cancers and chronic diseases including rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis. In addition to cytotoxic activity on tumor cells, CP exerts similar toxicity on rapidly proliferating normal cells which leads to damage of tissues and functions such as spermatogenesis and reproduction (Emadi *et al.*, 2009).

CP has already been proven by a large number of published reports to have toxicity that results in damage of the male reproductive system in humans and experimental animals (Fraiser *et al.*, 1991). A diminished sperm count and absence of the spermatogenic cycle in the testes were observed in CP treated adult male patients (Howell *et al.*, 1998). It has also been reported that chronic low-dose administration of CP in male rats results in oligospermia and azoospermia and also causes associated biochemical and histological changes in testes and epididymitis (Meistrich *et al.*, 1995; Kaur *et al.*, 1997; Le *et al.*, 2015). Decreased testicular weight and fertility, as well as reduced growth and development of offspring have also been shown in the animals exposed to CP (Trasler *et al.*, 1986; Higuchi *et al.*, 1995; Das *et al.*, 2002). The apparent adverse effects of CP, including reproductive toxicity, restrict the clinical dose of CP to below that can be tolerated by most patients, reducing its efficacy as a chemotherapeutic drug. There is an urgent need for further studies to identify cytoprotective agents that can selectively protect normal tissues without reducing the antitumor effects of chemotherapeutic drug.

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Currently, most studies using functional biological polysaccharides have mainly focused on immuno-modulatory, anti-tumor and other bioactivities. Few studies have attempted to determine the chemoprophylactic effects of polysaccharides against chemotherapeutic drugs, especially using marine derived bioactive polysaccharides. The bioactivities of squid ink are recognized and polysaccharides from black ink have been shown to have anti-tumor (Zong *et al.*, 2013), anti-oxidant (Zuo *et al.*, 2015) and chemoprophylactic activities (Zuo *et al.*, 2015; Le *et al.*, 2015; Liu *et al.*, 2016). Report showed that squid ink polysaccharide (SIP) can effectively protect the intestine of mice from chemotherapy induced injury (Zuo *et al.*, 2015). Our previous reports have shown that SIP alleviated the toxicity of CP on mouse testis via Nrf2/ARE signal pathway (Le *et al.*, 2015a, 2015b) and on ovary through PI3K/Akt/mTOR and p38 MAPK pathways(Liu *et al.*, 2016).

Based on our previous findings that SIP prevents mice testes from chemotherapy induced injury (Le *et al.*, 2015a, 2015b), in the current study, the protective effects of SIP from ink of *Sepia esculenta* on CP-induced damage in the male reproductive system of mice were determined. To further investigate the protective effects of SIP on the damage of male reproduction induced by CP, the present study was conducted to evaluate the bioactivities of SIP on reproduction of mice.

MATERIALS AND METHODS

Animals and experimental protocol

Sexually mature male Kunming mice were allocated into four groups: a control group (CONT, administered orally

with normal saline and injected abdominally with normal saline), a CP-treated group (CP, administered orally with normal saline and injected abdominally with CP in normal saline), a SIP-treated group (SIP, administered orally with SIP and injected abdominally with normal saline) and a co-treated group (SIP+CP, 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 for a continuous ten week period.

Biochemical analysis

The testes were prepared to 10% homogenate with normal saline for the assessment of biochemical parameters including activity of glutathione reductase (GR), the content of vitamin C (Vc) and total anti-oxidant capability (T-AOC). Detection was determined with kits developed by a Bioengineering Institute from China, according to the manufacturer's protocols.

Sperm parameters

Epididymis was cut using scissors into 1mL of normal saline and incubated for 5 min at 37°C to allow sperms to swim out of the epididymis tubule. 10µl of sperm suspension was mixed with an equal volume of 2% eosin and a sperm slide prepared by staining for 1 h at room temperature. Two hundred sperms were examined to determine abnormal rate of sperm in morphology.

