



Role of Endoplasmic reticulum apoptotic pathway in testicular Sertoli cells injury induced by Carbon disulfide

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HIGHLIGHTS

- Ultrastructures of Endoplasmic reticulum (ER) were damaged after CS₂ exposure.
- CS₂ exposure increased apoptotic Sertoli cells.
- Signals of ER apoptotic pathway were obviously changed.
- Endoplasmic reticulum apoptotic pathway plays a crucial role in CS₂-induced injury.

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ABSTRACT

The exposure of Carbon disulfide (CS₂) is associated with germ cell injury and male infertility in animals and humans. However, the molecular mechanism is currently unknown. This study show here that CS₂-induced Sertoli cells injury via Endoplasmic reticulum (ER) apoptotic pathway. SD male rats were exposed to doses of CS₂ (0, 50, 250, 1250 mg m⁻³) for 4 weeks. After treatment, loose structures of seminiferous tubules and disordered cell arrangements were observed by light microscopy. Ultrastructural lesions, deformed chromatin and vacuoles formed from swollen ER were observed by electron microscopy. After primary culture of Sertoli cells, a dose-dependent increased apoptosis were found. The increased activity of Caspase 3, accumulation of intracellular Ca²⁺, up-regulation of mRNA and protein expressions of ER apoptotic relative molecules (Calpain 2, Cleaved-Caspase 12, GRP78 and CHOP) were also found in this study. Altogether, our findings indicated that ER apoptotic pathway played an important role in CS₂-induced Sertoli cell impairment.

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1. Introduction

Carbon disulfide (CS₂), as important solvent and reagent, has long been used in the industry and daily life, such as synthetic fibers, rubber manufacture, dry cleaners production and many other fields. In recent years, the amounts of CS₂ usage are becoming even more, due to the existence of a considerable number of viscose rayon manufactures. However, in developed countries, CS₂ is a major concern of detectable chemicals in the effluent (Chou et al., 2005). It was even ranked as a high priority hazardous air pollutant in Clean Air Act in the United States (Vanhoorne et al., 1994). This is because CS₂ has toxic effects on almost all systems,

particularly on nervous system, cardiovascular system and reproductive system (Tan et al., 2003; Gelbke et al., 2009; Luo et al., 2011). Among those toxic effects, the injury on male reproductive system has recently raised public attention.

CS₂ was the first industrial solvent to be considered as a testicular toxicant (Gondzik, 1970). High-level CS₂ exposure has been well documented to produce change of seminal characteristics in male workers and animal studies (Balabaeva et al., 1982; Tepe and Zenick, 1984). The Chinese Society for Occupational Health recommended a standardized occupational exposure limits (OELs) for CS₂ to 10 mg m⁻³ (PC-STEL) and 5 mg m⁻³ (PC-TWA) (GBZ2.1-2007). And to the worldwide extent, OELs for Carbon disulfide vary between 1 and 10 ppm (Gelbke et al., 2009). However, as some researchers reported, numerous factories had not followed this rule for technical reasons and economic interests (Chen and Tan, 2004;

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Zhang, 2008). An earlier study of ours indicated that lesions on the male sexual function and semen quality were found in Chinese rayon manufacturing workers when the average exposure level was 10 mg m^{-3} (Ma et al., 2010). Moreover, as shown in another study of our laboratory, long-term exposure to low-dose of CS_2 could also significantly impact the male sexual function, semen quality and quantity in experimental animals (Chen et al., 2005).

As known to all, normal testicular structure and enough spermatocyte are important prerequisites for normal testicular function (Tousson et al., 2012). Sertoli cells play crucial role in regulating spermatogenesis. Sertoli cells perform the functions of nourishing and supporting spermatocyte, secreting growth factors and building the blood-testis barrier (Zhang et al., 2010; Chen et al., 2013). Therefore, excessive injury of Sertoli cells would result in abnormal spermatogenesis, even male reproductive dysfunction (Lin et al., 1997; Jiang et al., 2013). Some previous animal studies had demonstrated that exposure of reproductive toxicants could cause male reproductive dysfunction in rats by increasing Sertoli cells apoptosis (Li et al., 2000; Song et al., 2011).

Apoptosis, as a tightly controlled process in cell death, is executed for maintaining steady state. In testis, 75% cells are eliminated by apoptosis (Allan et al., 1992). The intrinsic signaling events leading to apoptosis could be divided into two major pathway, mitochondrial apoptotic pathway and Endoplasmic reticulum (ER) apoptotic pathway (Ji et al., 2011). In our previous study, we have demonstrated that mitochondrial apoptotic pathway played an important role in CS_2 -induced apoptosis (Guo et al., 2014). However, the results implied that there could be some other pathways involved in the CS_2 -induced Sertoli cell apoptosis. Many recent researches reported that ER stress was observed in several apoptotic mechanisms (Giorgi et al., 2010; Tabas and Ron, 2011). Therefore, we presumed that ER apoptotic pathway may take part in CS_2 -induced Sertoli cell apoptosis. To verify this hypothesis, Caspase 3, Calpain 2, Caspase 12, Glucose regulated protein (GRP78) and CHOP, as important molecules of ER apoptotic pathway, were introduced in this study (Seimon et al., 2010; Tsukano et al., 2010; Li et al., 2011).

