

Endocrine disruptor, dioxin (TCDD)-induced mitochondrial dysfunction and apoptosis in human trophoblast-like JAR cells

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ABSTRACT: The endocrine disruptor 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been demonstrated to disrupt hormone signalling, reduce fertility, interfere with embryo development and cause spontaneous miscarriage in humans. The precise mechanisms of its effects on early implantation in humans are still unclear. In this study, we examined the relationship between mitochondrial function and dioxin-induced toxicity in JAR cells, a human trophoblast-like cell line. Several experiments were performed to address the effects of TCDD on cell viability, reactive oxygen species (ROS) generation, oxidative damage (indicated by the presence of lipoperoxides and oxidized DNA bases), mitochondrial DNA (mtDNA) copy number, ATP content, mtDNA mutations and the protein levels of p53, Bax, Bcl2, cytochrome *c* and caspase 3. Increased oxidative damage and mitochondrial dysfunction in TCDD-treated trophoblast-like cells was demonstrated. A 2.58-fold increase in lipid peroxides was detected in cells treated with 2 nM TCDD for 4 h. The oxidative DNA damage marker 8-hydroxy-2'-deoxyguanosine was significantly increased by TCDD treatment in a time-dependent manner. Meanwhile, reductions in mtDNA copy number and ATP content and an increase in mtDNA deletions were found. Furthermore, we observed increased apoptosis, p53 accumulation, Bax overexpression, cytochrome *c* release and sequential caspase 3 activation after TCDD exposure. These results indicate that oxidative damage and mitochondrial dysfunction may be responsible for the apoptotic effects of TCDD.

Key words: dioxin / mitochondrial dysfunction / oxidative damage / trophoblast-like cell

Introduction

The placenta is the regulator of nutrient composition and supply from mother to fetus and is the source of hormonal signals that affect maternal and fetal metabolism. The appropriate development of the placenta is crucial to normal fetal programming (Myatt, 2006). As a result of the establishment of placental development and circulation, increased mitochondrial capacity is required to compensate for the increasing needs of the embryo. Placental mitochondria might play an important role in the maintenance of pregnancy and neonatal development through the regulation of metabolic activity and ATP production (Martinez *et al.*, 2002), hormone synthesis (Tuckey, 2005) and trophoblast oxygen sensing (De Marco and Caniggia, 2002). Mitochondrial dysfunction causes cell damage and death by

compromising ATP production and calcium homeostasis and increasing oxidative stress.

Exposure to the endocrine disruptor 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), commonly known as dioxin, has been shown to disrupt endocrine signalling (Halperin *et al.*, 1998), leading to alterations in ovarian steroidogenesis, inhibition of follicle development (Heiden *et al.*, 2006), suppression of placental vascular remodelling (Ishimura *et al.*, 2006) and an increase in the rate of spontaneous miscarriage in humans and animals (Schnorr *et al.*, 2001). Dioxin has been also demonstrated to cause the pre- and/or post-implantation loss of fertilized oocytes in humans (Kitajima *et al.*, 2004). *In utero* exposure to TCDD has been shown to cause placental toxicity (Augustowska *et al.*, 2003).

In fact, Ishimura *et al.* (2002) have hypothesized a mechanism of TCDD toxicity in the placenta, the primary response to which is

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oxidative stress-mediated toxicity. Following TCDD treatment, oxidative stress is induced in the mitochondrial fraction of testis, in epididymal sperm (Latchoumycandane and Mathur, 2002) and in mouse liver mitochondria (Stohs et al., 1991; Senft et al., 2002; Shen et al., 2005). In addition, oxidative stress and mitochondrial dysfunction are proposed to contribute to common placental-related disorders in pregnancy, such as pre-eclampsia and miscarriage (Hassoun et al., 1997; Jauniaux et al., 2006). The goal of this study was to identify the molecular targets of TCDD by investigating the production of reactive oxygen species (ROS), decrease in ATP synthesis, mitochondrial damage and trophoblast-like cell death.

Materials and Methods

Cell culture

The JAR (ATCC number: HTB-144TM) cell line was derived from a human trophoblastic tumour of the placenta. Cells were maintained at 37°C in humidified air with 5% CO₂ in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10% (v/v) fetal calf serum.

Cell viability determination using trypan blue exclusion

Different concentrations of TCDD (Sigma-Aldrich) were added to trophoblast-like cells to reach final concentrations of 0.2, 0.6, 2 or 6 nM, whereas using 0.1% dimethyl sulphoxide (DMSO) as the control group. To determine cell viability, both attached and floating cells were harvested and tested for trypan blue exclusion. Briefly, ~10 µl of cell suspension in PBS was mixed with 40 µl of trypan blue (Gibco, Grand Island, NY, USA), and the numbers of stained (dead) and unstained (live) cells were counted using a haemocytometer.

Cell viability determination using the MTT assay

The MTT assay is a colorimetric assay based on the tetrazolium salt MTT, which turns from yellow to purple in living cells. The optical density (OD) of the MTT-treated cell suspension at 570 nm is directly proportional to the number of living cells. In brief, 200-µl suspensions of 2×10^4 TCDD-treated cells were aliquoted into wells of microtitre plates. Untreated cells were utilized as controls for non-specific dye reduction. After incubation with 0.2, 0.6, 2 or 6 nM TCDD at 37°C for 2, 4, 6, 12, 24, 36 or 48 h in a humidified 5% CO₂ atmosphere, the plates were spun at 700g, the supernatants were discarded and 20 µl of the 10 µM MTT solution was added to each well. After an additional hour of incubation, 150 µl of DMSO was added to the wells to extract the formazan. The formazan absorbance was measured at 565 nm, and the values represent the OD (value) per mg of protein.

Flow cytometric analysis for ROS generation and cell apoptosis

Aliquots of 1×10^6 cells were stained with fluorescent probes in the dark for 15 min at room temperature. After staining, cells were washed with PBS and analysed by flow cytometry. All analyses were performed by a FACScan (Becton Dickinson, San Jose, CA, USA). A minimum of 30 000 cells per sample were analysed. Debris was gated out based on light-scatter measurements. Data were acquired in the list mode, and the relative proportions of cells within different areas of the fluorescence profile

were quantified using the LYSYS II software program (Becton Dickinson, Franklin Lakes, NJ, USA). Intracellular ROS were stained with dichlorodihydrofluorescein diacetate (H₂DCFDA; Molecular Probes, Eugene, OR, USA). In the analysis of apoptosis, a combination of annexin V-FITC and PI (TACSTM Annexin V-FIT Apoptosis Detection Kits, R&D systems, Minneapolis, MN, USA) was also used for simultaneous flow-cytometric analysis of the stages of cell apoptosis. Following TCDD treatment, both attached and floating cells were harvested and stained with annexin V-FITC and propidium iodide (PI) labelling solution. Except for the cell viability and apoptosis analyses, all of the preceding experiments were performed on attached cells.

Mitochondrial membrane potential ($\Delta\psi$) by confocal microscope

JC-1 is a cationic mitochondrial vital dye that is lipophilic and becomes concentrated in the mitochondria in proportion to their $\Delta\psi$; more dye accumulates in mitochondria with greater $\Delta\psi$ and ATP-generating capacity (Smiley et al., 1991). The dye exists as a monomer at low concentrations (emission, 530 nm, green fluorescence) but at higher concentrations forms J aggregates (emission, 590 nm, red fluorescence). Briefly, after different treatments, cells were collected and incubated for 10 min with 5 µM JC-1 (Molecular Probes) at 37°C, washed, resuspended in medium, mounted onto slides in PBS:glycerol (1:1 v/v), and topped with cover slips. The slides were viewed under an Olympus OLS 3000 laser scanning confocal microscope (Tokyo, Japan) equipped with a krypton/argon laser (excitation 488 nm, emission 490–550 and 590–630 nm).