Reproduction capability

At the end of the 10 week experimental period, 5 males per group were mated 1:3 with sexually mature natural females for 4 days. Pessary was examined and sperms were observed by microscopic smear at 7 a.m. every day. The day that a vaginal plug or sperms were found was considered day 0 of gestation. On day 18 the mated females were killed, the ovaries were removed and the total number of fetuses was counted. Abnormal fetuses were discriminated to calculate the rate of abnormalities.

Date analysis

Data were expressed as the mean ± standard deviation. Differences between groups were analyzed by ANOVA using the JMP statistical software and separated by Duncan's multiple range test where $p<0.05$ or $p<0.01$ was considered to be significant level.

RESULTS

Antioxidant ability of the testes

The data in fig. 1 show the activity of GR and level of Vc and T-AOC of testes in mice declined significantly with CP treatment in comparison with the control mice by 66.76%, 16% and 27.73% respectively. However, compared with the model mice treated with CP, co-treated of mice with SIP and CP showed significant improvement in the three detected parameters, there were no obvious differences between the co-treated and control mice. This phenomenon suggested that SIP could efficiently relieve CP-induced disruption of anti-oxidant capability and maintain the redox balance of testicular tissue.

Abnormal sperm

The results of the abnormal sperm analysis are presented in table 1 indicating an increased abnormal rate of sperm in the CP administered mice at 75.42% of that observed in the control mice. The percentages of all forms of abnormal sperm such as hook less, neck broken, mid-piece broken and others were all significantly higher than those in the control group, indicating adverse effects of CP on sperm. Pre-treatment with SIP caused significant decreases in the abnormality rates of several types of the abnormal sperm types except for mid-piece broken, and minimized sperm damage induced by CP. In all kinds of abnormal sperm induced by CP, the hook less and the neck broken forms accounted for more than 70% of the abnormalities.

Fertility and general reproductive performance

Data presented in table 2 and fig. 2 showed a strongly negative impact of CP on fertility and reproductive performance in male mice. Pregnancy index, the total number of fetuses and litter size markedly decreased in the CP-exposed mice whilst the percentage of fetal malformation was shown to increase to some extent. However, in the co-administered mice, the significant changes that occurred in CP treated mice disappeared when accompanied by SIP pretreatment. Pregnancy index, total number of fetuses, litter size and the percentage of fetal malformation were shown to be improved in mice pretreated with CP and SIP. Compared with the model mice, the total number of fetuses examined and litter size increased by 51.51% and 26.21% respectively and the percentage of fetal malformation decreased.

DISCUSSION

It has been reported that CP can cause oligospermia, azoospermia and the destruction of reproductive function (Anderson *et al.*, 1995). CP administration causes a decrease in body weight and reproductive organ mass, an increase in the abnormal rate of sperm, alteration of sperm acrosome morphology and damage of deoxyribonucleic acid in male mice (Selvakumar *et al.*, 2005). In this study, CP exposed mice showed a reduction in sperm quality and an increase in the percentage of abnormal sperm resulting in reduced reproductive ability, a decrease in the total number of fetuses and an increase in fetal malformation. The results were consistent with observations from previous report (Selvakumar *et al.*, 2005).

The mechanism by which CP mediates sperm damage may be correlated to CP inducing a sharp increase in reactive oxygen species (ROS). CP's metabolite acrolein

Table 1: Effects of SIP on sperm in CP-exposed mice

Items	CONT	CP	SIP	CP+SIP
Hookless / %	1.60 ± 2.13^{ABb}	4.63 ± 1.73^{Aa}	2.94 ± 2.51^{ABab}	1.86 ± 0.87^{Bb}
Neck broken / %	7.20 ± 1.99^{Bb}	12.13 ± 4.63^{Aa}	6.44 ± 2.56^{Bbc}	4.68 ± 1.63^{Bc}
Midpiece broken / %	9.80 ± 2.35^{Ab}	15.88 ± 4.92^{Aa}	9.81 ± 3.35^{Ab}	15.50 ± 2.88^{Aa}
Others / %	1.85 ± 1.17^{Bb}	6.06 ± 1.80^{Aa}	2.94 ± 1.92^{Bb}	3.14 ± 2.43^{Bb}
Abnormal sperm / %	21.28 ± 2.41^{Bb}	37.33 ± 3.74^{Aa}	22.00 ± 3.51^{Bb}	25.27 ± 4.28^{Bb}

