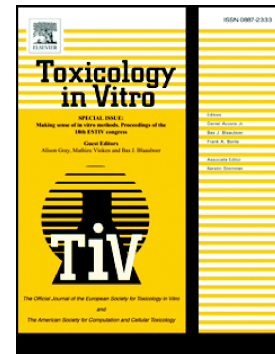


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TCDD-induced mitochondrial superoxide production does not lead to mitochondrial degeneration or genomic instability in human SH-SY5Y neuroblastoma cells

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Abstract

Several genotoxic and non-genotoxic agents have been reported to cause delayed genetic damage in the progeny of the exposed cells. Such induced genomic instability (IGI) may be a driving force in carcinogenesis, and it is thus highly important to understand the cellular events accompanying it. The aim of this study was to investigate whether 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) affects mitochondrial integrity and can consequently induce genomic instability. Mitochondrial integrity was evaluated by measuring mitochondrial superoxide production, mitochondrial membrane potential, and mitochondrial activity. Micronucleus formation was used to assess immediate genetic damage and IGI. The assays were performed either immediately, 8 or 15 d after the exposure. Mitochondrial superoxide production was increased by TCDD immediately after the exposure. No consistent effects on mitochondrial integrity were observed at later time points, although slightly decreased mitochondrial membrane potential at 8 d and increased mitochondrial superoxide potential production at 15 after exposure were observed in the TCDD-exposed cells. TCDD did not cause immediate genetic damage, and significant IGI was not observed. In conclusion, the present results suggest that immediate TCDD-induced increase in mitochondrial superoxide level does not lead to persistent loss of mitochondrial integrity or IGI in human SH-SY5Y neuroblastoma cells.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the model compound and the most toxic congener of polychlorinated dibenzo-*p*-dioxins and -furans, collectively called “dioxins”. These compounds are environmental contaminants found as impurities of certain chlorophenols or chlorophenoxy acid herbicides and formed also in the burning of organic materials in the presence of chlorine. TCDD has been classified as carcinogenic to humans (class 1) by the International Agency for Research on Cancer (IARC, 1997). The present understanding is that TCDD does not induce any direct DNA damage, but it rather acts as a tumour promoter (Dragan and Schrenk, 2000). Most of the toxic effects of TCDD are mediated by the aryl hydrocarbon receptor (AhR), to which TCDD binds with high affinity (Mimura and Fujii-Kuriyama, 2003).

Numerous studies have shown that ionizing radiation is able to cause induced genomic instability (IGI), a state of persistent or increased genetic damage manifesting multiple cell generations after the initial insult (Morgan et al., 1996; Baverstock 2000). This phenomenon could play a highly significant role in the carcinogenesis process, as it may explain the accumulation of mutations required for carcinogenesis (Negrini et al., 2010; Burrell et al., 2013). Although IGI was originally found in cells exposed to ionizing radiation, there are more recent findings suggesting that it can be induced by several other agents, such as extremely low frequency magnetic fields, UV radiation, and heavy metals (O'Reilly and Mothersill, 1997; Brennan and Schiestl, 2001; Coen et al., 2001; Li et al., 2001; Phillipson et al., 2002; Cheng 2009; Luukkonen et al., 2014). We have previously shown that TCDD can cause IGI in C3H10T1/2 mouse embryonic fibroblasts (Korkalainen et al., 2012).

Cancer cells have been postulated to have dysfunctional mitochondria (de Moura et al., 2010; Gasparre et al., 2013). Normal function of mitochondria is critical for cells as mitochondria are their primary energy producers. Mitochondria are also major producers of reactive oxygen species (ROS) in the cell and important mediators of several cancer-relevant processes, such

as apoptosis, proliferation, and cellular differentiation (de Moura et al., 2010; Ward and Thompson, 2012). Interestingly, the effects of TCDD have been suggested to be mediated through mitochondria (Aly and Domenech 2009; Biswas et al., 2009). Furthermore, AhR has been shown to regulate both TCDD-induced changes in mitochondrial function (Fisher et al., 2005; Tappenden et al., 2011; Martino et al., 2013) and mitochondrial oxidative stress (Senft et al., 2002; Shen et al., 2005; He et al., 2013). Importantly to this study, the loss of mitochondrial integrity has been reported to be related to IGI (Kim et al., 2006a, b), but the available data are insufficient for assessing this possible association.