Determination of lipid peroxidation in TCDD-treated cells

The degree of lipid peroxidation was determined in the form of malondialdehyde (MDA) as a thiobarbituric acid-reactive substance (TBARS). In brief, an aliquot of 50 µl of $3-5 \times 10^6$ cells was pipetted into a test tube containing 0.6 ml of 0.44 M phosphoric acid. After mixing with 0.2 ml of a 42 mM thiobarbituric acid solution, it was then placed in a 95°C dry bath for 1 h. The samples were then cooled and neutralized with 1 N NaOH in methanol before the high performance liquid chromatography (HPLC) analysis. An aliquot of 20 µl of supernatant was injected into a C₁₈ column (4.6 × 250 mm, with a particle size of 5 µm) using a Jasco PU-980 pump (Easton, MD) with a solvent system composed of methanol and 50 mM phosphate buffer (pH 6.8; 4:6, v/v) at a flow rate of 1 ml/min. The eluent was monitored with a fluorescence detector with the excitation wavelength at 525 nm and emission wavelength at 550 nm.

DNA extraction

An aliquot of $3-5 \times 10^6$ cells was incubated at 56°C for 2 h in 1.5 ml lysis buffer containing 10 mM Tris-HCl EDTA (pH 8.0), 1.5% SDS, and 10 mg/ml proteinase K. After digestion, the lysate was extracted once each with phenol and chloroform, precipitated with isopropanol (1:1, v/v) and a 1/10 volume of 3 M sodium acetate (pH 5.6) and incubated at -20°C overnight. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.3).

Determination of 8-hydroxy-2'-deoxyguanosine

An aliquot of 100 µg of cellular DNA dissolved in 100 µl of 10 mM Tris-HCl (pH 7.4) was digested by incubation with 1 µl of DNase I (20 units/µl) and 11 µl of a 0.1 M MgCl₂ solution at 37°C for 30 min. After adjusting the pH to 5.0 by adding 4.8 µl of 1 M sodium acetate (pH

5.3) and 1.2 μ l of 0.1 M ZnSO_4 , the DNA was hydrolysed with 5 μ l of nuclease PI (1 unit/3 μ l) at 65°C for 10 min and then hydrolysed by incubation with 5 μ l of 1 U/ μ l alkaline phosphatase for 30 min at 37°C. Processed DNA samples were separated on a C18 column (200 $\times$ 4.6 mm, with a particle size of 5 μ m, JT Baker, Phillipsburg, NJ, USA) on an HPLC system (Jasco) connected in series with an ECD detector (Bioanalytical Systems, West Lafayette, IN, USA) and a UV detector set at 254 nm. Elution was performed at a flow rate of 0.8 ml/min for 40 min, with the mobile phase consisting of 12.5 mM citric acid, 25 mM sodium acetate and 10 mM acetic acid containing 6% methanol (pH 5.8). The amount of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the DNA sample is expressed as a percentage relative to the amount of the total dG.

ATP assay

Cellular ATP levels were determined by a luciferin and luciferase-based assay. Cells were washed with PBS and lysed with ATP-releasing buffer [100 mM potassium phosphate buffer (pH 7.8): 1% Triton X-100, 2 mM EDTA and 1 mM dithiothreitol]. ATP concentrations in lysate were quantified using an ATP assay kit (A22066, Molecular Probe, Eugene, OR, USA) according to the manufacturer's instructions. The supernatant was collected and then read on a Wallac 1420 multilabel counter (Perkin Elmer, MA, USA). ATP levels were calculated as nM ATP per mg protein and normalized to the ATP levels of untreated control cells.

Determination of mitochondrial DNA copy number by real-time PCR

The copy number determination was based on the region in the NADH dehydrogenase gene ND1 of the mitochondrial genome. The PCR was performed in a Roche Light Cycler apparatus using the Faststart DNA master SYBR Green kit (Roche Mannheim, Germany). The reactions were performed as follows: initial denaturation at 95°C for 300 s followed by 40 cycles of 1 s at 95°C, 6 s at 58°C and 18 s at 72°C. The threshold cycle number (Ct) values of the β -globin gene and the mitochondrial ND1 gene were determined, respectively, for each individual quantitative PCR run. The primers used to perform the PCR are listed in Table I.

Identification of mitochondrial DNA deletions by long-extension PCR assay

A long-extension PCR was performed to identify large-scale deletions of mitochondrial DNA (mtDNA). Desired target sequences of mtDNA

were amplified from 15 to 20 ng of each DNA sample in a 50- μ l reaction mixture containing 200 μ M of each dNTP, 0.4 μ M of each primer, 1 unit of Ampli-Taq DNA polymerase (Perkin-Elmer/Cetus, Roche Molecular System, Branchburg, NJ, USA), 50 mM KCl, 1.5 mM MgCl_2 and 10 mM Tris-HCl (pH 8.3). The thermal profile was: denaturation at 94°C for 2 min, annealing at 68°C for 1 min, and primer extension at 72°C for 2 min. The primers used to perform the PCR are listed in Table I.

Primer-shift PCR and nucleotide sequencing of the mutated mtDNA

To avoid artefacts in the detection of mtDNA deletions, a primer-shift PCR method was employed to verify that the amplified DNA fragment was not due to the mis-annealing of primers to the DNA template (Kao *et al.*, 1998). The primers used to perform the primer-shift PCR are listed in Table I. The desired target sequence of mtDNA was amplified from 500 fmol of each DNA sample in a 20- μ l reaction mixture containing the A-dye terminator, T-dye terminator, C-dye terminator, G-dye terminator, dITP, dATP, dCTP, dTTP, 3.2 pmol primer, 10 units of Ampli-Taq DNA polymerase (Epicentric Technologies, Oldendorf, Germany) and 1 $\times$ sequencing buffer. The PCR was carried out for 30 cycles of denaturation at 96°C for 30 s and 50°C for 15 s and then primer extension at 60°C for 4 min. PCR products were then separated by electrophoresis on 6% polyacrylamide gels containing 8 M urea at 65 W for 4 h.

Mitochondrial fractionation

To prepare mitochondrial and cytosolic fractions, cells were harvested, washed with ice-cold PBS and resuspended in five volumes of isolation buffer [20 mmol/l HEPES (pH 7.4), 10 mmol/l KCl, 1.5 mmol/l MgCl_2 , 1 mmol/l EDTA, 1 mmol/l DTT, 250 mmol/l sucrose and a cocktail of protease inhibitors). The cells were homogenized with 10 strokes of a Teflon homogenizer, and the homogenates were centrifuged at 750g for 10 min at 4°C. The supernatants were subjected to further centrifugation at 10 000g for 15 min at 4°C. The resultant supernatant fractions were taken as crude cytosolic extracts, and the pellets saved as the mitochondrial fractions and washed with isolation buffer before use. The crude cytosolic extracts were further centrifuged at 10 000g for 1 h at 4°C, and the resultant supernatants were saved as the cytosolic fractions.

