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# Carbon disulfide induces rat testicular injury via mitochondrial apoptotic pathway

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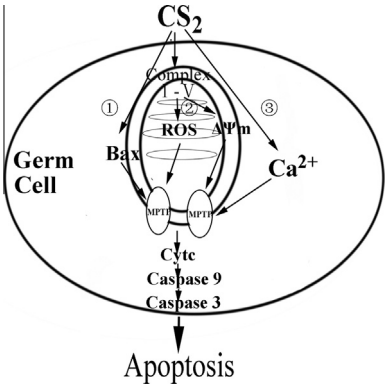
## HIGHLIGHTS

- The exposure of $CS_2$ induced testicular germ cells apoptosis.
- All factors of mitochondrial apoptotic pathway changed after $CS_2$ exposure.
- Inhibitor of MPTP could efficiently reverse the apoptosis.
- Mitochondrial apoptotic pathway plays a role in $CS_2$-induced testicular injury.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Carbon disulfide ($CS_2$), one of the most important volatile organic chemicals, was shown to have serious impairment to male reproductive system. But the underline mechanism is still unclear. In the present study, we aim to investigate the male germ cell apoptosis induced by $CS_2$ exposure alone and by co-administration with cyclosporin A (CsA), which is the inhibitor of membrane permeability transition pore (MPTP). It was shown that $CS_2$ exposure impaired ultrastructure of germ cells, increased the numbers of apoptotic germ cells, accumulated intracellular level of calcium, elevated ROS level, and increased activities of complexes of respiratory chain. Meanwhile, exposure to $CS_2$ dramatically decreased the mitochondrial transmembrane potential ($\Delta\Psi m$) and levels of ATP and MPTP opening. Exposure to $CS_2$ can also cause a significantly dose-dependent increase in the expression levels of Bax, Cytc, Caspase-9, and Caspase-3, but decreased the expression level of Bcl-2. Moreover, co-administration of CsA with $CS_2$ can reverse or alleviate the above apoptotic damage effects of $CS_2$ on testicular germ cells. Taken together, our findings suggested that $CS_2$ can cause damage to testicular germ cells via mitochondrial apoptotic pathway, and MPTP play a crucial role in this process.



## 1. Introduction

Carbon disulfide ($CS_2$) is one of the typical VOCs, which is frequently used in daily life as dry cleaning and insecticide. It is also widely used as an indispensable organic solvent in industrial

*Abbreviations:* $CS_2$, carbon disulfide; CsA, cyclosporin A; Cytc, Cytochrome c; MPTP, mitochondrial membrane permeability transition pore; $\Delta\Psi m$, mitochondrial transmembrane potential; ROS, reactive oxygen species; VOCs, volatile organic chemicals.

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productions. Therefore, humans as well as wildlife are easy to be exposed to $CS_2$. Large numbers of experiments have indicated that extensive exposure to $CS_2$ is associated with multisystem disturbance (Morvai et al., 2005; Ding et al., 2011), especially with the reproductive system (Xuan et al., 2009; Wang et al., 2011). Many human occupational hazard surveys suggest that $CS_2$ exposure is related to sexual dysfunction and decreased sperm count and motility (Cirla et al., 1978; Ma et al., 2010). However, there are limited studies to elucidate how $CS_2$ impairs the male reproductive system.

Apoptosis is a form of programmed cell death which is crucial for mammalian development (Sheridan and Martin, 2010). However, excess germ cell apoptosis may lead to testicular dysfunction and decreased sperm count (Sawhney et al., 2005). Mitochondrial apoptotic pathway, also known as the intrinsic apoptotic pathway, is involved in germ cell apoptosis. A variety of key events are involved in this pathway, such as the participation of Bcl-2 family, changes of $\Delta\Psi m$, the decrease of ATP, the production of ROS, and the MPTP opening (Green and Reed, 1998). The MPTP opening plays a core role in all events above, since it allows the release of pro-apoptotic factors, such as Cytochrome c (Cytc) and Apaf 1, that can induce the process of apoptosis (Tsujimoto and Shimizu, 2007; Kinnally et al., 2011). Thus, in order to clarify the role of MPTP in the mechanism of $CS_2$-induced apoptosis, CsA was introduced in this study.

The objective of this study is to investigate the ultrastructural changes caused by $CS_2$, and to elucidate whether $CS_2$ induces male germ cell apoptosis via the mitochondrial apoptotic pathway, especially through MPTP opening, and how mitochondrial apoptotic pathway exerts its functions in this process.

## 2. Materials and methods

### 2.1. Animal model of $CS_2$ toxicity and drug treatment

Male Sprague–Dawley rats were obtained from the Experimental Animal Center of Huazhong University of Science &Technology, Tongji Medical College, China (animal protocols No. 4209800122). Forty-eight rats, aged 7 weeks, were randomly divided into six groups ($n = 8$ for each group) and housed in polycarbonate cages under controlled laboratory conditions (12 h light/dark cycle photoperiod, 25 °C, standard diets were received and tap water was accessible ad libitum).