This study aims to investigate whether TCDD affects mitochondrial integrity and can consequently cause IGI. The exposures were performed on human SH-SY5Y neuroblastoma cells, as we wanted to compare our previous results (Korkalainen et al., 2012) to a cell line of human origin. We have also recently shown that extremely low frequency magnetic fields and menadione can cause IGI in this cell line (Luukkonen et al., 2014). Micronucleus frequency was used to assess both immediate genetic damage and IGI 8 and 15 d after the initial exposure. Increased level of micronuclei is a widely used marker for IGI in a variety of cell types (Jamali and Trott 1996; Seoane et al., 2007; Yokota et al., 2010; Sciandrello 2011; Korkalainen et al., 2012; Luukkonen et al., 2014). Mitochondrial integrity was evaluated by the measurement of mitochondrial superoxide ($O_2^{\cdot-}$) formation, mitochondrial membrane potential (MMP), and mitochondrial activity.

Materials and methods

Cell culture and exposure to chemicals

Human SH-SY5Y neuroblastoma cells (obtained from Dr. Sven Pålman, University of Uppsala, Sweden) were used as an experimental model. The SH-SY5Y cells were grown in Dulbecco's Modified Eagle Medium (DMEM, 4.5 g/l glucose), supplemented with 10 % inactivated FBS, 50 U/ml penicillin and 50 µg/ml streptomycin. Cell cultures were maintained in plastic bottles (75 cm², Nunc, Denmark) that were kept in a humidified cell culture incubator (Heraeus HERAcell, Germany) at +37 °C with 5 % CO₂. For the exposures, cells were cultured either in 48-well plates (Costar, USA) for the experiments measuring immediate effects or Petri dishes (Ø 60 mm, Nunc, Denmark) for measuring delayed effects. Number of cells used for each experiment is presented in Table 1. Before exposures, cells were cultivated for 20 h. An overview of the experimental scheme is presented in Figure 1. After exposure, cells in the 48-well plates were assayed immediately, except for the cells used in micronucleus assay, which were allowed to divide for 72 h and then assayed. In the experiments for delayed time points, cells were cultivated for 8 or 15 d after exposure. During the 8 and 15 d incubations for mitochondrial endpoints, cells were reseeded to new dishes once or twice, respectively. For micronucleus frequency assay, cells were reseeded to new dishes once during the 15 d incubation. Before assays, the cells were transferred to 48-well plates. For micronucleus assays, this transfer was performed 4 d before assay (0.15×10^6 cells/well). In assays related to mitochondrial endpoints, cells were transferred to 48-well plates 24 h before the assay (0.2×10^6 cells/well).

Cell cultures were exposed to TCDD (purchased from Ufa-Institute, Ufa, Russia, over 99 % purity as assessed by gas chromatography-mass spectrometry) for 48 h. The concentrations of TCDD used in different experiments are presented in Table 1. The concentrations and exposure

times for immediate mitochondrial endpoints were selected based on preliminary experiment and literature. TCDD was solubilised in DMSO and the maximum concentration of DMSO in samples was 0.25 %, which was used also in controls (0.1 % in the delayed endpoints). Menadione (20 μ M for 48 h) was used as a positive control in mitochondrial superoxide production, MMP, and mitochondrial activity assays. In micronucleus frequency analysis, 10 μ g/ml methyl methane sulphonate (MMS, Sigma, Germany) for 48 h was used as a positive control.

Mitochondrial superoxide production

Mitochondrial $O_2^{\cdot-}$ production was measured using a fluorescent probe, MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes, Invitrogen, Paisley, UK). After exposure, 1 μ M MitoSOX in HBSS was added to the cells on 48 well plates and incubated at +37°C for 30 min. The fluorescence was measured at an excitation wavelength of 492 nm and emission wavelength of 595 nm (Tecan Infinite F200 pro, Grödig, Austria).

Mitochondrial membrane potential

Mitochondrial membrane potential was measured using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Eugene, Oregon, USA). In the assay, 5 μ g/ml JC-1 in DMEM was added to the wells of 48-well plate and incubated at +37 °C for 15 min. After that, cells were washed twice with warm (+37 °C) PBS and 500 μ l of PBS was added to each well. The fluorescence was measured at an excitation wavelength of 540 nm and emission wavelength of 595 nm (Tecan Infinite F200 pro, Grödig, Austria).

Mitochondrial activity

Mitochondrial activity was assayed using thiazolyl blue tetrazolium bromide (MTT, Sigma, St. Louis, USA). During the experiments 30 μ l of 5 mg/ml MTT was added to 300 μ l of medium 2 h before the end of the incubation or exposure period. After 2 h incubation, medium and

MTT were removed from the wells of a 48-well plate and 450 μ l of DMSO was added into the wells. This was followed by shaking the samples on a plate shaker for 1 min and measurement of absorbance at 550 nm (Tecan Infinite F200 pro, Grödig, Austria).