Western blots

Whole-cell lysates were either used directly from TCDD-treated samples or prepared separately in whole-cell extract buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1 mM phenazine methosulphate and complete protease inhibitor; Roche Molecular Biochemicals, Basel, Switzerland). Equal amounts of protein were electrophoresed on an acrylamide gel, transferred to nitrocellulose (Bio-Rad, Hercules, CA, USA), and immunoblotted according to standard protocols using 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20. Blots were stained with Ponceau S to ensure equal loading. Membranes were probed with primary antibodies against cytochrome c (1:1000 dilution), p53 (1:2000), Bax (1:2000), Bcl-2 (1:2000), caspase3 (1:2000) and α -tubulin (1:2000; all purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were anti-mouse IgG conjugated to alkaline phosphatase (Santa Cruz Biotech) or anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signaling Technologies, Beverly, MA, USA). Reactive proteins were visualized by enhanced chemiluminescence detection with ECL plus (Amersham Biosciences, Uppsala, Sweden).

Data are expressed as the mean $\pm$ standard deviation (SD). Data were assessed using the *t*-test, and *P* < 0.05 was considered statistically significant.

Table I The DNA sequences of oligonucleotide primers used for the analysis of mtDNA deletion.

Primer pair	Oligonucleotide sequence
β -globin forward	5'-GATCCTGAGACTTCCACACT-3'
β -globin reverse	5'-ACCTGACTGAGGAGAAG-3'
ND1 forward	5'-TGGGTACAATGAGGAGTAGG-3'
ND1 reverse	5'-GGAGTAATCCAGGTCGGT-3'
L7901	5'-TGAACCTACGAGTACACCGA-3'
L8150	5'-CCGGGGGTATACTACGGTCA-3'
L8251	5'-GCCCGTATTTACCCTATAGC-3'
L8531	5'-ACGAAAATCTGTTCGCTTCA-3'
H16255	5'-CTTTGGAGTTGCAAGTTGATG-3'
H16450	5'-CGAGGAGAGTAGCACTCTTG-3'

Results

Reduced cell viability in TCDD-treated trophoblast-like cells

The viability of cells exposed to TCDD was determined by both the trypan blue exclusion assay and the MTT assay. Cells were cultured with different doses of TCDD for 24 h (Fig. 1A and C), or with 2 nM TCDD for various time periods (Fig. 1B and D). TCDD showed adverse effects on cell viability. In the trypan blue dye exclusion assay, cell viability was reduced in a dose-dependent manner ($r = -0.98$, $n = 5$, $P < 0.01$). Viability was reduced to $89.2 \pm 1.8\%$ (mean $\pm$ SD, $P < 0.01$), $76.3 \pm 4.3\%$ ($P < 0.01$), $66.3 \pm 3.4\%$ ($P < 0.01$) and $62.1 \pm 5.4\%$ ($P < 0.01$) in cells treated with 0.2, 0.6, 2 and 6 nM TCDD, respectively (Fig. 1A). The viability was $97.4 \pm 0.4\%$ and $92.6 \pm 0.6\%$ in the PBS and DMSO groups, respectively (Fig. 1A). There was no significant difference in viability between the PBS and DMSO groups. A significant decrease in cell viability was found in the 2 nM TCDD-treated cells after 12, 24 and 48 h (Fig. 1B). Meanwhile, a dose- and time-dependent reduction of cell viability was also found in the MTT assay (Fig. 1C and D). Viability was reduced to $54.5 \pm 8.2\%$ ($P < 0.01$) in cells treated with 2 nM TCDD for 24 h. According to the effect of TCDD on cell viability,

2 nM TCDD was chosen as the dose with which to address the time-dependent effects.

Elevated ROS generation and oxidative damage in TCDD-treated cells

The intracellular formation of ROS was established based on the oxidation of H_2DCFDA , which yields an intracellular trapped fluorescent compound. As shown in Fig. 2A, intracellular ROS levels significantly increased by 20.3 ± 1.5 -fold in cells exposed to 2 nM TCDD for 6 h (red histogram, $P < 0.01$) relative to the control group (pink histogram). TCDD-induced ROS generation (purple histogram) was blocked by pre-treatment of the cells with α -NF (aryl hydrocarbon receptor (AhR) antagonist) and the ROS (red histogram) was maintained at the level of the normal control (green histogram; Fig. 2B). A 2.80 ± 0.26 -fold increase in lipid peroxide [thiobarbituric acid (TBA)-reacted MDA] was found in the cells treated with 2 nM TCDD for 6 h ($P < 0.001$) (Fig. 2C). A gradual increase in lipid peroxide was detected 2–6 h after TCDD treatment. After 6 h, the level of lipid peroxide declined with time. It returned to the control level by 48 h. The lower levels of lipid peroxides that were found in the remaining survival cells might indicate protective effects against oxidative stress, and this needs further evaluation. In addition, the

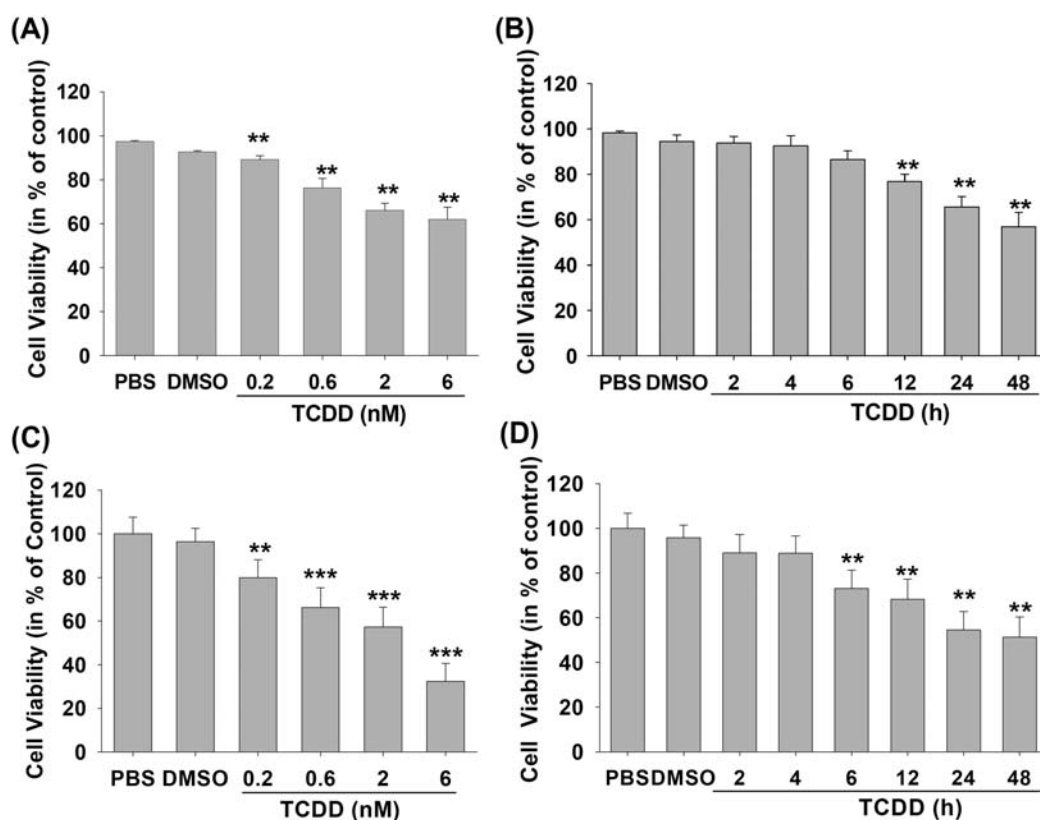


Figure 1 Viability of dioxin (TCDD)-treated trophoblast-like cells by trypan blue exclusion and MTT assay. Cells were cultured with different doses of TCDD for 24 h (A and C) or with 2 nM TCDD for various time periods (B and D). Cell viability was assayed by the dye-exclusion method (A and B) and MTT assay (C and D). Dose- and time-dependent reductions in cell viability were observed. The mean data from independent experiments were normalized to results obtained in the absence of TCDD (the PBS group). DMSO (0.1% v/v) alone was used as the solvent control. Data are presented as the mean $\pm$ SD ($n = 5$). ** $P < 0.01$ compared with the control group.

