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2,3,7,8-Tetrachlorobenzo-*p*-dioxin Inhibits Proliferation of SK-N-SH Human Neuronal Cells Through Decreased Production of Reactive Oxygen Species

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Oxidative stress has been known to be involved in the mechanism of toxic effects of various agents on many cellular systems. In this study we investigated the role of reactive oxygen species (ROS) in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced neuronal cell toxicity using SK-N-SH human neuroblastoma cells. TCDD inhibited proliferation of the cells in a dose-dependent manner, which was revealed by MTT staining, counting of cells stained with trypan blue and [^{3}H]thymidine uptake assay. TCDD also suppressed the basal generation of ROS in a time- and concentration-dependent manner assessed by 2′,7′-dichlorofluorescein fluorescence. In addition, TCDD induced a dose-dependent inhibition of lipid peroxidation, a biomarker of oxidative stress, whereas it significantly increased the level of glutathione (GSH), an intracellular free radical scavenger in the cells. Moreover, TCDD altered the activities of major antioxidant enzymes; increase in superoxide dismutase (SOD) and catalase, but decrease in glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Red). Pretreatment with L-buthionine-*S*,*R*-sulfoximine (BSO, 50 μM), an inhibitor of GSH synthesis, significantly prevented the TCDD-induced reduction in lipid peroxidation and cell proliferation. Interestingly, exogenous application of an oxidant, H_2O_2 (50 μM) markedly restored the inhibited cell proliferation induced by TCDD. Taken together, these results suggest that alteration of cellular redox balance may mediate the TCDD-induced inhibition of proliferation in human neuronal cells.

Keywords: TCDD; Proliferation; Reactive oxygen species; Glutathione; SK-N-SH cells

Abbreviations: BSO, L-buthionine-*S*,*R*-sulfoximine; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; GSH, glutathione; GSH-Px, glutathione peroxidase; GSH-Red, glutathione reductase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the best characterized dioxin congeners and the most toxic environmental pollutants.[1] Because of its lipophilic nature, TCDD easily enters body via a food chain but is eliminated very slowly from the body. Lactation is the most important route of excretion for TCDD among women.[2] A wide range of toxic effects of TCDD has been reported in various tissues in animal species and humans including endocrine disruption,[3] immune toxicity,[4] carcinogenicity[5] and chloracne,[6] developmental teratogenicity[7] that causes cleft palate and craniofacial abnormalities. Even though TCDD is mostly accumulated in liver and adipose tissue but low in brain,[8] central nervous system especially in newborn infants whose brain is under maturation process has higher chance of TCDD intoxication by

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being exposed to highly concentrated TCDD during gestation and lactation. In fact, TCDD shows developmental neurotoxicity such as brain asymmetry,[9] decrease in neurotransmitters,[10] neuronal calcium uptake,[11] cognitive deficits[12] and death of neurons.[13]

The toxic effect of TCDD is thought to be mediated through alteration of redox balance in hepatic and extrahepatic tissues of mice and rats.[14,15] Most of these reports demonstrated induction of reactive oxygen species (ROS), increased lipid peroxidation and decreased GSH content in liver, testis and macrophages following exposure to TCDD. However, recent reports demonstrated a reduced peroxide level and increase in GSH contents in TCDD-treated hepatocytes.[16,17]

Excessively produced ROS may result in cellular damage through their interaction with cellular macromolecules and structures. The mechanisms of these actions of ROS include activation of proteases and nucleases,[18] altered gene expression,[19] and changes in membrane permeability.[17] In contrast to such well recognized cell injury, ROS have been shown to act as an important signaling molecule in the processes of cell proliferation, differentiation[20] and tumor formation possibly through activation of growth factor receptor and downstream signaling pathway.[21,22] Particularly, it has been demonstrated that ROS may be involved in neuronal development.[23]

Thus, the main purpose of this study was to investigate whether ROS play a role in TCDD-induced neurotoxicity using SK-N-SH human neuroblastoma cells as a model for developing human neuronal system.