To gain an insight on whether ER apoptotic pathway played a role in CS_2 -induced apoptosis in Sertoli cells, we carried out this study. The results would deepen our understanding on the mechanism by which CS_2 impaired male reproductive systems.

2. Materials and methods

2.1. Materials

Carbon disulfide (purity $\geq 98\%$) was purchased from Shanghai Sihewihua chemical plant (China). All other chemicals used were analytical grade. Intracellular Ca^{2+} assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Antibodies were obtained from SANTA CRUZ (USA). Organic glass exposure cabinets were self-developed (Tongji Medical College).

2.2. Animal treatment

Forty mature male Sprague-Dawley rats (the Experimental Animal Center of Huazhong University of Science & Technology, Tongji Medical College, China), aged 7 weeks (w), housed in cages with free access to food and water in an environment maintained on a 12:12 h light-dark cycle, in a controlled temperature ($20\text{--}25^\circ\text{C}$) and humidity ($50 \pm 5\%$) environment. Animals were randomly divided into four groups ($n = 10$ for each). The rats were subjected to the following treatments: (1) animals from group I were ventilated with fresh air; (2) animals from group II, III and IV were statically ventilated with air containing CS_2 at

concentrations of 50, 250 and 1250 mg m^{-3} , respectively. Control and the three concentrations of CS_2 were chosen from no significant effect to maximum effect to observe the dose-dependent relationship of CS_2 exposure; and (3) the administration lasted 2 h d^{-1} , 5 d w^{-1} . The exposure frequency of CS_2 was set to simulate the real exposure of workers in workshop. Rats were sacrificed after 4 weeks treatment. The testes were removed and weighted. A part of testes were treated for microscopy. And the rest of the testes were treated into primary cells.

All procedures of animal experiments in our study followed the Guide for the Care and Use of Laboratory Animals established by Ministry of Health of People's Republic of China. Animal protocols No. 4209800122.

2.3. Hematoxylin and eosin (HE) staining

The testes were fixed in 4% formaldehyde for 1 h, and then embedded in paraffin. After that, the blocks were sliced into $5 \mu\text{m}$, stained with a standard HE staining, and then examined by light microscopy.

2.4. Transmission electron microscopy

For ultrastructural studies, testes were cut into small pieces (1 mm^3), fixed immediately in 2.5% glutaraldehyde, and washed three times with phosphate buffer. After post-fixed in 1% OsO_4 , the slices were washed with phosphate buffer, dehydrated in acetone, infiltrated and embedded in low-viscosity spur media and polymerized. Semithin sections were stained with toluidine blue, cut into 90 nm , mounted on 200 mesh copper grids and then stained with uranyl acetate and lead citrate. Finally, ultra thin sections were observed under a transmission electron microscope (FEI, Tecnai G212, The Netherlands).

2.5. Primary cultures

The rest of testes were treated into primary culture for further study. Briefly, tunicae and blood vessel of the testes were rejected. The tissues were washed, minced and transferred to a flask. And then, tissues were digested with trypsinization and collagenase I in turn, for 30 min respectively. After filtered through a 200-mesh stainless steel filter, primary cells were seeded in 6-well plates at a density of 10^6 cells per well. Cells were cultured in 20% fetal bovine serum-DMEM in a 35°C , 5% CO_2 humidified incubator. After 24 h, germ cells were suspended in the diurn and removed by changing the medium. Meanwhile, Sertoli cells were adherent to the bottom. The final purity of Sertoli cells were more than 95%.

2.6. Feulgen staining analysis

Sertoli cell grew on sterilized glass coverslips in 6-well plates (1×10^6 cells/well) for 48 h, fixed in 95% ethanol for 15 min at room temperature. Coverslips were treated with 1 mol L^{-1} HCl for 1 min at room temperature and preheated 1 mol L^{-1} HCl for 10 min at 60°C . And then cells were cultured with Schiff reagent for 1 h at room temperature in dark room. After that, coverslips were washed with 0.5% Na_2SO_3 and H_2O for three times, respectively. Following being dehydrated using graded ethanol, vitrified by dimethylbenzene and deposited in holly oil.

2.7. Apoptosis assay

Apoptotic Sertoli cells were analyzed by Annexin V-FITC Detection kit (Beyotime, Jiangsu, China). Cells were plated at the density of 1×10^6 cells/well and cultured for 48 h. And then cells were resuspended in binding buffer and then incubated with

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