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Inhibition of acrolein-induced autophagy and apoptosis by a glycosaminoglycan from *Sepia esculenta* ink in mouse Leydig cells

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Propidium iodide (PubChem CID: 104981)
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ABSTRACT

In our recent reports, a squid ink polysaccharide (SIP) was found having preventive activity against cyclophosphamide induced damage in mouse testis and ovary. Here we further reveal the regulative mechanism of SIP against chemical toxicity on testis. Leydig cells exposed to acrolein (ACR) underwent apoptosis at 12 h and 24 h. Before apoptosis, cells occurred autophagy that was confirmed by high autophagic rate and Beclin-1 protein content at 3 h. PI3K/Akt and p38 MAPK signal pathways involved in the regulatory mechanisms. These outcomes of ACR were recovered completely by SIP, which was demonstrated by attenuated disruption of redox equilibrium and increased testosterone production, through suppressing ACR-caused autophagy and apoptosis regulated by PI3K/Akt and p38 MAPK signal pathways in Leydig cells. Summarily, autophagy occurred before apoptosis caused by ACR-activated p38 MAPK and PI3K/Akt pathways were blocked by SIP, resulting in survival and functional maintenance of Leydig cells.

1. Introduction

As one of the most commonly used chemotherapeutic agents, cyclophosphamide (CP) is widely used for clinical management on several cancers (Emadi, Jones, & Brodsky, 2009). However, a series of undesirably systematic side effects on normal tissues/organs (Emadi et al., 2009) result in reduction of dosage for escaping systematic toxicity and in consequent decrease of therapeutic effects. In order to attenuate the systematic side effects and elevate the efficacy of chemotherapy, complementary and alternative medicine, particularly natural products, has been studied for decades.

A squid ink polysaccharide (SIP), a kind of glycosaminoglycan, has been isolated recently from *Sepia esculenta* ink and verified to be effective in preventing testis and ovary of mice from CP-mediated toxicity in our laboratory (Le, Luo, Gu, Tao, & Liu, 2015a; Le, Luo, Gu, Tao, & Liu, 2015b; Liu et al., 2016). SIP protected mouse ovaries exposed to CP through weakening autophagy to inhibit the apoptosis of ovarian cells, such as granulosa cells, or supressing autophagy-associated cell death to reduce programmed cell death (Liu et al., 2016). The work also revealed that the preventive mechanisms of SIP on mouse ovary were correlated with p38 mitogen-activated protein kinase and Akt-associated signaling pathways (Liu et al., 2016). Interestingly, our another simultaneous investigation found that testicular oxidative stress damage

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occurred in the mice exposed to CP and was interfered by SIP via altering protein contents of keap-1 and histone deacetylase 2 to trigger Nrf2/ARE signaling pathway to up-regulate expression of the downstream phase II enzymes and antioxidative enzymes (Le et al., 2015a, 2015b).

It is well known that CP must be metabolized to acrolein (ACR) and phosphoramide mustard by liver-originated enzymes before exerting killing effects on tumor or normal cells (Emadi et al., 2009). So further investigation of the accurate molecular mechanisms for CP to injure cells should be based on *in vitro* experiments of cells treated with ACR or phosphoramide mustard. In order to approach the mechanisms, ACR was used in this study. Key reason of employing ACR is that ACR is a biotransformation product of CP and is also the product of endogenous lipid peroxidation reactions, consumption of cigarettes and intake of automobile exhausts, overheated cooking oils and residual herbicides, which are everywhere in our surroundings and has been proved fully to have toxicity on reproductive system (Kern & Kehrer, 2002; Lewars & Liebman, 2013; Tully, Zheng, Acosta, Tian, & Shi, 2014). Another critical cause is different functions of the two metabolites, i.e., phosphoramide mustard functions in anticancer and ACR is mainly responsible for toxic side effects on normal cells of tissues/organs (Emadi et al., 2009). Therefore, this study aimed to investigate the protective effects of SIP against ACR toxicity on survival and function of mouse Leydig cells, as well as the possible mechanisms.

2. Materials and methods

2.1. Preparation of SIP

According to our methods (Liu et al., 2016), the procedure is modified slightly and briefly discribed as follows. Fresh ink derived from squid sacs (*Sepia esculenta*) was stored at −70 °C and was thawed at 4 °C followed by dilution with PBS and then ultrasonicated. The suspension was stored at 4 °C for more than 8 h and then subjected to centrifugation at 4 °C, 8000 rpm. The supernatant was hydrolyzed with papain for 90 min and then heated to denature the enzyme. The deproteinized ink solution was mixed with four volumes of ethanol. Crude polysaccharides were obtained through recovery of the precipitate and then further separated into three fractions by DEAE-52 cellulose column chromatography. SIP was in the first fraction, that fraction was collected and then dialyzed, concentrated under vacuum, freeze-dried, and stored at −20 °C.