Micronucleus frequency

After exposure, cells were incubated further for 3, 8 or 15 d to allow micronuclei to form during the subsequent cell divisions. The number of micronuclei and relative cell survival was assessed using flow cytometry (Bryce et al., 2007). Cells were stained as described previously (Luukkonen et al., 2011). Briefly, ethidium monoatside (EMA, 8.5 μ g/ml, Invitrogen Corporation/Molecular Probes, Eugene, Oregon, USA) was used to stain nuclei from cells with compromised cell membrane (necrotic and apoptotic cells). The second staining with SYTOX Green (0.4 μ M, Invitrogen Corporation/Molecular Probes, Eugene, Oregon, USA) after lysing the cells tinted all chromatin. Thus, micronuclei and nuclei from healthy cells were stained only with SYTOX green and we were able to distinguish them from the nuclei and pieces of nuclei from dying cells stained with EMA. Fluorescent counting beads (Peak Flow, Green Flow cytometry reference beads, 6 μ m; Invitrogen Corporation, Eugene, Oregon, USA) were included in all samples; therefore relative cell survival was calculated from nuclei to beads ratios and we were able to monitor that no overtly toxic exposures were performed (data not shown). The samples were analysed using a flow cytometer (Becton Dickinson FACSCalibur, San Jose, CA, USA) equipped with Cell Quest Pro (v. 5.2.1, BD Biosciences, USA) and 20 000 gated nuclei were acquired from each sample.

Statistical analysis

The two-way ANOVA was used for the statistical analysis, with TCDD concentration as a fixed factor and replicate as a random factor. Replicate was included as a random factor in the analysis because the replicates differed from each other statistically significantly in several cases. Post-hoc

tests were performed using the least significant difference (LSD) test. The test for linear trend was used to analyse dose-dependency of TCDD. The analysis was carried out using the general linear model procedure of SPSS for Windows release 19 (SPSS Inc., Chicago, Illinois, USA) using raw or logarithm-transformed (micronucleus data) values. All experiments were replicated three times (each experiment consisted of three parallel samples, except for micronuclei experiments with two parallel samples). A p -value less than 0.05 was considered as a statistically significant difference.

Results

This study investigated the effects of TCDD on mitochondrial integrity and genomic stability (measured by the changes in the micronucleus frequency). Mitochondria-related endpoints were the level of mitochondrial superoxide, MMP, and mitochondrial activity. The mitochondrial integrity and the stability of the genome were assayed immediately, 8 and 15 d after exposure. As micronucleus formation requires at least one cell division, the time point of 72 h was considered as an immediate effect for micronucleus frequency.

Effects of TCDD immediately after the exposure

A significant trend for increased mitochondrial superoxide levels was observed immediately after TCDD exposure ($p < 0.001$, Figure 2 A) and of superoxide levels were significantly increased at 0.1, 50, and 250 nM TCDD ($p = 0.015$, $p = 0.001$, and $p < 0.001$, respectively). As expected, the positive control increased significantly the level of mitochondrial superoxide (20 μ M menadione for 48 h, 4.3-fold increase, $p < 0.001$).

All TCDD concentrations caused a modest decrease in the MMP level, but the dose-response was not significant immediately after the exposure ($p = 0.175$, Figure 2 B). The performance of the MMP assay was verified by 20 μ M menadione exposure for 48 h, which caused a decrease of 56.7% in MMP level ($p = 0.002$).

The mitochondrial activity of the cells was moderately elevated at all TCDD concentrations immediately after exposure, but the dose-response did not reach statistical significance ($p = 0.057$, Figure 2 C). The positive control, 20 μ M menadione for 48 h, decreased mitochondrial activity (49.6 % of the control, $p = 0.019$).

The micronucleus frequency was not altered by TCDD in the measurements reflecting the immediate responses ($p = 0.340$, Figure 2 D). However, as expected, the positive control

increased significantly the level of micronuclei (10 $\mu\text{g/ml}$ MMS for 48 h, 3.2-fold increase compared to controls, $p = 0.016$).

Effects of TCDD 8 d after the exposure

The level of mitochondrial superoxide was not altered by TCDD at 8 d post exposure ($p = 0.402$, Figure 3 A). However, TCDD concentration-dependently decreased the MMP level ($p = 0.048$, Figure 3 B), and the decrease was significant at 10 and 100 nM TCDD ($p = 0.031$, $p = 0.023$, respectively). In contrast, mitochondrial activity was not affected by TCDD treatment (Figure 3 C, $p = 0.456$). In addition, the effect of TCDD on micronucleus frequency was not significant (Figure 3 D, $p = 0.077$).

