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In vitro Cytotoxicity of TCDD on SPC-A1 Cells[1]

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Objective The toxicology of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) has been studied mainly with regard to the carcinogenicity of its metabolites, but its phototoxicity is not well understood. Although some studies have indicated the lethal phototoxicity of TCDD, this study was designed to investigate its effect on SPC-A1 cells. M**ethods** SPC-A1 cells were cultured in 1640 medium and treated with 10 nmol/L, 0.1 μmol/L, 1 μmol/L TCDD for either 24 h or 96 h at each concentration. SPC-A1 cells were co-cultured with TCDD at different concentrations. Then the cell morphology, DNA fragment electrophoresis, and cell cycle were analyzed by flow cytometry, and enzyme assays were used to observe the effect of TCDD on the morphology, growth rate, and enxyme change of SPC-A1 cells. **Results** With the increasing concentrations of TCDD and prolongation of culture time, the morphology of SPC-A1 cells was changed from round shape to spindle, and the ability of SPC-A1 cells to adhere to wall was decreased. With debris emitted around the cells, the morphologic changes included reduction in cell volume. Nuclear chromatin condensation and PI were observed. With the increasing concentrations of TCDD, DNA ladder occurred. After treatment with TCDD, extraction of cancer cells exhibited typical DNA fragmentation, and flow cytometry analysis showed apoptosis in a dose-dependent manner. As the concentration of TCDD rose from 10 nmol/L to 1 μmol/L, the ratio of apoptotic cells increased from 10.76% to 21.82%. **Conclusions** TCDD has *in vitro* cytotoxicity on SPC-A1 cells, and the cytotoxicity is positively related to its concentration and culture time. TCDD may inhibit the growth and proliferation of SPC-A1 cells through the pathway of apoptosis introduction.

Key words: TCDD; SPC-A1 cells; Cytotoxicity; Apoptosis

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is an environmental contaminant originating from industrial waste and also a highly toxic environmental pollutant formed as a result of incineration of wastes and over-treatment with some herbicides and as a by-product of paper processing and plastics manufacturing, particularly in the production of polyvinyl chloride[1]. It is one of the most potent toxins and tumor promoters which can elicit a variety of toxic, teratogenic, and carcinogenic responses in exposed animals and humans.

Humans are passively exposed to TCDD through the diet and gradually accumulate them throughout the life-time. TCDD exerts a wide variety of adverse effects on regulation of gene expression and cellular signal transduction pathways. Injury to cells by TCDD leads to a series of events that can culminate in the death of cells. It has been reported that this pollutant induces apoptosis in many cell lines. However, the morphological characteristics leading to apoptosis or subsequent regeneration of cells exposed to TCDD have not been reported in SPC-A1 cells. We investigated whether human SPC-A1 cells could undergo apoptosis when exposed to TCDD.

MATERIALS AND METHODS

Materials

RPMI 1640 medium and fetal bovine serum (FBS) were from Hycone, dimethylaulfonyl oxide (DMSO) and 5-zac were from Sigma. Propidium iodide, RNase A, proteinase K, phenol: chloroform: isoamyl alcohol (25:24:1) saturated with 10 mmol/L Tris, ethylenediamine tetraacetic acid (EDTA), bromophenol blue and ethidium bromide were

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purchased from Sigma. Molecular biology certified agarose and DNA-standard AmpliSize molecular ruler, 50-2000 bp ladder were procured from Bio-Rad (Hercules, CA). TCDD was a kind gift from Dr. Shun-Qing XU, SPC-A1 cell was from our laboratory. All other chemicals purchased from Lingfei, Hubei (China) were of analytical grade.

Cell Culture and Treatment

The human hepatoma cell line SPC-A1 was incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum containing 100 units penicillin/mL and 100 μg streptomycin/mL. For the whole process of the study, SPC-A1 cells were seeded onto sterilized poly-L-lysin (40 ng/mL) coated glass coverslips. The cultures were incubated at 37℃ in a humidified 5% CO_2 incubator. SPC-A1 cells were seeded at a density of 1.0×10^6 onto 25 cm^2 tissue flask and thereafter grown to subconfluence (as judged from light microscopy) for 3 days. The medium was changed every 24 h. Subsequently, SPC-A1 cells were exposed to different concentrations of TCDD (0, 10 nmol/L, 0.1 μmol/L, 1 μmol/L) by adding an appropriate volume of its stock solution in dimethyl sulfoxide (DMSO) to the culture medium and added to the medium, so that DMSO concentration in the medium was less than 0.1% (v/v). The period of incubation with TCDD was routinely 24 h. Control cells were cultured in medium containing 0.1% DMSO. SPC-A1 cells were treated with 10.00 nmol/L TCDD for 12 h, 24 h, and 48 h .

