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DNA damage and apoptosis of endometrial cells cause loss of the early embryo in mice exposed to carbon disulfide

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ABSTRACT

Carbon disulfide (CS_2) may lead to spontaneous abortion and very early pregnancy loss in women exposed in the workplace, but the mechanism remains unclear. We designed an animal model in which gestating Kunming strain mice were exposed to CS_2 via i.p. on gestational day 4 (GD4). We found that the number of implanted blastocysts on GD8 was significantly reduced by each dose of 0.1 LD_{50} (157.85 mg/kg), 0.2 LD_{50} (315.7 mg/kg) and 0.4 LD_{50} (631.4 mg/kg). In addition, both the level of DNA damage and apoptosis rates of endometrial cells on GD4.5 were increased, showed definite dose–response relationships, and inversely related to the number of implanted blastocysts. The expressions of mRNA and protein for the Bax and caspase-3 genes in the uterine tissues on GD4.5 were up-regulated, while the expressions of mRNA and protein for the Bcl-2 gene were dose-dependently down-regulated. Our results indicated that DNA damage and apoptosis of endometrial cells were important reasons for the loss of implanted blastocysts induced by CS_2.

Introduction

Carbon disulfide (CS_2), a raw material, is widely used in various industrial processes, such as vulcanizing rubber, fumigating grain, extracting oil, manufacturing viscose rayon fibers, and in agriculture as dithiocarbamate fungicides (Ma et al., 2010). In China and some other developing countries, a large number of female employees working in the production of synthetic fibers are exposed to CS_2. CS_2 has multiple-system toxicity (Bortkiewicz et al., 2001; Jhun et al., 2009; Song et al., 2009; Sun et al., 2009; Wang et al., 2009). Many epidemiological studies have focused on the relationship between CS_2 exposure and human reproductive health and have found that CS_2 exposure is associated with various types of reproductive disturbance (Beauchamp et al., 1983; Bezversenko, 1984; Cai et al., 1990; Chen et al., 2001; Deng et al., 1998; Lancranjan, 1972; Pieleszek, 1997; Vanhoorne et al., 1994; Wang et al., 1999a). Testicular atrophy, dyszoospermia, sexual dysfunction, testosterone decline, and significant decrease in the quality of semen were found in CS_2-exposed male workers (Cai et al., 1990; Deng et al., 1998; Lancranjan, 1972; Vanhoorne et al., 1994). On the other hand, previous studies reported that CS_2 could induce menstrual disorders (Bezversenko, 1984; Chen et al., 2001; Wang et al., 1999a) and abnormal labor, such as spontaneous abortion (Pieleszek, 1997) and premature birth (Wang et al., 1999a), among female employees. Our previous cohort study showed that the rate of very early pregnancy loss, confirmed by detecting the level of human chorionic gonadotropin in the urine of each menstrual cycle, was 48.7% in the CS_2-exposed group compared with 26.3% in the control (Wang et al., 1999b). At the same time, we found that the time-to-pregnancy in the exposed group was longer than the control, in both the retrospective (Wang et al., 1997) and prospective (Wang et al., 2000) studies. These results indicated that the period of early embryo development was sensitive to CS_2 exposure. Furthermore, we previously showed (Wang et al., 2005a, 2005b) that CS_2 exposure for three consecutive days during the peri-implantation period could disrupt the process, leading to the loss of implanted blastocysts.

A successful implantation depends on two important factors: embryo quality and endometrial receptivity, which are responsible for the embryo–maternal interaction necessary for the attachment and invasion of the blastocyst into the endometrium (Cavagna and Mantese, 2003). Timely modifications in the endometrium to make it receptive to the developing embryo are crucial for successful implantation (Navot et al., 1991). Alterations in the expression of cell surface molecules have been observed during conversion of the endometrial surface from a non-receptive to a receptive state (Lessey and Castelbaum, 2002). The intactness of the endometrial cells undoubtedly plays a crucial role in the development of a receptive endometrium. DNA damage and apoptosis of the endometrial cells can certainly affect the endometrial function, which may induce the conversion of endometrial surface from a receptive to a non-receptive state. Moreover, impaired endometrial receptivity is considered to be a major limiting factor for the establishment of pregnancy (Edwards, 1995). Hence, we hypothesized that CS_2 exposure during the period of blastocyst implantation could induce DNA damage and endometrial cell apoptosis, which would affect endometrial receptivity. Therefore, our objectives in this study were (1) to establish an animal

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model in which gestating mice were exposed to CS_2 during the period of blastocyst implantation and (2) to explore the role of DNA damage and endometrial cell apoptosis in the interference of intrauterine implantation by CS_2.

Materials and methods

Animals. Sexually-mature 8–12-week-old Kunming mice were obtained from the Experimental Animals Production Center, Shandong University (Jinan, China. Batch No. SCXK (LU) 20090001). The animals weighed approximately 27–30 g for females and 30–35 g for males. They were raised in a specific-pathogen-free (SPF) animal room and maintained under controlled conditions of temperature (20 ± 2 °C) and humidity (50%–60%) under a 12-h light/dark cycle. Standard laboratory animal feed (purchased from a commercial supplier) and water were provided ad libitum. All animals had at least 1 week of acclimatization prior to treatment. Following mating, the day of sperm plug detection was designated gestational day 0 (GD0). All animal experimental protocols were approved by the Institution's Animal Ethics Committee with their prior approval for using the animals.