Effects of TCDD 15 d after the exposure

Contrary to the findings of measurements at 8 d post exposure, the trend analysis of TCDD effects showed an increased level of mitochondrial superoxide at 15 d after exposure ($p = 0.027$, Figure 4 A). Superoxide levels were also significantly increased at 1 nM TCDD ($p = 0.011$). However, neither MMP level (Figure 4 B, $p = 0.147$) nor mitochondrial activity (Figure 4 C, $p = 0.472$) indicated overall disturbance of the mitochondrial integrity. Micronucleus frequency was not significantly altered by TCDD exposure (Figure 4 D, $p = 0.599$).

Discussion

This study investigated whether TCDD affects mitochondrial integrity and whether possible changes in mitochondria play a role in genomic instability. Mitochondrial superoxide formation, MMP, and mitochondrial activity were used to assess the integrity of mitochondria, while micronucleus frequency was measured to evaluate genetic damage and genomic instability.

TCDD treatment increased mitochondrial superoxide production immediately after the exposure, while MMP or mitochondrial activity (measured by MTT) were not significantly altered at this time point. The finding of TCDD-induced increase in mitochondrial superoxide level by TCDD corroborates previous findings (Senft et al., 2002; Shen et al., 2005; Aly and Domènech, 2009; Aly and Khafagy, 2011). However, contrary to the present findings, there are several studies (Aly and Khafagy, 2011; Chen et al., 2010; Morales-Hernández et al., 2012) indicating both decreased MMP and MTT levels in TCDD-treated cells. One of these investigations (Morales-Hernández et al., 2012) is of particular interest, as it utilized the same cell line and TCDD concentration (250 nM) as the present study. Morales-Hernández et al. (2012) found that 250 nM TCDD for 90 min reduced MTT level by ~30 % (measured between 18 and 24 h after TCDD treatment), while tetramethyl rhodamine methyl ester fluorescence, reflecting MMP level, was reduced by ~30–35 %. In contrast, the present results indicate ~27 % increase in MTT level and only ~13 % decrease in MMP level after TCDD treatment for 48 h (non-significant findings). A possible explanation for the discrepancy lies most likely in the difference between the TCDD treatment times (90 min vs. 48 h). The consideration of different treatment times provides two candidates for the explanation of the results. As loss of MMP is an indication of apoptosis (Kroemer et al., 2007), the results of the Morales-Hernández et al. (2012) and present studies could imply that a subgroup of SH-SY5Y cells undergoes apoptosis during the 48 h treatment, leaving behind a more resistant cell population. Another possible explanation is that SH-SY5Y cells are able to respond to the initial TCDD damage (90 min), thus resulting in a higher MMP level, and rebound of

mitochondrial activity when the exposure time is 48 h. Further studies are needed to clarify cellular mechanisms of TCDD effects on mitochondria.

At the delayed time points, TCDD decreased MMP level at 8 d and increased mitochondrial superoxide production at 15 d post exposure. However, the reproducibility of these findings needs to be tested in an independent confirmatory study because neither of the findings were accompanied by the other mitochondrial endpoints measured. In addition, the results at 8 d post exposure have to be interpreted with caution due to internal inconsistency; MMP was decreased significantly by 10 and 100 nM TCDD (Fig. 3 B) while, inconsistently, both highest and lowest MTT values were recorded with these same concentrations (Fig. 3 C, non-significant findings). Thus, as no consistent pattern of TCDD-induced effects was noted, the present results do not support delayed mitochondrial dysfunction.

Mitochondria play an important role in multiple cellular functions such as energy production, apoptosis, and proliferation, thus making the normal function of mitochondria essential for the cells. Dysfunction of mitochondria have been linked both to cancer formation (de Moura et al., 2010; Gasparre et al., 2013) and to genomic instability (Kim et al., 2006 a, b). Many research groups (Ishii et al. 2006; Van Houten et al. 2006; Ott et al. 2007) have suggested that the malfunctioning of the electron transport chain, located in the inner mitochondrial membrane, might trigger a “vicious cycle of ROS production”. According to this hypothesis, the dysfunctional electron transport chain would lead to the subsequent increase in ROS, which would result in further damage to mitochondria (i.e. the loss of MMP), leading to further increase in ROS production and degeneration of mitochondrial functionality. Although this study was not designed to test the hypothesis on the “vicious cycle of ROS production”, the present results point out that TCDD-induced increased mitochondrial ROS levels do not lead to further enhanced ROS levels nor to persistent degeneration of mitochondrial function. This indicates that TCDD-induced radicals do

not trigger the subsequent events in human SH-SY5Y neuroblastoma cells, as described in the hypothesis above.