Morphological Examination by Electron Microscopy (HE Staining)

After SPC-A1 cells were treated, the cells (1×10^6) remaining on the plate were washed twice with PBS (freshly prepared) and 0.25% trypsinized, and the cell suspension was immediately used for the test. The cells were fixed in this solution for 15 min at room temperature with 4% paraformaldehyde, and stained with HE. The pellets were then dehydrated in ethanol and examined using a photic microscope.

Detection of DNA Fragmentation by Agarose Gel Electrophoresis

Low molecular weight DNA was extracted using a modified classical procedure described by Wyllie[2]. The DNA fragmentation pattern (DNA laddering) was also assessed by agarose gel electrophoresis. Cells (1×10^6) were washed twice with ice-cold phosphate-buffered saline (PBS) and 0.25% trypsinized, and the cell suspension was immediately used for the test. After centrifugation at 1200 rpm for 10 min, the cell pellets were resuspended in 1 mL of lysis buffer containing 10 mmol/L Tris·HCl, pH 7.4, 10 mmol/L NaCl, 10 mmol/L EDTA, 100 μg/mL proteinase K, 0.5% SDS and incubated for 1 h at 50℃. DNA was first extracted with 2 mL phenol [balanced with TE buffer (50 mmol/L Tris, 1 mmol/L EDTA, pH 7.5)], followed by extraction with 1 mL of chloroform/isoamylalcohol (24:1). The aqueous phase was precipitated with 2.5 volumes of ice-cold ethanol and 10% volume of 3mol/L sodium acetate, pH 5.2, at -20℃ overnight. The precipitates were collected by centrifugation at 13 000 × *g* for 10 min. The pellets were air-dried and resuspended with 50 μL TE buffer supplemented with 0.1 μg/mL RNase A. DNA was loaded onto 1.5% agarose gel, electrophoresed in TE buffer containing 2 μg/mL ethidium bromide for 90 min at 90 V, before electrophoresis in 1% agarose gels containing ethidium bromide and photographed under UV illumination.

Flow Cytometry for Apoptosis Detection in SPC-A1 Cells

The percentage of subdiploid apoptotic nuclei was determined by staining with propidium iodide (PI). SPC-A1 cells were washed with PBS and fixed in 70% ethanol, resuspended in 70% cold (-20℃) ethanol and transferred to the freezer, where they were stored for up to 20 h or 3 days. The cells were washed again and resuspended in PBS containing PI (50 g/mL). Following incubation for 10 min at room temperature, the percentage of apoptotic nuclei recognized by their subdiploid DNA content, was determined using the FCM.

These samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon-ion laser emitting at 488 nm. The green fluorescence emitted by fluorescein-12-dUTP in DNA of individual cells was collected through a 530/30 nm bandpass. The data from 10 000 cells were stored and analyzed on a logarithmic scale using CellQuest Software.

Statistical Analysis

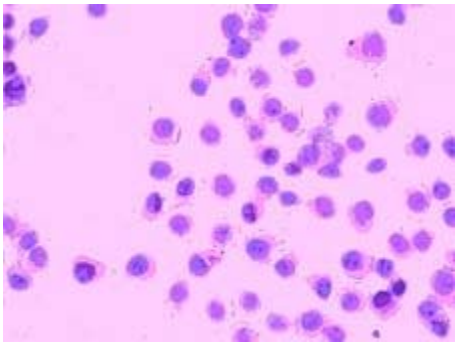
The statistical significance of the difference between the control and treated groups was evaluated by SPSS 11.0. $P<0.05$ was considered statistically significant.

