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Down-regulation of uterine LIF expression induced by the hormonal level disorder causes embryo implantation loss after mice exposed to carbon disulfide at peri-implantation

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ABSTRACT

Carbon disulfide (CS_2) exposure can cause embryo implantation loss but the mechanism remains unclear. Earlier study revealed that the 4th day of gestation (GD4) and GD5 were the most sensitive exposure time on which the number of implanted embryos decreased obviously in mice. Leukemia inhibitory factor (LIF) in maternal uterine tissue is involved in embryo implantation, which is produced by endometrium and Th2 cells that participate in cellular adhesion of maternal–fetal interface. We herein investigated the effect of CS_2 on the expression of LIF in uterine tissue and its regulatory mechanism in Kunming mice. Exposure was on GD3, GD4, GD5 and GD6, respectively, single administration (631.4 mg/kg), and the indexes were arranged in time series after exposure. The results showed that LIF gene breakage was captured at the 18th hour after exposure by Comet-FISH and the protein and mRNA of LIF in uterine tissue were down-regulated after exposure through the peri-implantation period. In addition, sex steroid hormones, progesterone (P_4) and oestrogen (E_2) were detected since they can stimulate synthesis of LIF from endometrial cells. Results showed that P_4 and E_2 in serum were down-regulated at all the endpoints of CS_2 exposure groups. These findings suggested that the down-regulated LIF induced by the decreased P_4 and E_2 after mice exposure to CS_2 might be important reasons for implantation disorders.

1. Introduction

Carbon disulfide (CS_2), a volatile organic solvent and raw material, is widely used in various industrial processes, such as vulcanizing rubber, fumigating grain, extracting oil, and manufacturing viscose rayon fibers, and in the agriculture environment as dithiocarbamate fungicides [1]. CS_2 is toxic to many organs and physiologic processes [2,3], including the reproductive system [4,5]. Many epidemiological studies suggested that the incidence of spontaneous abortion, birth defects in offspring [6] and very early pregnancy loss of women workers exposed to CS_2 was higher [7] than those in the control group. Although CS_2 compromises pregnancy outcomes in femaleapparently, the underlying mechanism remains unclear. Therefore, exploring its effects on reproduction, especially in early pregnancy, would be valuable. When mice were exposed to CS_2 at the phase of embryo implantation, especially at 4th day of gestation (GD4) and GD5 [8,9], we found that the number of implanted embryos decreased significantly. Thereby, an animal model of implantation disorders in mice induced by CS_2 was successfully built to explore the mechanism of implantation loss by CS_2.

Abbreviations: CS_2, carbon disulfide; GD, gestational day; DEPC, diethylpyrocarbonate; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT-PCR, real time-polymerase chain reaction; IOD, integrated optical density; LMA, low-melting-point agarose; NMA, normal-melting-point agarose; P_4, progesterone; E_2, oestrogen.

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Embryo implantation is the group of processes in which the blastocyst adheres to, and embeds into the receptive endometrium [10]. Endometrial receptivity is the key to successful implantation, which is rigorously controlled both temporally and spatially and embraces various factors under the influence of ovarian hormones, including morphological features, cellular adhesion molecules family, cytokines and immunological regulation [11,12]. In the previous study, we observed that CS_2 single exposure during the peri-implantation period could lead to the decreased integrin β_3, a kind of cell adhesion molecules in uterine tissue, but the mechanism was not clear [9]. According to embryology, as the upstream regulatory protein of integrin β_3, leukemia inhibitory factor (LIF) expresses in the endometrial tissue and regulates endometrial receptivity [13]. LIF is expressed on endometrium and maximal expression is observed on day 4 post-coitus in mice, and it is on the day that implantation occurs in mice [14]. In women, the low expression of LIF in endometrium might be relevant to unexplained infertility with multiple implantation failures [15]. Now it has been accepted as a molecular biomarker of the embryo implantation process [16,17]. Thus, LIF might be the reason for the decreased integrin β_3 which was found in our earlier study. In addition, sex steroid hormones, such as progesterone (P_4) and oestrogen (E_2), stimulate synthesis of LIF from endometrial epithelial cells [18].

Looking at the change of LIF relevant to the disturbed cellular adhesion and immunological regulation process after CS_2 exposure would unravel the mechanism of embryo implantation loss induced by CS_2.

Therefore, we investigated the effect of CS_2 exposure on the LIF, sex steroid hormones in serum, including P_4 and E_2 after CS_2 exposure during the implantation period in this study, which provides new insights for the molecular mechanisms of embryo implantation loss induced by CS_2 in relation to the cellular adhesion and immunological regulation.