2.2. Cell culture

Primary Leydig cells of mice (purchased from ATCC) were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified incubator.

2.3. Cell viability and biochemistry detection

Cell viability was detected with WST-1 assay kit. Testosterone (T) content in medium was measured with enzyme-linked immunosorbent assay kit. Intracellular activity of superoxide dismutase (SOD), total antioxidative ability (TAOC) and content of malondialdehyde (MDA) were determined with kits respectively. The kits were purchased from a bioengineering institute in China.

2.4. Ultrastructure observation

Cells were fixed with 5% glutaraldehyde at 4 °C for 2 h, and then washed three times with PBS followed by post-fixing with 1% osmium tetroxide for 2 h at 4 °C. After washing twice with PBS, cells were dehydrated with ethanol and acetone, embedded in Epon812 and sectioned with an ultra-microtome. The stained sections were observed using transmission electron microscope.

2.5. Double staining of Leydig cells with Annexin V-FITC/PI

Leydig cells were washed twice to remove medium, and added to 500 μl of Binging buffer with 5 μl of Annexin V-FITC and 5 μl of PI. Cells were incubated for 10 min in dark at room temperature. The confocal laser scanning microscope was used to observe apoptosis of Leydig cells.

2.6. Monodansylcadaverin staining of Leydig cells

Leydig cells were washed twice and added monodansylcadaverin (MDC) staining solution before incubating for 30 min in dark at room temperature. The confocal laser scanning microscope was used to observe autophagy of Leydig cells. Image Pro Plus 6.0 software was utilized to analyze MDC-positive cells.

2.7. Flow cytometry assay

Cells were stained with 50 μM MDC for 30 min at 37 °C, trypsinized, washed, and collected in PBS. Cells illuminated with

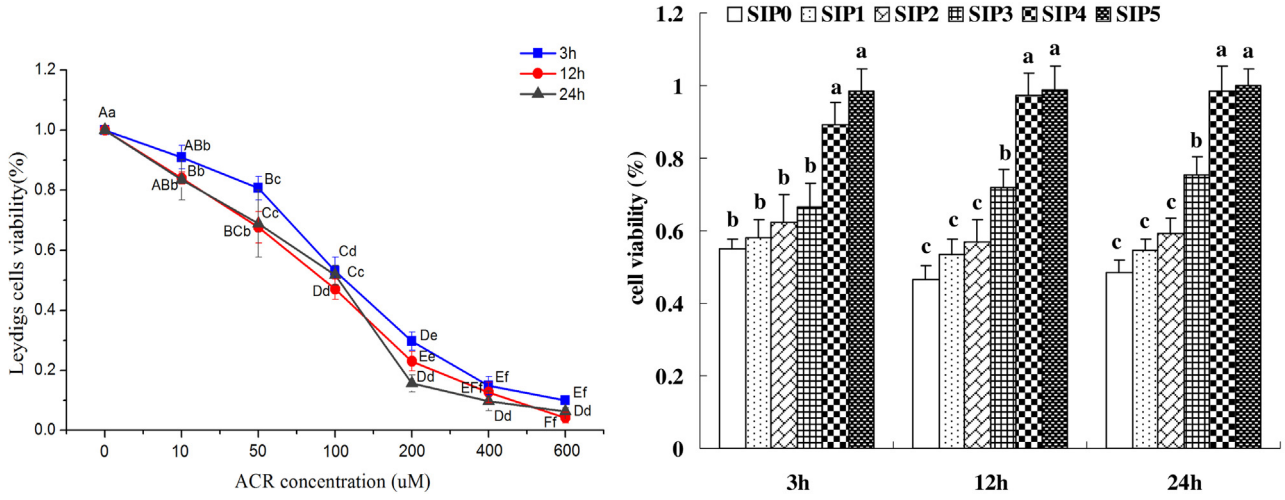

Fig. 1. SIP inhibited ACR mediated death of Leydig cells. Primary Leydig cells of mice grown to 70% confluence were exposed to ACR (0, 10, 50, 100, 200, 400 and 600 μmol/L) and different concentrations of SIP (0, 0.1, 0.2, 0.5, 1 and 2 mg/ml, corresponding to SIP0, SIP1, SIP2, SIP3, SIP4 and SIP5 respectively) for 3, 12 and 24 h respectively. Cell viability was measured with WST-1 assay. $^{abcdef}p < 0.05$.