Animals from group I, II, III, IV, V and VI were statically inhaled air containing $CS_2$ (purity $\geqslant$ 98%, Siheweihua chemical plant, China) in organic glass exposure cabinets (self-developed by Tongji Medical College). The concentrations of $CS_2$ were 0, 50, 250, 1250, 0, 1250 mg $m^{-3}$, respectively. During the inhalation period, eight rats of the same group were put in an exposure cabinet. Then, according to our experimental design, different doses of $CS_2$ were dropwise added in the plate just in the middle of two fans. The airflow through the fan ensured that the concentration of $CS_2$ remained constant in the exposure cabinets, during the 2 h $d^{-1}$ exposure. Animals were deprived of food and water during the treatment. The exposure time was 2 h $d^{-1}$, 5 d $w^{-1}$, for 10 weeks. In the last 6 weeks, animals were subjected to the following treatments by oral intake: group I, II, III and IV received milk; group V and VI received 12.5 $mg^{-1}$ $kg^{-1}$ $d^{-1}$ CsA (Dalian Meilun Biotechnology Co., Ltd.), using milk as solvent.

Body weights of rats were measured twice per week. All procedures of animal experiments in our study followed the Guide for the Care and Use of Laboratory Animals established by Tongji Medical College.

### 2.2. Sample preparation

After 10-week exposure, animals were sacrificed by decapitation. After weighting, testes were isolated, and then fixed in 4% formaldehyde, treated into primary cells or stored at −80 °C till later analysis.

#### 2.2.1. Hematoxylin and eosin (HE) staining

After fixed, the testes were embedded in paraffin and sliced. The sections were stained with hematoxylin and eosin, and then examined by light microscope.

#### 2.2.2. Immunohistochemistry staining

We performed immunohistochemistry staining with antibodies to Bcl-2, Bax, and Cytc (Golden Bridge, Beijing, China). Specimens were fixed in 10% deparaffinized formalin. After microwave antigen retrieval, sections were deparaffinized and rehydrated. And then, sections were blocked in 3% goat serum and incubated with rabbit antibody (dilution 1:500) for 1 h. After that, the sections were incubated with anti-rabbit secondary (dilution 1:200) for 30 min. Slides labeled 3,3′-diaminobenzidine (Sigma, US). Pictures were acquired by an Olympus DP12 microscope (Olympus, US).

#### 2.2.3. Electron microscopy

After fixed, blocks of testes were diced. The slices were washed in ice-cold cacodylate buffer, and then postfixed in 1% $OsO_4$ in phosphate buffer. Sections were examined with FEI Tecnai $12G^2$ transmission electron microscope (FEI, Eindhoven, Netherlands).

### 2.3. TUNEL assay

The TUNEL assay was carried out according to the manufacturer's protocol (Roche, Mannheim, Germany). After deparaffinized with 4% paraformaldehyde, the sections were rinsed with PBS and then incubated and permeabilized with 0.1% Triton X-100 for 15 min for FITC endlabeling the fragmented DNA. After washed with PBS, sections were incubated with 50 μL of TUNEL inspection fluid for 60 min at 37 °C in dark and then rinsed for three times. The TUNEL-positive cells were acquired by Olympus fluorescent microscopy with 488 nm/530 nm wavelengths.

### 2.4. Measurement of cell level

#### 2.4.1. Primary culture

Parts of testes from $CS_2$-exposed rats were treated into primary culture for investigations in cell level. Briefly, tunicae and blood vessel of the testes were rejected. And then cut into small pieces and transferred into a flask. After digestion with trypsinization and collagenase I for 30 min, cells were seeded in 6-well plates at a density of $10^5$ cells per well. Then cells were cultured in DMEM supplemented with 20% fetal bovine serum at 35 °C in a 5% $CO_2$ humidified incubator, and experimental cells were assessed after incubated for 24 h.

#### 2.4.2. Mitochondrial membrane potential determination

JC-1 probe was employed to measure $\Delta\Psi$. Primary cells were incubated with 1 mL JC-1 staining solution (5 μg $mL^{-1}$) at 37 °C for 30 min and rinsed for three times. Then cells were placed in fresh medium without serum. The $\Delta\Psi$ were monitored by an Olympus fluorescent microscope at 514 nm/585 nm. Mitochondrial depolarization was indicated by an increase in the ratio of red/green fluorescence intensity.

#### 2.4.3. Measurement of intracellular $Ca^{2+}$ and ROS

To monitor the release of $Ca^{2+}$, germ cells were loaded with 5 μM of $Ca^{2+}$ indicator Fluo-3/AM (Beyotime, China) for 30 min at 37 °C. Cells were washed with D-Hank's, and then incubated for 20 min at 37 °C. Changes in $Ca^{2+}$ levels were measured by an Olympus Fluoview system (Olympus IX71, Germany). The mitochondrial mediated influx of $Ca^{2+}$ was obtained by Image Pro Plus 6.0.

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