Micronucleus frequency was not altered immediately after the exposure to TCDD, which is also consistent with our previous study (Korkalainen et al., 2012). This result is also concordant with the other previous findings (Hernandez et al., 2009; Katic et al., 2010). Furthermore, micronucleus frequency was not significantly altered by TCDD 8 or 15 d after the initial exposure. Contrary to this finding, we have previously reported an induction of genomic instability in mouse embryonic fibroblasts C3H10T1/2 6 days after exposure to 10 and 100 nM TCDD (Korkalainen et al., 2012). The explanation for the inconsistency between these two studies lies most probably in the use of vastly different cell lines; C3H10T1/2 are pluripotent contact-inhibited embryonic cells whereas SH-SY5Y are non-pluripotent neuroblastoma cells exhibiting no contact inhibition.

In conclusion, the main finding of this study was increased level of mitochondrial superoxide immediately after exposure to TCDD. The results do not support TCDD-induced genomic instability or persistently impaired mitochondrial integrity in human SH-SY5Y neuroblastoma cells.

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Figure legends

Figure 1. An overview of the experiments.

Figure 2. Mitochondrial superoxide level (A), mitochondrial membrane potential (B), mitochondrial activity (C), and micronucleus frequency (D) in human SH-SY5Y neuroblastoma cells immediate after the TCDD exposure for 48 h. As the formation of micronuclei requires at least one cell division, 72 h was considered as an immediate effect for this assay. TCDD exposure increased the level of mitochondrial superoxide ($p < 0.001$). Significant differences of individual TCDD concentrations from controls are: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. The data are shown as mean \pm SEM, $n = 3$ (each experiment consisted of three parallel samples, except for micronuclei experiments with two parallel samples), RFU = Relative Fluorescence Unit, RAU = Relative Absorbance Unit.

Figure 3. Mitochondrial superoxide level (A), mitochondrial membrane potential (B), mitochondrial activity (C), and micronucleus frequency (D) in human SH-SY5Y neuroblastoma cells 8 d after the TCDD exposure for 48 h. TCDD exposure decreased the level of mitochondrial membrane potential ($p = 0.048$) while the other measured mitochondrial endpoints remained unaffected. Significant differences of individual TCDD concentrations from controls are: * = $p < 0.05$. The data are shown as mean \pm SEM, $n = 3$ (each experiment consisted of three parallel samples, except for micronuclei experiments with two parallel samples), RFU = Relative Fluorescence Unit, RAU = Relative Absorbance Unit.

Figure 4. Mitochondrial superoxide level (A), mitochondrial membrane potential (B), mitochondrial activity (C), and micronucleus frequency (D) in human SH-SY5Y neuroblastoma cells 15 d after the TCDD exposure for 48 h. TCDD exposure increased the level of mitochondrial superoxide ($p = 0.027$), while the other measured mitochondrial endpoints and micronucleus

frequency were not altered. Significant differences of individual TCDD concentrations from controls are: * = $p < 0.05$. The data are shown as mean \pm SEM, $n = 3$ (each experiment consisted of three parallel samples, except for micronuclei experiments with two parallel samples), RFU = Relative Fluorescence Unit, RAU = Relative Absorbance Unit.

