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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Mediated Cleft palate by Mouse Embryonic Palate Mesenchymal Cells

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ABSTRACT

Objective: To evaluate the effects of mouse embryonic palatal mesenchymal (MEPM) cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induced.
Study design: An experimental study at Xinxiang Medical University.
Methods: Primary MEPM cells were derived from palatal tissue on 30 pregnant C57BL/6 female mice (embryonic day13, GD13). The MEPM cells were placed in a humidified incubator at 37 °C with 5% CO_2 atmosphere with media replaced every other day. The third passage cells were seeded, and one part of cells were treated with 10 nM TCDD (TCDD group). And others were treated with DMSO (≤0.05%, as control group). After 72 h, MTT assay was analysed cell viability. Scratch wound-healing was analysed cell motility. Flow cytometry was analysed cell apoptosis and cycle. Western blot was analysed the expression of cyclinE, CDK2, P16 and P21.
Results: TCDD inhibited the growth and migration of MEPM cells, while increased cell apoptosis. TCDD exposure inhibited the progression of cells from G1 to S phase and tended to reduce the number of cells entering the G2/M phase. TCDD inhibited expression of cyclinE and CDK2 at the protein level, instead increased the expression of P16 and p21 proteins.
Conclusions: TCDD might induce cleft palate by altering MEPM cells.

1. Introduction

In the whole process of palatogenesis, the palatal mesenchymal cells are very important for normal palatal proliferation (Takechi, Taniguchi, Ebara, Fukui, & Watanabe, 2008). Mesenchymal cells growth are disturbed, which is believed that one of the primary causes is failure of palate to elevate. Various environmental teratogenic agent may interfere with the growth of mesenchymal cells (He, 2014). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a widespread environmental pollutant, which causes a variety of severe health effects such as immunosuppression, hepatotoxicity, carcinogenesis, birth defect (Wesselink et al., 2014; Yamada et al., 2014; Haarmann-Stemmann, Bothe, & Abel, 2009). TCDD interfere with embryogenesis by regulating cell proliferation, differentiation, and extracellular cartilage formation (Li et al., 2014; Zhang et al., 2010; Dong, Hinton, & Kullman, 2011). TCDD is teratogenic and can induce cleft palate (Hu, Gao, Liao, Tang, & Lu, 2015). Interestingly, TCDD reduced proliferation of mouse embryonic palatal mesenchymal (MEPM) cells can contribute to smaller shelves size (Yu et al., 2005). We supposed that TCDD might have detrimental side effects on the MEPM cellular processes. So far, the role of TCDD in MEPM cells remained unknown. The goals of the present study were to find the possible mechanism of TCDD-induced cleft palate.

2. Materials and methods

2.1. Animals

30 pregnant C57BL/6 female mice (embryonic day0, E0) were obtained from Henan Laboratory Animal Center. The mice were housed at constant temperature (22 ± 2 °C) and relative humidity

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(50 ± 10%) with a 12 h light/dark cycle. All experiments were carried out in accordance with the Experimental Animal Center Guide for the Care and Use of Laboratory Animals, and the Institutional Ethical Guidelines for Experiments with Animals (SCXK).

2.2. Cell culture and treatment

Primary MEPM cells were derived from palatal tissue on the embryos of GD13 C57BL/6 mice. The method of MEPM cells culture were according to the detail by Feng et al. (2013). Primary MEPM cells were cultured in flasks with DMEM/F12 medium (Hyclon, Logan, UT, USA) supplemented with 10% fetal calf serum (FBS, Sijiqing, Hangzhou, China). The MEPM cells were placed in a humidified incubator at 37 °C with 5% CO^2 atmosphere with media replaced every other day. The third passage cells were seeded, and the cells were treated with TCDD (Sigma-Aldrich, MO, USA). After 72 h, 10 nM TCDD were prepared by dilution with DMSO. Control cells were treated with DMSO (≤0.05%).

2.3. Cell viability assay

To evaluate the effect of TCDD, MEPM cell viability was determined by MTT (JT343, Genview, CA, USA) assay. The third passage of MEPM cells (5×10^3 cells per well) were seeded in 96-well plates (Nunc, Denmark). The cells were treated with 10 nM TCDD and DMSO (≤0.05%). After 72 h incubation, the quantification of cell viability was detected by MTT assay according to the manufacturer's protocols.

2.4. Scratch wound-healing motility assay

MEPM cells were seeded on 6-well plates and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip, and washed with PBS three times to remove the cell debris. 10 nM TCDD and DMSO (≤0.05%) treated MEPM cells as the experiment group and control group respectively. After maintained under standard conditions for 24 h, plates were washed once with fresh medium to remove nonadherent cells and then photographed. The percentages of open spaces covered by migrated cells were determined using ImageJ software (http://rsb.info.nih.gov/ij/).