RESULTS

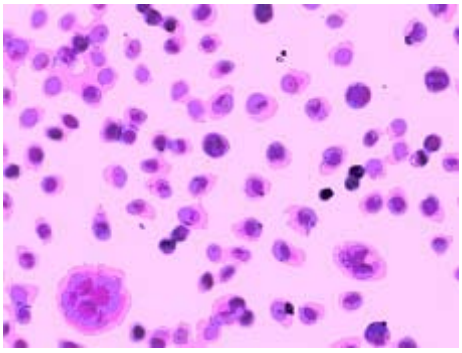
HE Staining Results

Morphologically the normal growing SPC-A1 cell strain was round-shaped, the cytoplasm was uniformly distributed and abundant, the nuclei were

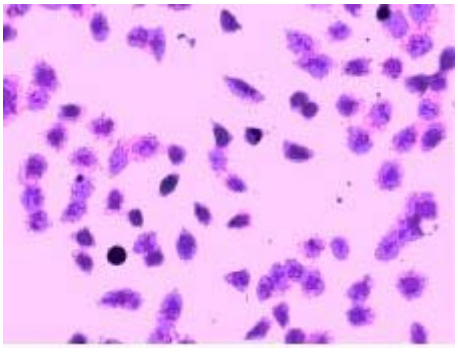
regularly arranged and blue in color, and the in-nucleus staining was even, while cell strains of the SPC-A1 treated with TCDD were all changed in comparison with their intrinsic morphological feature; as a result the cells became shuttle in shape, the nucleus withered or broke and the apoptotic bodies could be noticed. These changes became more prominent with the concentration of TCDD.



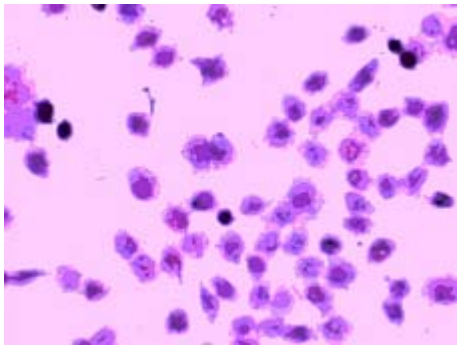
Control



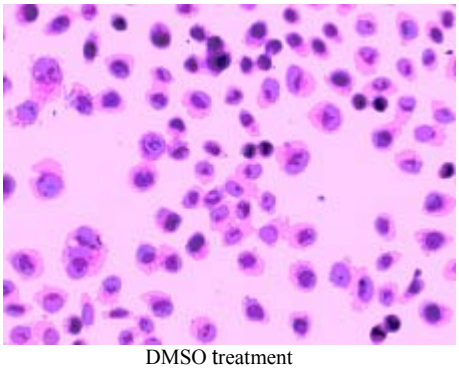
10 nmol/L TCDD treatment



0.1 μmol/L TCDD treatment



1 μmol/L TCDD treatment



DMSO treatment

FIG. 1. Analysis of cell morphology by conventional HE staining (original magnification ×200).

Demonstration of TCDD-induced Apoptosis by Electrophoresis to Obtain a 'DNA Ladder' (*Agarose Gel Electrophoresis Results*)

DNA fragments extracted from SPC-A1 cells treated with 0.2% DMSO or different concentrations of TCDD for 24 h were subjected to agarose gel electrophoresis (Fig. 2). A typical 'DNA ladder' was observed, with bands separated by multiples of about 200 bp. This indicates the presence of DNA fragments. The size of single nucleosomes and oligonucleosomes[3] is considered to be a characteristic biochemical marker for apoptotic cells[4]. Again, no DNA fragments were obtained from cells treated with DMSO.

Flow Cytometric Analysis of Apoptosis-associated DNA Strand Breaks Using Propidium Iodide Staining of Cellular DNA (Flow Cytometric Analysis Results)

The dose-dependence of TCDD inducing apoptosis in SPC-A1 cells was also examined using flow cytometric analysis of propidium iodide-stained cells (Fig. 3). The size of the hypodiploid peak varied with dose exposure to TCDD in the same manner as the DNA ladder pattern obtained by electrophoresis. These investigators were able to determine the phase of the cell cycle in which the DNA breaks occurred[5]. SPC-A1 cells were exposed to 0.2% DMSO (a), or 10 nmol/L TCDD (b), 0.1 μmol/L TCDD (c), or 1 μmol/L TCDD (d) for 24 h. The rate of apoptosis was 3.84%, 7.21%, 10.76%, 15.94%, and 21.82%, respectively. SPC-A1 cell apoptotic percentage induced by TCDD was enhanced in a concentration dependent pattern.