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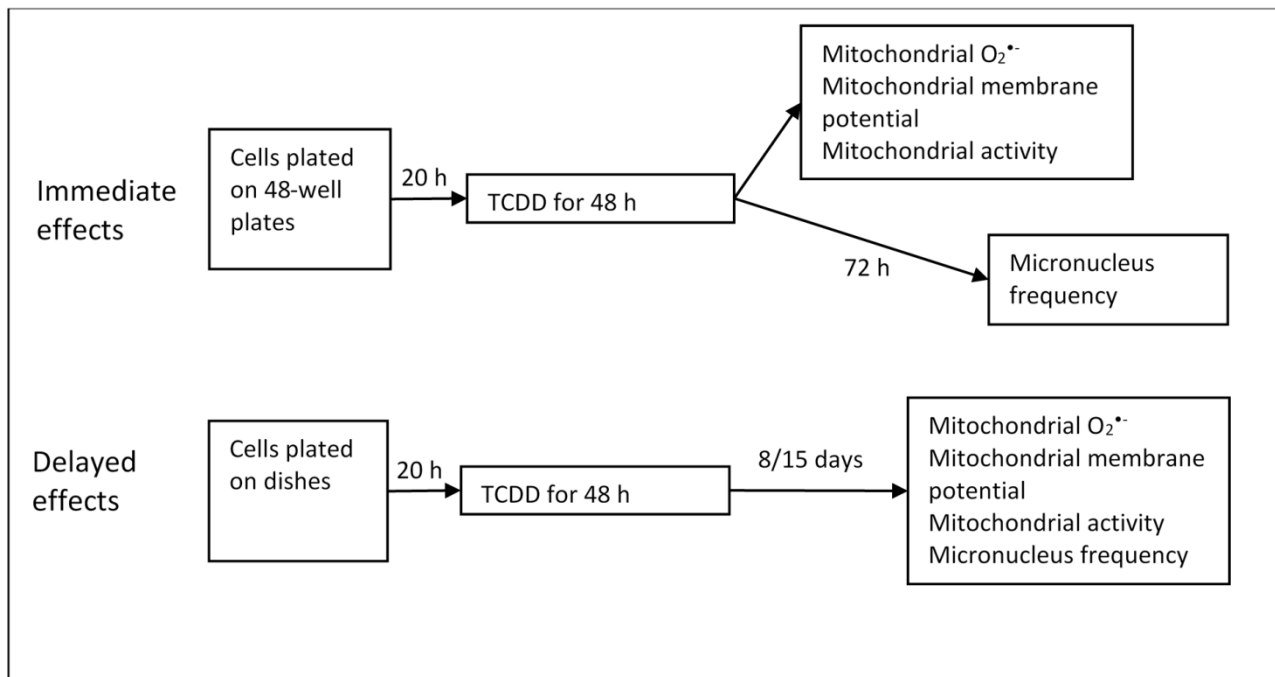