Chemicals. CS_2 (analytical reagent) was purchased from Guangcheng Chemical Reagent Corporation (Tianjin, China) and dissolved in olive oil before administration. Low-melting-point agarose (LMA), normal-melting-point agarose (NMA), Triton X-100, and dimethyl sulfoxide (DMSO) were purchased from Amresco Corporation (USA). Tris, trypan blue, ethidium bromide (EB), deoxyribonuclease I and diethylpyrocarbonate (DEPC) were obtained from Sigma-Aldrich Corporation (USA); bovine serum albumin (BSA) and proteinase K were purchased from Roche Diagnostics (UK). DNA marker from Fermentas (Canada) and protease inhibitor cocktail was purchased from Merck Biosciences, Inc. (Darmstadt, Germany). Rabbit anti-mouse Bax, caspase-3, Bcl-2 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (USA) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG as a secondary antibody was purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd. (China).

Experimental design. Gestating mice on GD0 were randomly assigned into 6 experimental groups. Each experimental group which consisted of 40 mice was randomly divided into 4 different dosage-groups: the control group and the doses of $0.1LD_{50}$, $0.2LD_{50}$ and $0.4LD_{50}$. Experimental groups 1–5 were euthanized 12 h after exposure for comet assay, flow cytometry, TUNEL, RT-PCR and Western blot measurements respectively. Experimental group 6 was euthanized on GD8 to count the number of implanted blastocysts and evaluate maternal toxicity.

Uteri excised from the body were weighed after blotting with filter paper and stripping away the attached tissues. Endometrial cells were collected and used for comet assay and flow cytometry. Uteri were frozen at −80 °C and used for RT-PCR and Western blot, or were fixed with neutral buffer formalin and made into paraffin sections for TUNEL. Samples from each mouse in each experiment were independently evaluated three times to ensure precision.

CS_2 exposure. Intraperitoneal injections of CS_2 dissolved in olive oil were administered on GD4. The doses in the exposed groups were 0.1 LD_{50} (157.85 mg/kg), 0.2 LD_{50} (315.7 mg/kg) and 0.4 LD_{50} (631.4 mg/kg) based on the data obtained from an acute toxicity test for female Kunming mice in our previous study (LD_{50} = 1578.5 mg/kg) (Wang et al., 2005b). The injection volume was 0.1 mL/10 g body weight. CS_2 was specially prepared immediately prior to administration. Mice in the control group were injected with body weight-dependent volumes of olive oil.

Blastocyst implantation counts. Uteri were excised from the mice on GD8 and blotted with filter paper. We counted the implanted blastocysts visually and included flesh-red humps in the counts.

Endometrial cells collection. Uteri were excised and gently washed three times with ice-cold Ca^{2+}, Mg^{2+}-free PBS in 5-mL hollow Petri dishes. The uteri were cut longitudinally and added into 1 mL of ice-cold Ca^{2+}, Mg^{2+}-free PBS. The endometrium was gently manually scraped with a sterile, flat bamboo stick and isolated into single cells by repeated mechanical percussion with a pipet, which was confirmed by the microscopic examination. Cell viability was detected by trypan blue exclusion and the number of cells was counted by automatic cell counter (Invitrogen Corporation, USA). Cell density was adjusted into $2 * 10^5$–$4 * 10^5$ cells/mL by ice-cold Ca^{2+}, Mg^{2+}-free PBS for comet assay and flow cytometry.

Comet assay. The comet assay was carried out with slight modifications of the standard protocol as described by Singh et al. (1988). Two solutions were prepared, one containing 0.8% NMA and the other 0.8% LMA dissolved in Ca^{2+}, Mg^{2+}-free PBS. Cell suspension (25 μL) and LMA (75 μL) were distributed onto microscope slides pre-coated with 100 μL of 0.8% NMA. After the agarose solidified, the slides were immersed in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10.0, containing 1% Triton X-100 and 10% DMSO) for 1 h to lyse the cells and allow DNA unfolding. The slides then were placed in a horizontal electrophoresis chamber and DNA was allowed to unwind for 20 min in an alkaline solution containing (300 mM NaOH and 1 mM Na_2EDTA, pH 13). Electrophoresis was performed at 20 V, 150 mA for 20 min. After electrophoresis, the slides were gently washed three times for 5 min with neutralization buffer (0.4 M Tris–base, pH 7.4). The slide was drained and EB (30 μL/slide) was added. All the above steps were run under red light to prevent additional DNA damage. Analyses were performed immediately under a fluorescence microscope (Nikon Instruments, Inc., Japan) and the photographs were individually measured at 200× magnification using a calibration scale. A total of 50 individual cells were evaluated per slide to determine comet length (CL), tail length (TL), head-DNA%, tail-DNA%, tail moment (TM), and olive tail moment (OTM) with image analysis software (CASP, Poland). All slides were coded and scored blindly without knowledge of the dose administered.

Flow cytometry. Detection of apoptosis by flow cytometry was undertaken with the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Jingmei BioTec Corporation, Shenzhen, China). Staining was performed according to the producer's manual. Five thousand cells per sample were analyzed using the BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS), translocated from the inner leaflet of the plasma membrane to the outer leaflet in apoptotic cells (Vermes et al., 1995). Annexin V-FITC is a sensitive probe for identifying cells that are undergoing apoptosis, since PS exposure occurs early in the apoptotic process (Koopman et al., 1994). PI is a nonspecific DNA dye that is excluded from living cells with intact plasma membranes but is incorporated into nonviable cells

Table 1
Primers designed for amplification of Bax, caspase-3, Bcl-2 and β-actin.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
Bax	AAGCTGAGCGAGTGTCTCCGG	CAGATGCCGGTTCAGGTACTCAGTC
Caspase-3	CGTGTATTGTGTCCATGCTCAC	CCATCATTGACAGTTACTTGCTCC
Bcl-2	CTCGTCGCTACCGTCGTGACTTCG	CAGATGCCGGTTCAGGTACTCAGTC
β-Actin[a]	TGGAATCCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG

[a] β-Actin serves as loading control.