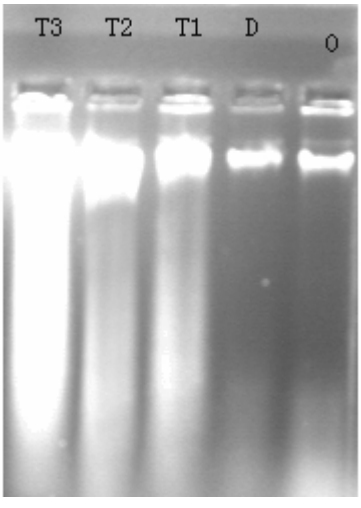


FIG. 2. Qualitative analysis of DNA fragments by agarose gel electrophoresis.

DISCUSSION

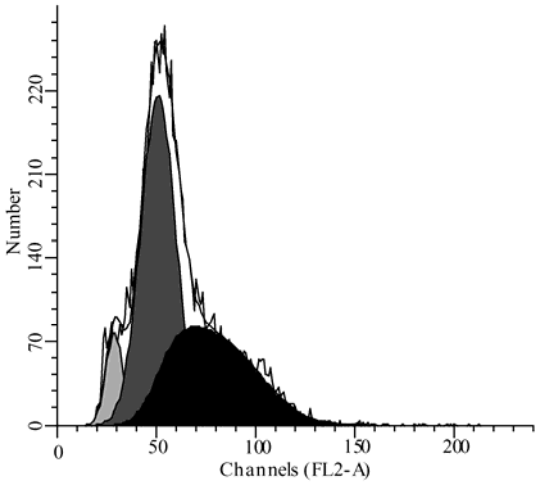
Many kinds of PAHs enter the aquatic and atmospheric environments as a result of human activities. TCDD is the most potent congener of the dioxin-like compounds present as ubiquitous environmental agents in the atmosphere, soil, water, and food. TCDD exerts diverse species-specific toxic effects in animals and humans, including immunological, reproductive, and developmental toxicity, carcinogenicity, which may cause wasting syndrome and even death[6].

Apoptosis is characterized by a series of distinct morphological and biochemical changes. An increased percentage of hepatocytes from TCDD-treated mice displayed classical apoptotic phenotype compared to the control mice. The most profoundly affected tissue appears to be the thymus. Kamath and colleagues[7] have demonstrated that TCDD induces thymic atrophy and apoptosis *in vivo* and apoptosis of thymocytes *in vitro*. Other reports indicate that TCDD induces apoptosis in a variety of tissues in fish embryos[8].

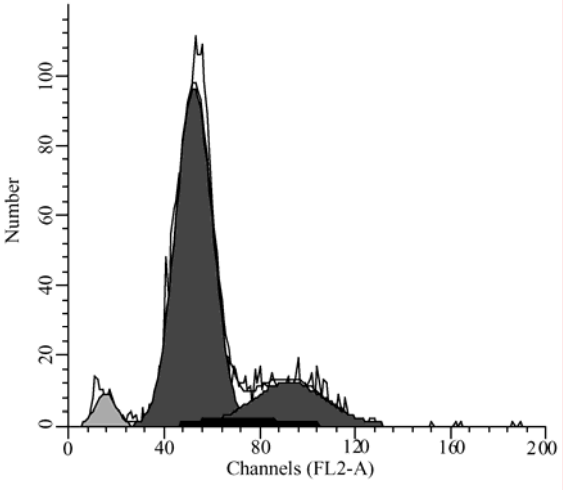
Handyside and Hunter detailed apoptosis, which is a normal feature of embryogenesis, in the mouse embryo. Acritical threshold number of inner cell mass blastomeres is required in the blastocyst for normal postimplantation development, and programmed cell death may be responsible for the control of this process[9]. TCDD is known to affect apoptosis in other reproductive tissues. Heimler *et al.*[10] reported TCDD's effects on apoptosis in human luteinized granulosa cells, and showed that higher concentrations of TCDD increase apoptosis in a dose-dependent fashion.