Figure 1

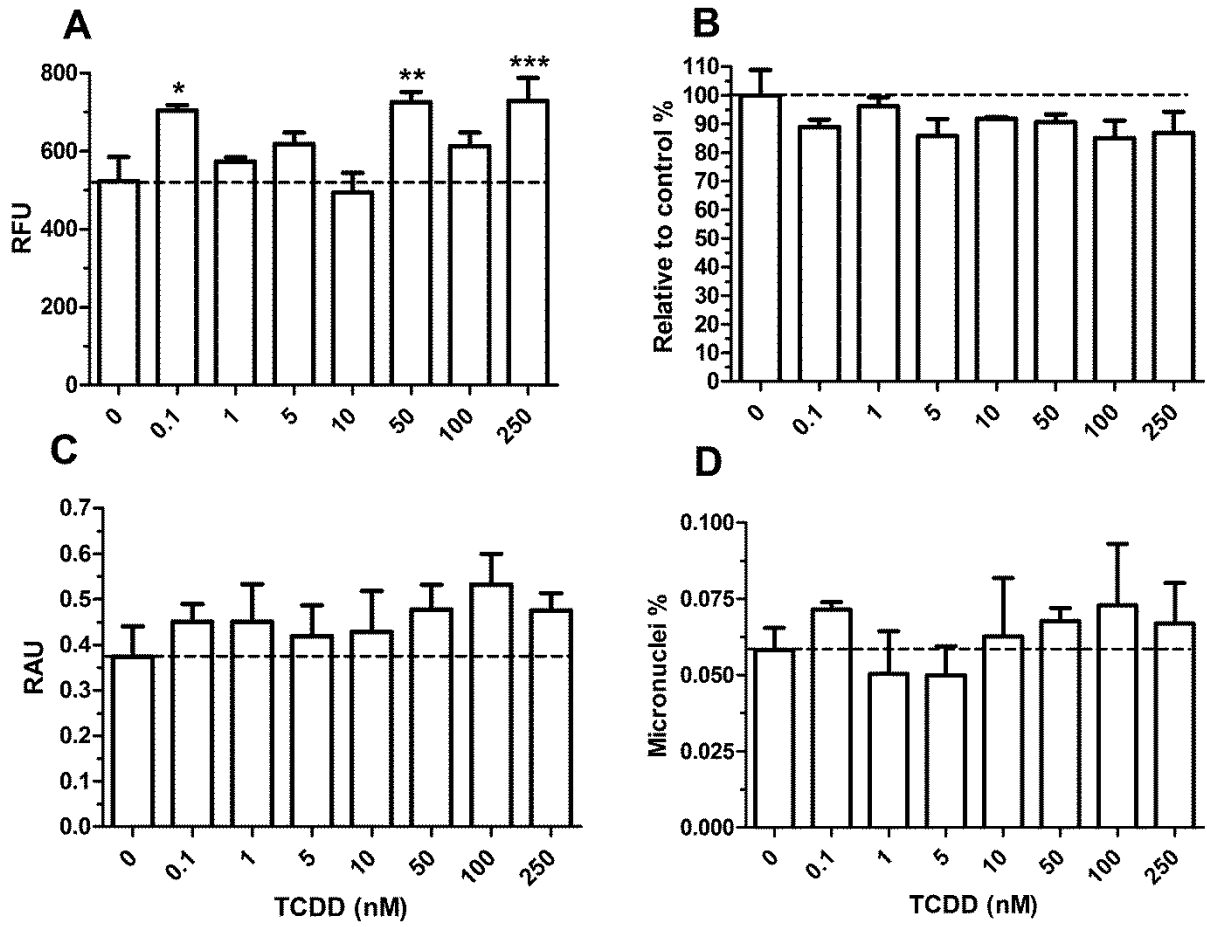


Figure 2

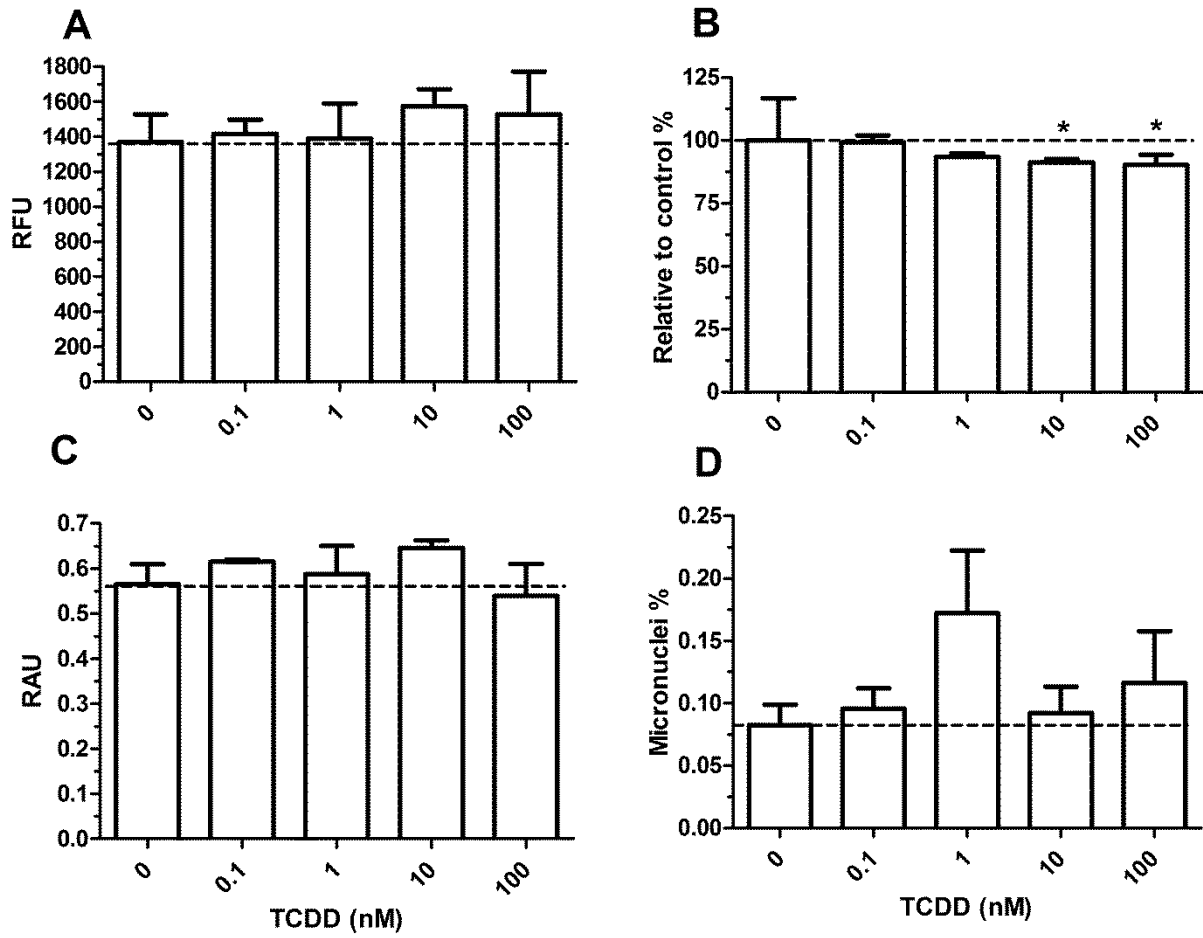


Figure 3

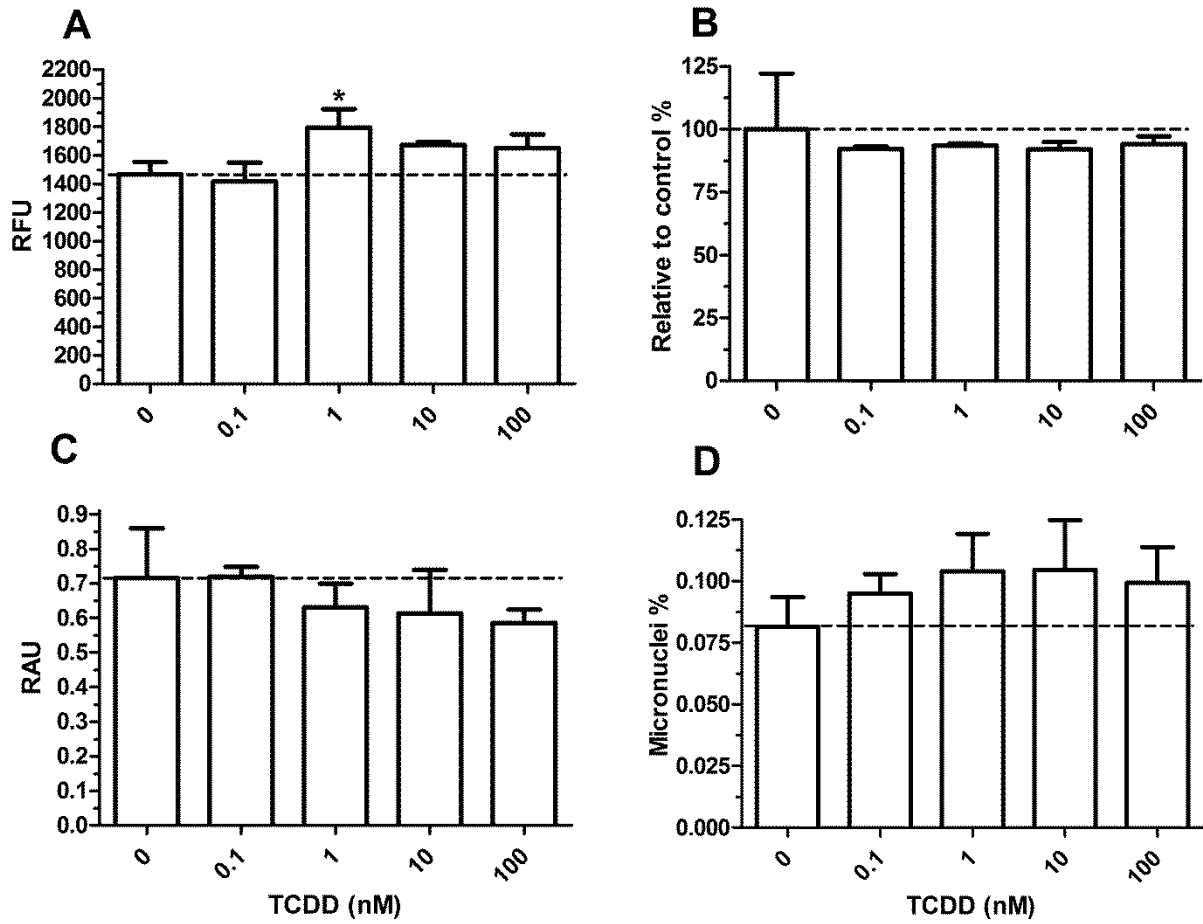


Figure 4

Table 1. The concentrations of TCDD used and the number of cells seeded in the experiments.

	Immediate		Delayed	
	Mitochondrial endpoints	Micronucleus assay	Mitochondrial endpoints	Micronucleus assay
TCDD ¹ , nM (48 h)	0.1, 1, 5, 10, 50, 100, 250	0.1, 1, 5, 10, 50, 100, 250	0.1, 1, 10, 100	0.1, 1, 10, 100
Cell numbers (* 10 ⁶)	0.075 (48-well plates)	0.10 (48-well plates)	1.8 (Petri dishes)	0.4 (Petri dishes)

¹TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Highlights:

- TCDD increased mitochondrial superoxide production immediately after exposure
- TCDD-induced radicals did not lead to persistent degeneration of mitochondria
- TCDD did not cause genomic instability in human SH-SY5Y neuroblastoma cells

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