MATERIALS AND METHODS

Materials

The SK-N-SH human neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders for Eagle's minimum essential medium, trypsin solution, sodium pyruvate, BSO, MTT and all salt powders were obtained from Sigma Chemical Co. (St Louis, MO). DCFH-DA was from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). The stock solutions of drugs were sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell Culture

SK-N-SH cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in an Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

Cell Viability Assay (MTT Staining)

Cell viability was assessed by the MTT staining method.[24] Cells from 4- to 5-day-old cultures were seeded in 24-well plates at the density of 5×10^4 cells/well. The volume of the medium in the wells was 1 ml. In control experiments, cells were grown in the same media containing drug-free vehicle. After incubation with drug for 48 h, 100 μl of MTT (5 g MTT/l in H_2O) were added and cells incubated for a further 4 h. Two hundred microliters of DMSO were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with an ELISA reader (Molecular Devices, Menlo Park, CA) with a 540 nm filter.

[^{3}H]Thymidine Incorporation Assay

Cell proliferation was determined by measuring [^{3}H]thymidine uptake according to the method of El-Metwally and Adrian.[25] Cells were treated with TCDD and [^{3}H]thymidine (0.5 μCi/ml) was added 6 h before the termination of a 48 h incubation. After incubation, cells were washed with ice-cold PBS three times and fixed with glacial acetic acid/methanol (1:3, v/v) for 1 h. Cells were then washed with 10% ice-cold trichloroacetic acid and lysed with 0.25% sodium dodesyl sulfate and 0.1 N NaOH. The radioactivity was counted in a liquid scintillation counting.

Cell Death Assay (Trypan Blue Exclusion)

Cells from 4- to 5-day-old cultures were seeded in equivalent amounts in 35 × 10 mm culture dishes at the density of 2×10^5 cells/dish. The volume of the medium in the dishes was 2 ml. The drugs to be tested were added to cultures 1 day after seeding in order to ensure uniform attachment of cells at the onset of the experiments. The cells were grown for an additional 2 days. Drug and culture media were replaced every day. Cytotoxicity of the drug was assessed by counting the number of cells stained by trypan blue using a hemocytometer.

Intracellular ROS Measurement

Relative changes in intracellular ROS in the SK-N-SH cells were monitored using a fluorescent probe,

DCFH-DA.[26] DCFH-DA diffuses through the cell membrane readily and is hydrolyzed by intracellular esterases to nonfluorescent DCFH, which is then rapidly oxidized to highly fluorescent DCF in the presence of ROI. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly.[27] Cells were washed twice and resuspended at a concentration of 4×10^5 cells/ml in Hank's solution. For loading DCFH-DA into the cells, cells were incubated with the dye for 2 h at a final concentration of 5 μM at 37°C. Fluorescence (excitation wavelength set at 485 nm and the emission wavelength at 530 nm) was monitored in a well-stirred cuvette. Data are presented as relative fluorescence changes compared to control condition in which the cells were incubated without TCDD.

Enzyme Assays

For the measurement of enzyme activities crude cell homogenate after lysed by freeze/thaw three times was centrifuged at 600*g* for 10 min. The pellet was discarded and supernatant was re-centrifuged at 10,000*g* for 30 min. The supernatant was used for sources of glutathione reductase (GSH-Red), glutathione peroxidase (GSH-Px) and SOD (superoxide dismutase). The pellet was homogenized in 100 μl of 0.25 M sucrose and sonicated for 10 s at 4°C in an ultrasonic processor (power 150 W). The sonicated suspension was used for the source of catalase.

Catalase activity was measured according to Aebi.[28] This assay involves the change in absorbance at 240 nm due to the catalase-dependent decomposition of H_2O_2. The catalase source was mixed with 60 mM potassium phosphate buffer, pH 7.0, and 20 mM H_2O_2. The change in absorbance at 240 nm was measured for 30 s and the slope of the curve at linearity was calculated. Data are expressed as μmol of peroxide decomposed/s/mg protein.

SOD activity was assayed according to the methods of McCord and Fridovich.[29] The reaction mixture contained 50 mM of potassium phosphate, pH 7.8, 150 nM EDTA, 15 nM cytochrome C, 75 nM xanthine, 0.04 U xanthine oxidase and suitable amount of enzyme solution in a total volume of 1.5 ml. The activity was monitored at 550 nm at 25°C, and one unit of enzyme activity was defined as the amount of enzyme that inhibited 50% of the reduction of cytochrome C.