It has been proposed that the ability of TCDD to induce apoptosis may be a cell type/ tissue-specific phenomenon. Various methods of measuring apoptosis have been reported, each having different degrees of specificity, sensitivity, and accuracy. In all cases, it is strongly recommended that morphological examination under light microscopy be used to confirm apoptosis measured by any other method. In the event of ambiguous or conflicting results, the morphological data should be the deciding factor[11-12].

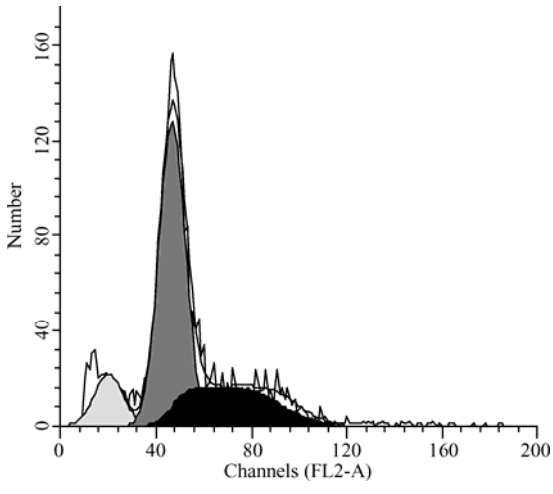
In our study, TCDD induced apoptosis in SPC-A1 cells, over the concentration range of 10 nmol/L, 0.1 μmol/L, 1 μmol/L, and the percentage pf apoptotic cells increased in a concentration-dependent manner. Evidence supporting this conclusion includes morphological features in which chromatin condensation is demonstrated by HE staining. DNA fragmentation by conventional agarose gel electrophoresis obtains a 'DNA ladder', TCDD causes cell DNA double strand breaks, and the size of the hypodiploid peak varies with dose exposure to TCDD in the same manner as the DNA ladder pattern obtained by electrophoresis. By combining the results of these three methods, we provide strong evidence to support the conclusion that TCDD causes programmed cell death (apoptosis) of SPC-A1 cells.



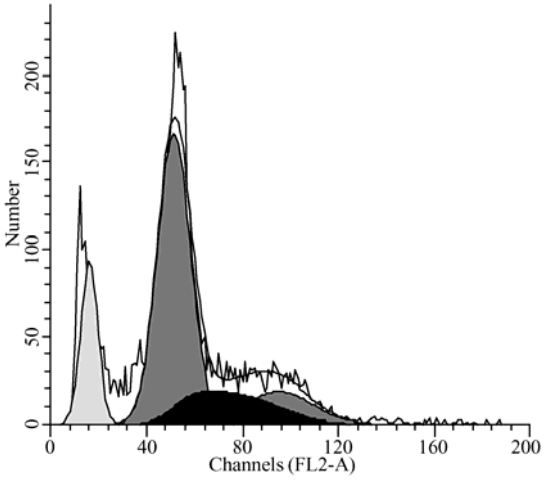
DMSO (a1), Apoptosis: 7.21%



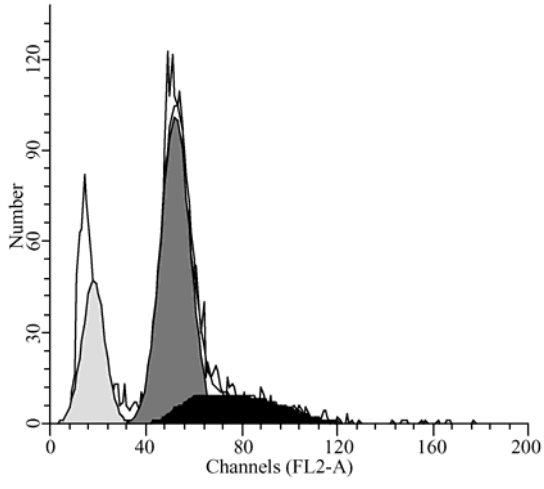
DMSO(a2), Apoptosis: 3.84 % control



10 nmol/L TCDD (b), Apoptosis: 10.76%



0.1 μmol/L TCDD (c), Apoptosis: 15.94%



1 μmol/L MTCDD (d), Apoptosis: 21.82%

FIG. 3. Analysis of cells in hypodiploid by flow cytometry.

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