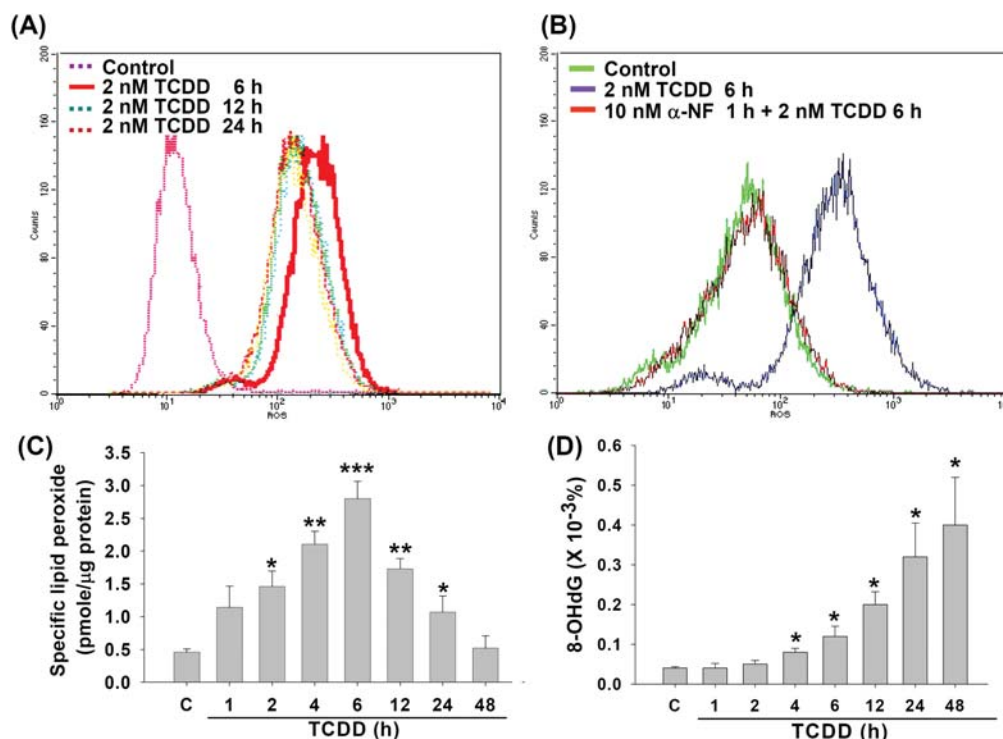


Figure 2 Dioxin (TCDD)-mediated ROS generation and oxidative damage in TCDD-treated trophoblast-like cells. Cells were treated with 2 nM TCDD at 37°C for different times. ROS were detected in TCDD-treated (A) and α -NF (AhR antagonist)-pretreated cells (B) by H₂DCFDA staining and flow cytometry. TCDD-induced ROS generation was blocked by pre-treatment of the cells with α -NF. Lipid peroxides were determined as TBARS by HPLC-fluorospectrometry (C). 8-OHdG was measured by HPLC-electrochemical detection (D). A significant increase in lipid peroxides was detected after 2–6 h of treatment. A time-related increase in 8-OHdG content was observed after 4 h of treatment. Data are presented as the mean $\pm$ SD ($n = 4$). * $P < 0.05$ compared with the control group; ** $P < 0.01$ compared with the control group; *** $P < 0.001$ compared with the control group.

8-OHdG content significantly increased in a time-dependent manner ($r = 0.83$, $P < 0.05$). 8-OHdG accumulated and persisted for long periods in the TCDD-treated cells. A significant increase in 8-OHdG was observed 6 h after treatment of trophoblast-like cells with 2 nM TCDD (Fig. 2D). An approximately 6.5-fold increase in the specific content of 8-OHdG was found 24 h after TCDD treatment. The 8-OHdG levels were 0.32 ± 0.09 and 0.04 ± 0.01 8-OHdG/ 10^5 dG in the TCDD-treated cells and the control group, respectively (Fig. 2D).

Decreases in ATP content and mtDNA copy number in TCDD-treated cells

Time-dependent ATP depletion was observed in the cells treated with TCDD for 1, 2, 4, 6, 12, 24 and 48 h (Fig. 3A). After 24 h of incubation, the intracellular ATP content decreased to $68.4 \pm 3.2\%$ of the control group ($P < 0.01$). mtDNA copy number is a limiting factor in cellular respiration and consequently it influences metabolic efficacy and cell fate. To measure the mtDNA copy number, the relative proportions of the NDI (mitochondrial genome) and β -globin genes were determined by real-time PCR. The mtDNA levels were investigated in five alternate cell passages to exclude the ageing effect of cells. A significant reduction in mtDNA copy number was found in cells treated with 2 nM

TCDD for 24 h (25.3%) and 48 h (50.3%). The mtDNA copy number was reduced to $50.3 \pm 3.7\%$ of the control group 48 h after treatment with TCDD ($P < 0.05$; Fig. 3B). In confocal images of JC-1 stained TCDD-treated trophoblast-like cells, JC-1 accumulated preferentially in polarized mitochondria, existing as green fluorescent monomers at low membrane potentials (excitation 485 nm, emission 535 nm) and as orange fluorescent aggregates at high membrane potentials (excitation 550 nm, emission 600 nm). Following TCDD treatment, mitochondrial JC-1 was primarily in the green-monomeric form and less in the orange-aggregated form.

Increased mtDNA mutations in TCDD-treated cells

Using the long-range PCR technique, we screened for large-scale deletions of mtDNA in TCDD-treated cells. Many types of mtDNA rearrangements were generated with 24 h and 48 h of treatment. Deletions in mtDNA reached a maximum 48 h after treatment, which is consistent with our findings regarding the specific content of 8-OHdG. Further analysis revealed five major DNA fragments (with approximate lengths of 8355, 3500, 2600, 1700 and 756 bp) on the agarose gel after ethidium bromide staining (Fig. 4). The 8355-bp band was the full-length PCR product generated from the wild-type mtDNA. The 756-bp