GSH-Px activity was determined by the method of Rybak *et al.*[30] Briefly, 500 μl of 0.05 M sodium phosphate buffer containing 1 mM EDTA, pH 7.0, were mixed with 100 μl of 0.01 M GSH, 100 μl of NADPH (1.5 mM), 100 μl of GSH-Red (20 units/ml), 100 μl of 0.25 mM H_2O_2 in the test tubes and incubated for 10 min at 25°C. The reaction was started by adding 100 μl of cell extract and the change in absorbance was monitored at 340 nm for 5 min in a recording spectrophotometer. The millimolar extinction coefficient of 6.22 was used to determine GSH-Px activity expressed as nmol NADPH oxidized/min/mg protein.

GSH-Red activity was measured according to the methods of Meiz *et al.*[31] The reaction was started by addition of 20 μl of sample into 2.98 ml of reaction mixture containing 0.9 mM EDTA, 0.54 mM GSSG, 0.2 mM NADPH and 0.1 M Tris–HCl pH 8.0, and then incubated at 25°C for 5 min. The GSH-Red activity was monitored by measuring the changes of optical density at 340 nm and converted to nmol of oxidized NADPH using the extinction coefficient $\Delta E340 = 6.22\,mM^{-1}\,cm^{-1}$.

Lipid Peroxidation Assay

The cells harvested were suspended in 1 ml of PBS and mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution (pH 3.5), and 1.5 ml of 0.8% thiobarbituric acid. The mixture was heated at 95°C for 1 h, chilled to room temperature, and extracted with 1 ml of ddH_2O and 2.5 ml of $n-$butanol × pyridinemixture (15:1, v/v). The upper organic layer containing malondialdehyde (MDA) produced by lipid peroxidation was measured at 532 nm. MDA was used as an external standard, and the level of lipid peroxides was expressed as nM of MDA. The protein concentration was measured by the method of Bradford.[32]

Determination of GSH Level

The level of GSH was assayed in SK-N-SH cells according to the method of Griffith.[33] Briefly, cells (1×10^5) were collected in 300 μl of PBS containing 0.1% Triton X-100. Two hundred microliters of this homogenate was used in each assay in the presence of NADPH (86 μM), GSH-Red (66 mU/M) in 50 mM phosphate buffer (pH 7.5). Reduction of 66 μM 5,5′-dithiobis-(2-nitrobenzoicacid (DTNB) by cellular reduced GSH was monitored at 412 nm for 5 min and the GSH was determined from a standard GSH curve (2.5–40 μM).

Data Analysis

All experiments were performed four times. Data were expressed as mean ± standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student–Newman–Keul's test for individual comparisons. *P* values less than 0.05 are considered statistically significant.

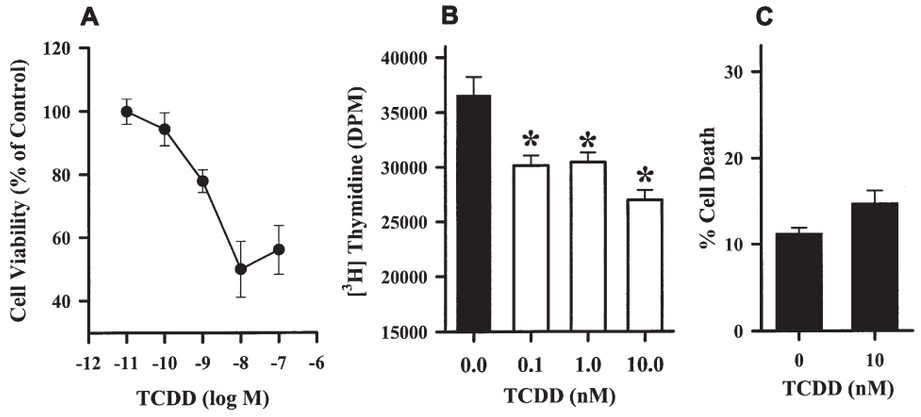

FIGURE 1 TCDD decreases cellular proliferation in SK-N-SH human neuronal cells. Cells treated for 48 h with or without each concentration of TCDD were analyzed for viability by MTT assay (A), for proliferation by [^{3}H]thymidine incorporation assay (B) and for cell death by trypan blue exclusion method. Data points (B and C) represent the mean values of four replications with bars indicating SEM. *$P < 0.05$ compared to control.

RESULTS

Inhibition of Neuronal Cell Proliferation by TCDD in SK-N-SH Cells

TCDD reduced the apparent viability of SK-N-SH cells in a dose-dependent manner as shown in Fig. 1A. This effect of TCDD may be predominantly due to suppressed cell proliferation, rather than cell death, since [^{3}H]thymidine uptake was decreased by TCDD in a concentration-dependent fashion (Fig. 1B), but the number of dead cells was not significantly altered assessed by trypan blue exclusion method (Fig. 1C).