Notes: others include bulb-head, looped-head, bicephalic, *etc*. Data are presented as mean±S.D. Values with different lowercase or uppercase superscripts mean significant difference ($p<0.05$ or $p<0.01$).

Table 2: Effects of SIP on fertility and reproductive performance in CP-treated mice

Items	CONT	CP	SIP	CP+SIP
Mating index[@]	90（9/10）	8(8/10)	8(8/10)	90(9/10)
Pregnancy index[&]	100(9/9)	75(6/8)	87.5(7/8)	100(9/9)
Total number of Fetuses examined	134	48	95	98
Litter size	14.89 ± 1.45^{Aa}	8 ± 2.45^{Cc}	13.57 ± 1.99^{ABa}	10.89 ± 2.26^{BCb}
Fetal deaths	1.22 ± 1.39^{Bc}	4.83 ± 3.06^{Aa}	1.43 ± 1.72^{Bbc}	3.22 ± 1.09^{ABb}
Live fetuses	13.67 ± 0.87^{Aa}	3.17 ± 1.17^{Cc}	12.14 ± 1.68^{Aa}	7.67 ± 2.29^{Bb}
Fetal malformations	0.44 ± 0.53^{Aa}	0.50 ± 0.55^{Aa}	0.86 ± 0.69^{Aa}	0.33 ± 0.50^{Aa}

Notes: Data are presented as mean±S.D. Values with different lowercase or uppercase superscripts mean significant difference($p<0.05$ or $p<0.01$). [@](Number of males producing a vaginal females/number of males cohoused with females) ×100, [&](Number of males producing pregnant females/number of males producing a vaginal plug in females)×100.

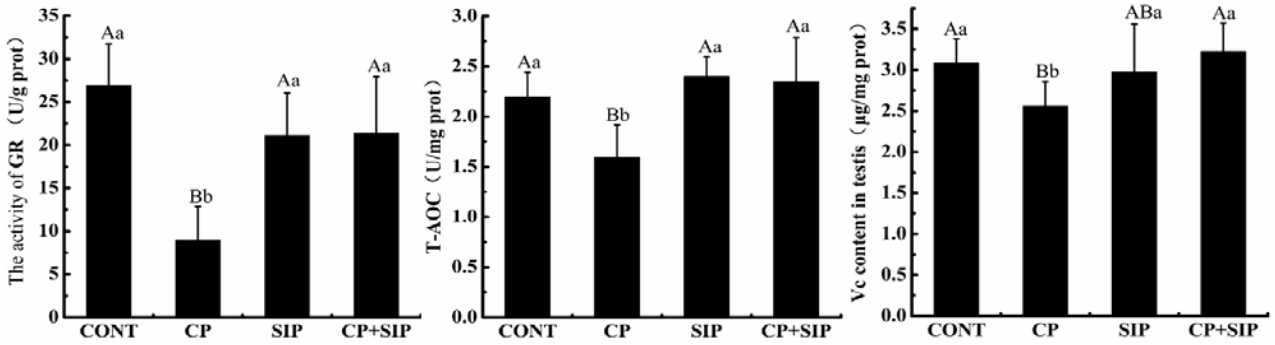

Fig. 1: The effects of SIP on antioxidant ability of testes in mice treated with CP. Data were determined with kits developed by a professional Bioengineering Institute from China. Bars indicate means ± standard deviations. Values with different lowercase or uppercase superscripts mean significant difference ($p<0.05$ or $p<0.01$).