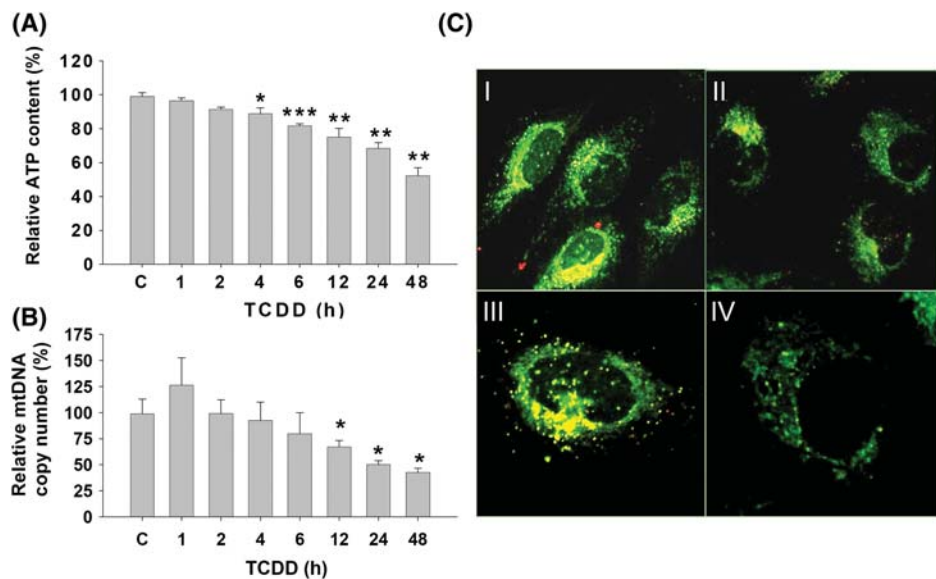


Figure 3 Effects of dioxin (TCDD) on mitochondrial function. Cells were incubated with 2 nM TCDD at 37°C for various time periods. **(A)** ATP levels were determined using the ATP-luciferin-luciferase assay **(B)** mtDNA copy number was analysed by real-time PCR. Declines in ATP level and mtDNA copy number were observed in TCDD-treated cells. **(C)** Fluorescent images of TCDD-treated cells by JC-1 staining. Cells were treated without (I and III) or with 2 nM TCDD for 24 h (II and IV). JC-1 accumulated preferentially in mitochondria, existing as green fluorescent monomers at low membrane potentials (I and III) and as orange fluorescent aggregates at high membrane potentials (II and IV). I and II were observed under 100× magnification, III and IV under 200×. Data are presented as the mean ± SD (*n* = 4). * *P* < 0.05 compared with the control group; ** *P* < 0.01 compared with the control group; *** *P* < 0.001 compared with the control group.

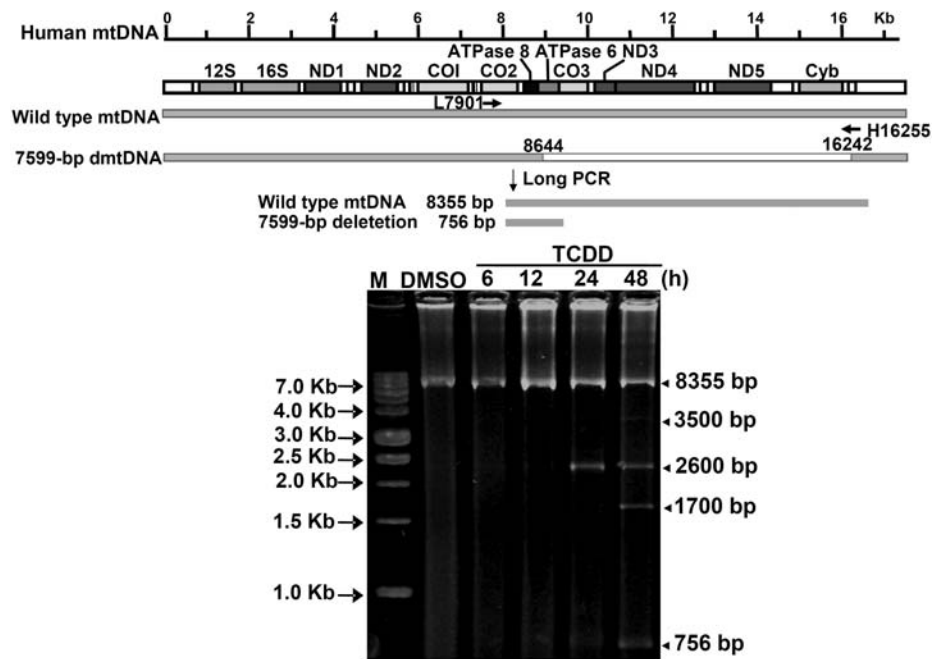


Figure 4 Electrophoretogram of the PCR products amplified from mtDNA in trophoblast-like cells. mtDNA deletions were found in TCDD-treated cells by long-range PCR with the primer-pair L7901/H16255. Cells were treated with 2 nM TCDD for 6, 12, 24 and 48 h. Five types of PCR products were amplified from cells treated with TCDD for 48 h, with approximate lengths of 8355, 3500, 2600, 1700 and 756 bp (arrowhead). An 8355-bp DNA product was amplified from wild-type mtDNA. The 756-bp PCR product was generated from 7599 bp-deleted mtDNA. M, 1-kb DNA ladder.

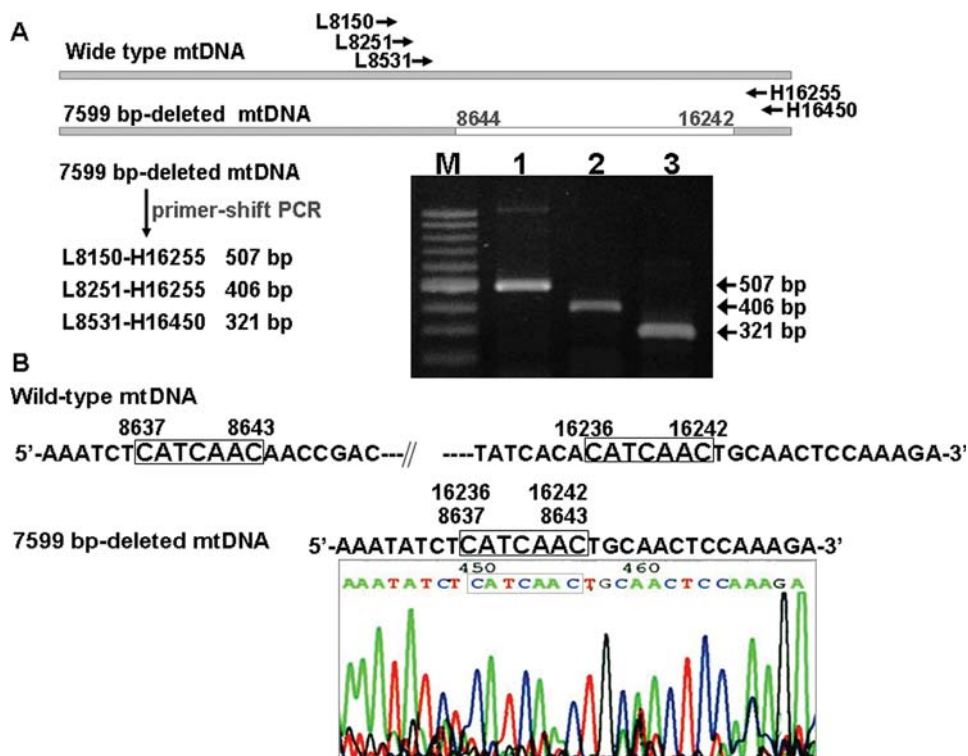


Figure 5 Nucleotide sequence at the breakpoint of the 7599-bp mtDNA deletion determined by primer-shift PCR and DNA sequencing. **(A)** Primer-shift PCR and an electrophoretogram of the PCR products amplified from mtDNA from TCDD-treated trophoblast-like cells with the specific 7599-bp deletion. DNA was prepared from 48 h TCDD-treated cells. Lanes 1–3 indicate the PCR products of 507 bp (L8150-H16255), 406 bp (L8251-H16255) and 321 bp (L8531-H16450) amplified from the 7599 bp-deleted mtDNA, respectively. Lane M is a 100-bp DNA ladder. **(B)** Nucleotide sequence of the light strand of mtDNA showing the breakpoint at the 5'-end of the 7599 bp-deleted mtDNA in TCDD-treated trophoblast-like cells. The analysis revealed a 7-bp direct repeat (5'-CATCAAC-3') located in the breakpoints at np 8637–8643 and np 16 236–16 242 (5'–3') of the light strand.