2.5. Apoptosis assay

After 10 nM TCDD exposed to MEMP cells for 72 h, the cells were harvested using 0.25% trypsin without EDTA and washed twice with ice-cold PBS, then resuspended in 500 μl binding buffer. For the Annexin V/FITC binding assay, the cells were stained with 5 μl Annexin V-FITC and 5 μl PI for 15 min in the dark at room temperature, and then analyzed by flow cytometry (Becton Dickinson, Franklin, NJ). Apoptosis was evaluated with the Annexin V-FITC/PI apoptosis kit (KeyGen Biotech, Nanjing, China). For the Annexin V/FITC total binding assay. Results were calculated as the percentage of apoptotic cells out of the total number of cells counted, and finally immediately analyzed by flow cytometry with FACScan (Becton Dickinson, Franklin, NJ).

2.6. Cell cycle assay

10 nM TCDD exposed MEPM cells for 72 h, and the cells were harvested and fixed in 70% ice-cold ethanol at 4 °C overnight, washed twice with 0.1% (vol/vol) Triton X-100 in phosphate buffer solution (PBS), treated with RNAse A (200 mg/L) at 37 °C for 30 min, stained with 20 mg/L propidium iodide, and finally immediately analyzed by flow cytometry with FACScan (Becton Dickinson). Data were gated using the FlowJo FACS analysis software (Tree Star Inc., Ashland, OR).

2.7. Western blot analysis

Total lysates from different treated MEPM cells using 5 x SDS-lysis buffer supplemented with proteases inhibitors (M250, Amresco, Ohio, USA). Protein concentration was determined using a standard BSA protein assay (Dingguo, Beijing, China). 40 μg protein was fractionated on 12% SDS-PAGE, transfered to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were immunoblotted with the primary antibodies: Cyclin E (BA0774, Boster Biotech, Wuhan, China), P21 (BA0272, Boster Biotech), CDK2 (sc-365846, Boster Biotech), P16 (sc-552, Boster Biotech), β-actin (WD03056, Weiao Biotech, Shanghai, China). β-actin was probed as a loading control. Then membrances were washed and incubated with HRP-conjugated secondary antibody (sc-2004 or sc-2005, Santa Cruz, CA, USA), Western blot analysis was performed using the Odyssey Infrared Imaging System (Li-Cor Lincoln, NE).

2.8. Statistical analysis

All statistical analyses were performed with SPSS 13 software (SPSS, Chicago, IL, USA). All data are presented as mean values ± SD (standard deviation), and all experiments were performed a minimum of three times. Differences between groups were analyzed using the double-sided Student's *t*-test for comparison of two groups, and multiple comparisons were evaluated by a one-way ANOVA. Results were considered significant at $P < 0.05$.

3. Results

3.1. Inhibition of cell viability by TCDD in MEPM cells

In this experiments, we evaluated the effect of viability in TCDD induced-MEPM cells. After treatment with 10 nM TCDD for 72 h, the cell viability was significant inhibition by MTT assay. As shown in Fig. 1, we found that the relative cell survival rate in TCDD group was significantly decreased by (13.23 ± 0.71%) compared with control cells that were treated only with vehicle (≤0.05% DMSO).

3.2. Inhibition of cell motility by TCDD in MEPM cells

To determine whether TCDD effects motility of MEPM cells. We used wound-scratch healing assay to detect cell motility. After

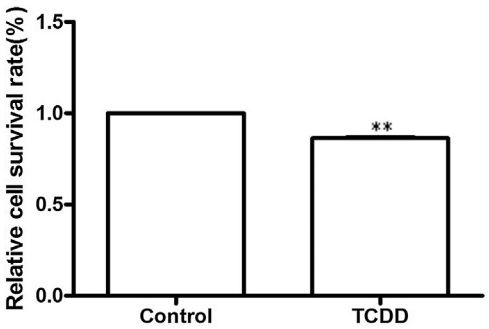

Fig. 1. The effects of cell viability by TCDD induced MEPM cells. 10 nM TCDD and DMSO(≤0.05%) treated MEPM cells as the experiment group and the control group respectively. After treated for 72 h, the cell viabilities were determined by MTT assay according to the manufacturer's protocols. Data were mean ± SD of three replicate experiments. **, $P < 0.01$ vs. the corresponding control values. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMSO, dimethyl sulphoxide; MEPM, mouse embryonic palatal mesenchymal; SD, standard deviation.