can promote ROS generation that results in lipid peroxidation and cell apoptosis in mice (Sikka, 2004). Lipid peroxidation is closely related to the impairment of membrane functioning such as decreased fluidity, inactivation of membrane bound receptors and an increase in the non-specific permeability to ions (Motawi *et al.*, 2010). This impairment plays a pivotal role in leading to the decline of sperm motility and viability, and an increase in acrosome reaction defects (Agarwal *et al.*, 2002). The anti-oxidative system consists of enzymatic and non-enzymatic reactions. Reduction of anti-oxidant enzymes activities and anti-oxidants contents in testes are linked with CP-induced oxidative stress which results in lipid peroxidation and oxidative damage in testicular tissue (Gutierrez-Correa *et al.*, 1997). To explain whether the reduction of sperm quality is associated with oxidative stress induced by CP, this study investigated the anti-oxidative capability of testicular tissue. The data showed a significant depletion of anti-oxidant ability in the testes of CP-exposed mice but the reduction of anti-oxidant ability that CP caused in testes was effectively abrogated by SIP directly implying that SIP acted as a cyto-protector to perform preventive effects on CP in the disruption of anti-oxidative ability and to maintain redox balance. These results were in agreement with to our previous reports and further validate the protective effect of SIP on CP-induced damage of sperm and reproduction in mice may be linked with SIP eliminating excess ROS induced by CP.

Fig. 2: Uteri containing fetuses from female mice of the four groups. At the end of trail period, female mice were sacrificed and decapitated to obtain the whole uteri. Fetuses of each uterus was counted and used to analyze the scheduled parameters.

It is well-known that different sperm parameters such as sperm concentration, motility and viability are closely linked to fertilization and growth of zygotes and that CP can result in decrease of sperm quality. The toxic effects of CP on sperm were confirmed again in this study as the deleterious impact was evident in the reduction of abnormal rates of sperm. Meanwhile, pregnancy index, total number of fetuses and litter size were all negatively affected by CP, which suggested a drop of fecundity in CP-exposed male mice. Additionally, a positive role of SIP on CP associated damage on sperm was demonstrated. The preventive effects of the marine polysaccharide observed in this paper, including survival rate, abnormality rate and quantity of sperm which resulted in improvement of pregnancy index, total number of fetuses and fetal quality, which must be originated from intervention of SIP against CP-mediated oxidative stress via activating Nrf2/ARE signal pathway (Le *et al.*, 2015a, 2015b).

CP exposure causes genotoxic damage and inhibits proliferation of spermatogenic cell prior to the meiotic pachytene stage during spermatogenesis resulting in the damage of sperm cells including decrease sperm vitality and viability and increased abnormal sperm (Schimenti *et al.*, 1997). In addition, spermatogenesis is limited by many other aspects such as cell apoptosis and endocrine disorder (Cai *et al.*, 1997; Oh *et al.*, 2007) caused by CP. Our previous report showed the *in vitro* preventive roles of SIP on DNA strand breakage caused by ultraviolet radiation and H_2O_2 (Luo *et al.*, 2013) which may be a potential for the alleviated reproductive toxicity in CP treated mice.

CONCLUSION

Based on our previous findings, SIP *in vivo* prevented testes of mice from CP-mediated damage via Nrf2/ARE signal pathway (Le *et al.*, 2015a, 2015b), and *in vitro* protected Leydig cells from acrolein induced oxidant stress by regulating autophagy and apoptosis through PI3K/Akt and p38 MAPK pathways (Gu *et al.*, 2017). This paper further revealed that SIP could effectively prevent testes and sperm of mice from CP caused oxidant damage through evaluating sperm quality indicators and reproductive performance, rescuing reproductive ability of male mice exposed to CP. These results indicate that SIP is a potential marine bioactive substance that can be developed to be a chemotherapy adjuvant drug to protect male reproductive ability of childbearing cancer patients.

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