PCR product was further confirmed by primer-shift PCR analysis and DNA sequencing. By using the primer pairs L8150-H16255, L8251-H16255, L8531-H16450, we obtained PCR products of 507, 406 and 321 bp from the deleted mtDNA (Fig. 5A). The sizes of the DNA fragments amplified from the different primer sets differed exactly by the shift of the distance between the two primer-pairs. After sequencing, a 7599-bp deletion of mtDNA was identified in TCDD-treated cells. Analysis of the nucleotide sequences flanking the breakpoint of the 7599-bp deletion revealed a 7-bp direct repeat (5'-CATCAAC-3') located in the junction sites at nucleotide position (np) 8637–8643 or np 16 236–16 242 on the light strand of mtDNA (Fig. 5B). The deletion region encompasses the following genes: part of the ATP synthase 6 (complex V); the entire cytochrome c oxidase gene COIII (complex IV); the NADH dehydrogenase genes ND3, ND4, ND4L, ND5, ND6 (complex I); and the cytochrome b gene Cytb (complex III).

Induction of cell death by TCDD treatment

To measure apoptosis, a combination of Annexin V and PI (AV/PI) dual staining was used. In the sample scatter graph (Fig. 6A) and stack diagram (Fig. 6B) based on the fluorescent intensity of each channel, the cell populations were segmented into three regions: low-intensity

AV and PI staining, indicating viable cells; cells stained primarily with AV, indicating that they were undergoing early apoptosis; and cells that had higher PI intensity combined with either higher AV or lower AV intensity, indicating a later stage of apoptosis and necrosis. By calculating the percentage of cells in each category, a relative apoptotic rate could be determined. A 4.8-fold increase in early apoptosis was found in the TCDD-treated cells at 6 h. Apoptosis was significantly elevated after 12 and 24 h of incubation in 2 nM TCDD ($P < 0.001$).

Induced cytochrome c release and caspase 3 activation in TCDD-treated cells

Cytochrome c in cytosolic and mitochondrial fractions of TCDD-treated cells was detected by western blot. α -tubulin and cytochrome c oxidase subunit IV (COX IV) served as controls for equal protein loading and as indicators of the purity of each fraction: tubulin for the cytosolic fraction and COX IV for the mitochondrial fraction. The absence of COX IV in the cytosolic fraction indicated it was not contaminated by mitochondrial components. Cytochrome c in the mitochondrial fraction decreased in a time-dependent manner during TCDD treatment (Fig. 7A). The percentage of cytochrome c that was in mitochondria dropped to $68.1 \pm 8.1\%$, $52.2 \pm 4.3\%$, $41.1 \pm 5.1\%$, $22.9 \pm 3.8\%$ and $3.1 \pm 0.3\%$ at 2, 4, 6, 12

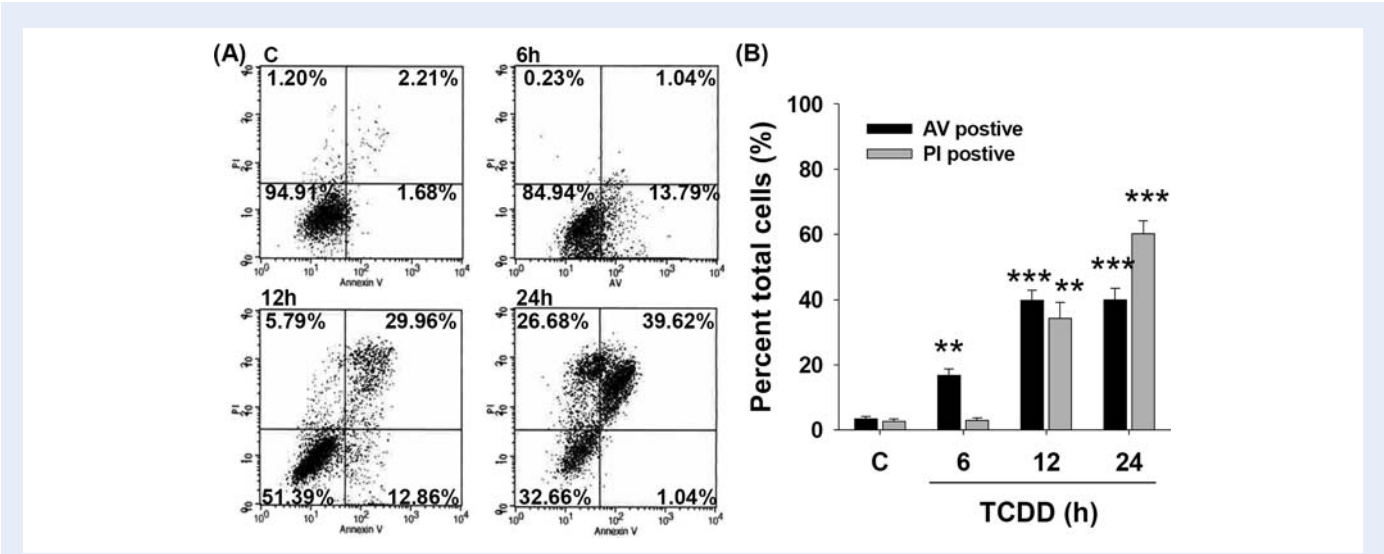


Figure 6 Apoptosis assay by flow cytometry after treatment of JAR cells with 2 nM TCDD. **(A)** Dot plot of cell apoptosis by flow cytometry shown in four groups (control and 2 nM TCDD for 6, 12 and 24 h). The percentage of cells at each different apoptotic stage is presented. The lower-left field represents viable cells, the lower-right field early-apoptotic cells (AV-positive and PI-negative), and the upper-left field late-apoptotic cells (PI-positive). **(B)** Bar graph representing the data flow of the three experiments showing the induction of apoptosis by TCDD treatment.

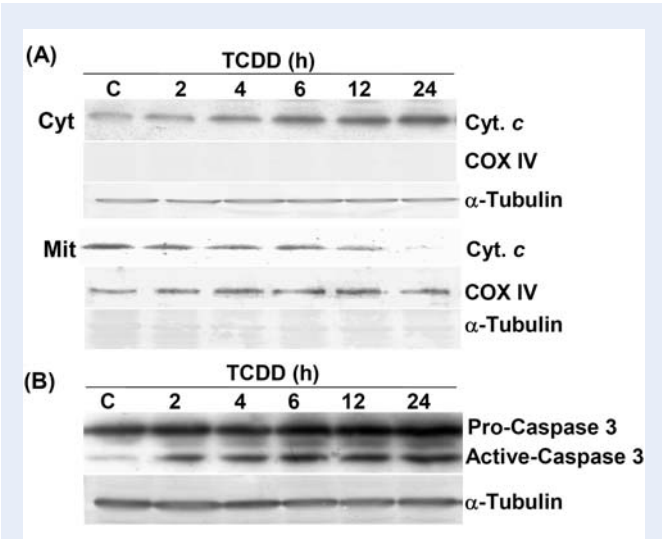


Figure 7 Analysis of cytochrome c release and caspase activation in TCDD-treated cells. **(A)** Time-dependent stimulation of cytochrome c release from mitochondria into the cytosol. α -tubulin and COX IV served as controls for protein loading and for the purity of each fraction. α -tubulin was the cytosolic marker and COX IV the mitochondrial marker. Cytochrome c was measured in TCDD-treated cells by western blot **(B)**. More active caspase 3 was found in the TCDD-treated cells.

and 24 h in 2 nM TCDD, respectively. The sequential changes in the cytochrome c levels in the cytosol and mitochondria are shown in Fig. 7A. A corresponding increase in the amount of cytochrome c in the cytosolic fraction was seen in TCDD-treated cells. The proportion of cytosolic cytochrome c was $17.3 \pm 2.9\%$, $30.9 \pm 2.6\%$, $47.8 \pm$

4.7% , $78.4 \pm 6.2\%$, $82.5 \pm 5.9\%$ and $98.5 \pm 8.4\%$ at 0, 2, 4, 6, 12 and 24 h treatment, respectively. In Fig. 7B, we examined the effects of caspase-3 activation in TCDD-treated cells.