2. Material and methods

2.1. Animals

Sexually-mature Kunming mice at 8–12 weeks of age were obtained from the Experimental Animals Production Center, Shandong University (Jinan, China. Batch No. SCXK (LU) 20030004). The weight of animals was approximate 27 g–30 g for females and 30 g–35 g for males. They were acclimated for 1 week before use and raised in SPF (specific pathogen free) grade animal room under controlled conditions of temperature (20 ± 2 °C) and humidity (50% ~ 60%) with 12 h light/dark cycle. Standard laboratory animal feed (purchased from commercial supplier) and water were provided *ad libitum*. After mating procedure, the day of sperm plug detection was designated as gestational day 1 (GD1). Animal protocols used in these studies were approved by the Medical Ethics Committee of Shandong University (MECSDU).

2.2. Experimental design

The experimental group was designed according to our previous research [9]. Four exposure groups (Group 1–4) were designed, with single intraperitoneal injection on GD3, GD4, GD5 and GD6 respectively. The number of examination endpoints in each group was different (shown in Fig. 1): Four endpoints in Group 1 were designed on GD4, 5, 6 and 7 respectively. Three endpoints in Group 2 were on GD5, 6 and 7. Two endpoints in Group 3 were on GD6 and 7. One endpoint in Group 4 was on GD7. Consequently, there were 10 endpoints all together. Mice on GD1 were randomized into the 10 endpoints-groups, in which each endpoints-group contained one CS_2 exposure sub-group and one olive oil exposure sub-group. Twelve mice were observed in each endpoint in which six were selected randomly to have CS_2 exposure and six olive oil exposure. These endpoints were designed to examine the level of LIF, P_4 and E_2.

Based on our previous study, GD4 was the most sensitive exposure time at which the number of implanted blastocysts decreased significantly [9], therefore LIF gene breakage at the 6th, 12th, 18th and 24th after GD4 exposure was detected by Comet-FISH.

The dosage of CS_2 was designed to be 631.4 mg/kg (0.4LD50) for the female, which is based on our previous study [19]. The injection volume was 0.1 ml/10 g body weight and the maximum volume for each animal was under 0.3 ml.

2.3. Tissue collection

Mice were sacrificed on the designated endpoints and the uterus samples were collected immediately. In each endpoint, each uterus was cut into two parts and one part was used for LIF protein analysis and the other for mRNA and DNA damage analysis. All experimental apparatus and freezing tubes were treated without RNA enzyme by 0.1% diethylpyrocarbonate (DEPC) solution. Serum P_4 and E_2 were measured on each endpoint.

2.4. Uterine protein extraction and western blotting for LIF protein expression

The expression of LIF protein was detected by western blot analysis as described previously [20]. After being quickly washed twice with ice-cold phosphate-buffered saline, the uterus samples were homogenized in ice-cold RIPA buffer containing 0.1% SDS, 50 mM Tris (PH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, protease inhibitor cocktail (50 μl/g tissue)and 2 mM phenylmethanesulfonyl fluoride (PMSF). Homogenates were centrifuged at 10,000 *g* for 30 min at 4 °C, and then supernatants recovered. Protein concentration was determined by the BCA protein quantification kit. Protein was loaded on a 12.5% SDS-polyacrylamide gel, electrophoresed, and transferred onto PVDF membranes. And then membrane was blocked with blocking buffer (TBS containing 5% milk powder and 0.1% Tween 20). After incubating overnight with primary antibody, anti-LIF polyclonal antibody (1:2000) and anti-β-actin polyclonal antibody (1:2000) in TBS–T (TBS containing 0.1% Tween 20) at 4 °C, the membrane was incubated with HRP-conjugated IgG secondary antibodies (1:2000) for 1 h. The signal was detected by enhanced chemiluminescence (Millipore, USA) after rinsing with TBS–T, and the relative optical densities of the bands were quantified by Kodak Imaging Program and Image-Pro Plus software.

2.5. Uterine RNA isolation and real-time PCR assay for LIF mRNA expression

Total RNA from uterine tissue was extracted using Trizol reagent and following the manufacturer's instructions. For cDNA synthesis, 200 ng RNA was used in a reaction volume of 20 μl with use of RT reagent Kit (Sangon, China) as recommended by the manufacturer.

Real-time PCR analysis was then performed for examining mRNA level of LIF using a LightCycler® 480 System (Roche Diagnostics, IN, USA) using LightCycler 480 SYBR Green I master mix (Roche Diagnostics, IN, USA). The sequences of the PCR primer pairs for LIF gene are 5′-CTTCTCCCTCTGGTCTCCAA-3′ and 5′-GGGTCAGGATGTTTCAGCAC-3′. The primers of β-actin were 5′-GTCCCTCACCCTCCCAAAAG-3′ and 5′-GCTGCCTCAACACCTCAACCC-3′. qRT-PCR determinations were performed on The reaction samples in a