Differential expression of p53, Bcl-2, and Bax proteins after TCDD treatment

The protein levels of p53, Bcl-2 and Bax were determined by western blot. The levels of p53 and Bax were significantly higher in the TCDD-treated cells than in the controls (Fig. 8). As a transcriptional regulator, p53 increases the transcription of several downstream genes in response to DNA damage. A 3.3-fold ($P < 0.05$ versus control) and 3.1-fold ($P < 0.001$ versus control) increase of p53 protein expression was observed at 12 and 24 h, respectively (Fig. 8A). This enhanced p53 expression is consistent with the increase in 8-OHdG and the mtDNA mutations. Bax and Bcl-2 regulate apoptosis downstream of p53. Bcl-2 blocks cell death following various stimuli, demonstrating a death-sparing effect; however, Bax counters the anti-apoptotic activity of Bcl-2, and over-expression of Bax has a pro-apoptotic effect. It has been proposed that the ratio of Bcl-2 to Bax may govern the sensitivity of cells to apoptotic stimuli. In our results, the protein levels of Bax and Bcl-2 varied co-ordinately. Bcl-2 protein was reduced to 71.1% of control levels after TCDD treatment for 24 h (Fig. 8B). A marked 1.7-fold increase of Bax protein ($P < 0.01$ versus control) was detected in the 12 h TCDD-treated cells. The pro-apoptotic effect that we observed was accompanied and possibly regulated by an imbalance between the anti-apoptotic Bcl-2 and the pro-apoptotic Bax.

Discussion

Dioxins are bioaccumulative endocrine disruptors found in many environmental pollutants. In prenatal exposure to TCDD, placental

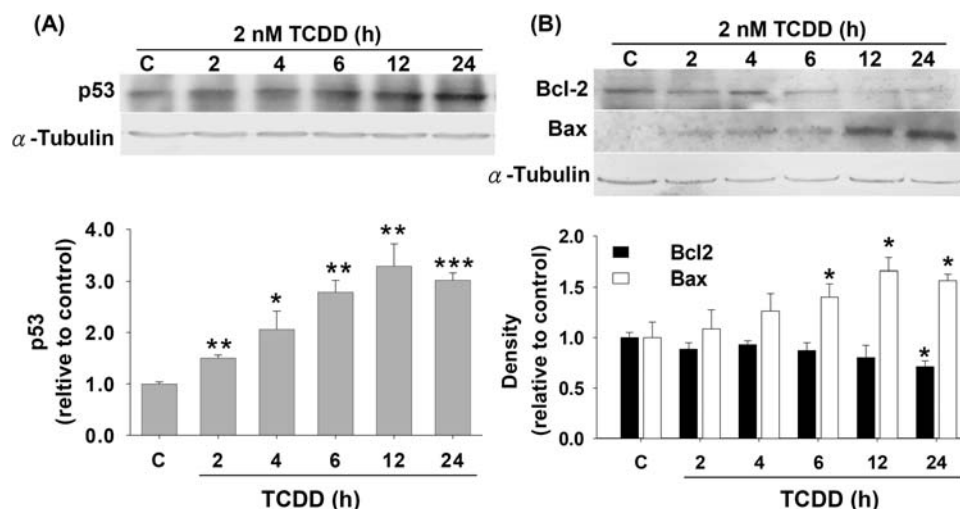


Figure 8 Western blot analysis of p53, Bax and Bcl-2 levels in TCDD-treated cells. Cells were incubated with 2 nM TCDD at 37°C for various time periods. The levels of p53 (A) and Bax/Bcl-2 (B) were determined by immunoblotting. Each value was measured by densitometric analysis of the immunoblots based on the density of the band in the vehicle control as 100%. Data are presented as the mean $\pm$ SD ($n = 4$). * $P < 0.05$ compared with the control group; ** $P < 0.01$ compared with the control group; *** $P < 0.001$ compared with the control group.

tissue is the foremost deposition site, with less accumulation in other fetal tissues such as the liver, head and urogenital tract (Hurst *et al.*, 2000). The accumulation of TCDD in placental tissue reduces estradiol secretion and blood flow through the placenta and causes abnormal fetal programming (Augustowska *et al.*, 2003).

Dioxin toxic equivalence values of 8.4–17.6 ng/kg have been reported in normal placental material (Schechter *et al.*, 1996), but higher concentrations have been found, in the range of 610–9010 ng/kg, in exposed mothers. Additionally, alterations in placental ultrastructure after maternal exposure to dioxin have been demonstrated in cases of induced abortion after the Seveso disaster (Remotti *et al.*, 1981), with numerous microprecipitates found in the trophoblastic basement membrane at various distances from the fetal capillaries, impairing the metabolic systems for *trans*-trophoblastic transfer. In the cynomolgus macaque, a well-documented reproductive and developmental model for humans, TCDD exposure increases early fetal loss, possibly due to an endocrine imbalance that leads to placental insufficiency and compromised embryonic circulation (Guo *et al.*, 1999). However, in late gestation, a reduction of more than 50% in trophoblast apoptosis was found in rats following exposure to dioxin *in vivo* (Ishimura *et al.*, 2006, 2009). On the other hand, Chen *et al.* (2003) observed no change in cell viability when primary trophoblast cultures derived from human term placentas were exposed to dioxin *in vitro*, and they found TCDD to have differential effects on early, late-gestational and term trophoblasts. The differential molecular regulation mediating these varied effects of TCDD require further study.

In the present study, TCDD at 2 nM reduced the viability of trophoblast-like JAR cells. After 24 h in culture, TCDD significantly increased apoptosis in JAR cells in a dose-dependent manner. However, there are differences in the reported doses of TCDD required to induce cell apoptosis. Heimler *et al.* (1998) found that TCDD perturbed steroid secretion in a dose- and time-dependent

fashion and induces apoptosis in human luteinized granulosa cells (23% apoptosis in controls, 45% in 3.1 nM TCDD and 62% in 3.1 μ M TCDD). Low concentrations of TCDD (100 pM to 1 nM) have also been demonstrated to elicit apoptosis in mouse primary hepatocytes (Christensen *et al.*, 1998). However, TCDD was found to accelerate the differentiation of the blastocyst at a concentration of 10 nM (Blankenship *et al.*, 1993), but it did not alter the development of the early mouse morula to the blastocyst stage nor did it significantly induce apoptosis at 3.1 μ M *in vitro* (Matthews *et al.*, 2001). The discrepancies between these studies may be due to species-specific effects, different cell types or the different concentrations of TCDD that were used.

The placenta is a highly vascularised tissue that develops during early gestation to facilitate the circulation of blood, oxygen, glucose and nutrients between the mother and fetus (Myatt, 2006). The metabolic activity of placental mitochondria leads to oxidative stress, which is exacerbated further in pregnancies complicated with intrauterine growth retardation (Myatt and Cui, 2004), pre-eclamptic pregnancies (Widschwendter *et al.*, 1998), gestational diabetes mellitus (Chen *et al.*, 2000) and gestational trophoblastic disease (Durand *et al.*, 2001). Mitochondria are suggested to be a major source of ROS and are enriched with polyunsaturated fatty acids that are susceptible to peroxidation (Wang and Walsh, 1998). In our study, a 2.58 ± 0.81 -fold increase in lipid peroxides (Fig. 2C) and 6.5-fold increase in 8-OHdG (Fig. 2D) was observed in trophoblast-like cells treated with 2 nM TCDD for 4 h ($P < 0.001$). After 48 h, a long-lasting accumulation of 8-OHdG was observed, but the maximal level of lipid peroxides was found at 6 h. TCDD has been suggested to impair cell function through lipid peroxide-induced changes in membrane fluidity and integrity, perturbation of mitochondria and disruption of steroidogenesis (Aitken and Baker, 2006) and to induce mutagenesis, as indicated by 8-OHdG levels (Nakabeppu *et al.*,

2006). If not repaired, 8-OHdG modifications in DNA are mutagenic, and they may cause embryonic loss, childhood cancers and inferior pregnancy outcomes (Loft et al., 2003).

Dioxins have been demonstrated to exert their effects via high-affinity binding to a specific cellular protein known as AhR. We found that TCDD-induced ROS generation was blocked by pre-treatment of the cells with an AhR antagonist (α -NF), and that ROS were maintained at control levels (Fig. 2A and B). AhR is thought to be a key regulatory protein in normal development and homeostasis (Andersson et al., 2002) and is associated with dioxin-induced mitochondrial ROS production (Senft et al., 2002; Schecter et al., 2006). The increased expression of AhR during the peri-implantation phase of the mouse uterus may indicate functional roles for this receptor in fetal–maternal interactions, and it increases the risk of exposure to chemicals such as dioxins during the reproductive period (Kitajima et al., 2004).

Human placental mitochondria are the main source of progesterone and they contribute to the production of ATP through oxidative phosphorylation (Uribe et al., 2003). An imbalance in the redox state of the developing embryo resulting from suboptimal conditions leads to altered gene expression and impaired ATP generation, which can impair placental and embryonic growth (Hyslop et al., 1988). TCDD-mediated declines in ATP levels and increases in ROS production have also been demonstrated in the liver tissue of mice. The lower tissue ATP levels are proposed to be mediated by damage to the mitochondrial F_0F_1 -ATP synthase and to ubiquinone (Shertzer et al., 2006). In our study, a $\sim 47.3\%$ reduction in ATP content was found in cells treated with 2 nM TCDD for 48 h ($P < 0.01$). A concomitant loss of mitochondrial membrane potential and mtDNA copy number was observed in the TCDD-treated cells. The maintenance of mitochondrial membrane potential, mitochondrial ATP synthesis and mitochondrial pH are all required for hormone biosynthesis (Midzak et al., 2007). In addition, lower progesterone production in MA-10 Leydig cells is accompanied by loss of mitochondrial membrane potential (Levine et al., 2007).

Our study revealed that TCDD reduces the integrity of the mitochondrial genome, as evidenced by reductions in mtDNA copy number and the presence of large-scale deletions in the mitochondrial genome. mtDNA copy number is a limiting factor in cell respiration, and it therefore influences metabolic efficiency and cell fate. Significantly, mtDNA depletion is also associated with benign and malignant trophoblast-like diseases, indicating a relationship with embryo survival (Durand et al., 2001). mtDNA depletion and deletions may contribute to reductions in ATP synthesis and in the efficiency of oxidative phosphorylation. Additionally, reduced mitochondrial respiratory enzyme activity (Matsubara et al., 1997) and the occurrence of multiple mtDNA mutations (Chiu et al., 2003) have been observed in the placentas of patients with pregnancy-related diseases. Mitochondrial dysfunction was also identified in the placentas of smoking mothers, who exhibited lower mitochondrial respiratory activity and mtDNA depletion (Bouhours-Nouet et al., 2005). Shertzer et al. (2006) reported that the primary TCDD-induced changes consist of an increase in the reduction state of glutathione, a decrease in CoQ associated with Complex III, and a defect in ATP synthesis that increases respiration but decreases tissue ATP levels. We observed an increase in mtDNA rearrangements in cells treated with TCDD for 48 h, including a 7599-bp mtDNA deletion. The deleted region

encompasses genes in mitochondrial respiratory complexes I, III, IV and V and might contribute to the ATP depletion. The breakpoints of 7599 bp deletions are located in the hot-regions that are prone to large-scale deletions. The nucleotide sequences within these regions were found to assume unusual structures, such as bent DNA and anti-bent DNA. These regions are proposed to be more vulnerable to attack by free radicals or serve as recognition motifs for certain recombination machinery involved in the large-scale deletions. Loss of mitochondrial genome integrity may be used as a biomarker to monitor the toxicity of TCDD in the management of hormone disruptors in environmental pollutants.

In our findings, TCDD-induced apoptosis was accompanied by p53 accumulation and an imbalance of Bax/Bcl2. The induction of apoptosis by TCDD was demonstrated through the modulation of Bcl2 and Bax, release of cytochrome c and subsequent caspase 3 activation. p53, normally expressed in response to DNA damage and/or hypoxia, arrests cells in the G1 phase of the cell cycle, allowing for DNA repair or promoting apoptosis if the damage is too severe (Robertson and Orrenius, 2000). The activation of a p53-dependent pathway provides evidence of oxidative stress and a response to DNA damage. A placental increase in the Bax/Bcl-2 ratio might contribute to the pathogenesis of pre- and post-term births (Daher et al., 2008). Similarly, increased apoptosis after TCDD exposure has been demonstrated in several kinds of cell, such as T-cells (Kobayashi et al., 2009), dendritic cells (Singh et al., 2008) and pituitary cells (Huang et al., 2005). Controversially, a distinct role of TCDD as tumour promoter has been proposed, as TCDD antagonizes DNA damage-induced apoptosis following a variety of treatments, such as UV-irradiation or H_2O_2 , in a human mammary epithelial cell line (Park and Matsumura, 2006) and in rat hepatocytes (Chopra et al., 2009).

We summarize and discuss the significance of TCDD-induced abnormalities, and finally, speculate about the TCDD induced oxidative damage and mitochondrial dysfunction may be detrimental for trophoblast-like cell survival. To address the main concern of whether the TCDD-induced detrimental effects on early implantation by impaired trophoblast cell survival and responsible for early pregnancy loss, the cell model of the primary cultures of first-trimester trophoblast or other cell types found at the materno-fetal interface in early pregnancy is needed to be set up. In the future, we plan to establish the first-trimester trophoblast cell model providing a framework for future investigations into the significant pathogenesis of TCDD in the placenta, particularly with respect to influencing normal pregnancy and fetal development.

Authors' roles

S.-C.C., C.-R.T., S.-H.K. involved in the conception and design of the study. S.-C.C., C.-R.T. provided administrative support. S.-C.C., C.-R.T. were responsible for provision of study materials or patients.

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