Alteration of Redox Balance by TCDD Treatment in the Neuronal Cells

To explore the role of ROS in the mechanism of the anti-proliferative action of TCDD, we first examined whether TCDD can alter the level of intracellular ROS in the cells using DCF fluorescence. TCDD suppressed the basal generation of ROS in a time- and concentration-dependent manner as shown in Fig. 2. We also detected lipid peroxidation, a biomarker of oxidative stress. TCDD suppressed basal lipid peroxidation in a dose-related manner as shown in Fig. 3A. In addition, the level of cellular GSH was significantly increased as depicted in Fig. 3B. To examine a possible relationship between reduced production of ROS and increased GSH level by TCDD, we investigated the activities of major antioxidant enzymes, SOD, catalase, GSH-Px and GSH-Red. As shown in Fig. 3C, the activities of GSH-Px and GSH-Red were decreased, while the activities of SOD and catalase were increased in a concentration-dependent manner by TCDD. These results imply that reduced production of ROS by TCDD may be due to increased cellular GSH level and enhanced activities of SOD and catalase.

Roles of Reduced Production of ROS and Increased GSH Level in the TCDD-induced Neurotoxicity

Since it has been reported that endogenously generated ROS and cellular GSH level regulate cell

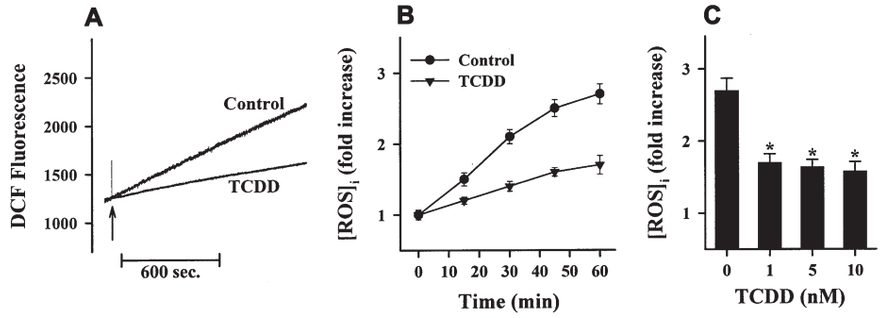

FIGURE 2 TCDD suppresses the basal generation of ROS in a time- (A and B) and dose-dependent (C) manner. The data (A) show changes in ROS levels as a function of time, which was measured by DCF fluorescence method. The arrow shows the time point for addition of TCDD (10 nM). In the data (B and C) results are expressed as fold increase compared to the initial DCF fluorescence intensity. Data points represent the mean values of four replications with bars indicating SEM. *$P < 0.05$ compared to control condition in which the cells were incubated with TCDD-free medium.

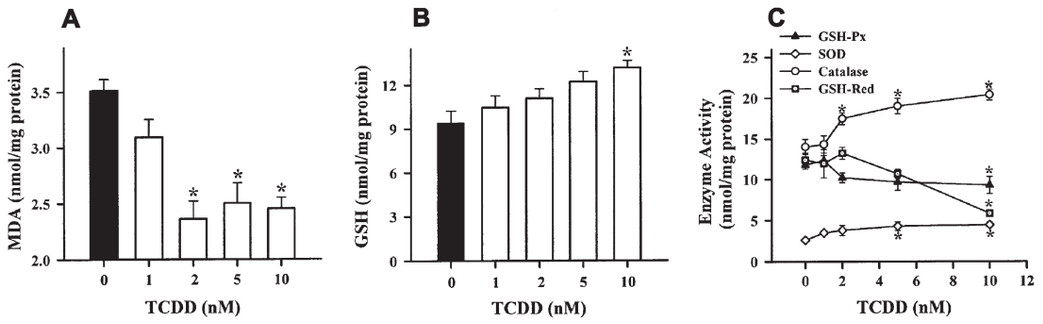

FIGURE 3 Effects of TCDD on the lipid peroxidation (A), cellular GSH level (B) and activities of antioxidant enzymes (C). Cells were treated for 48 h with or without each concentration of TCDD. Data points represent the mean values of four replications with bars indicating SEM. *$P < 0.05$ compared to control.

proliferation,[20] the possible role of reduced ROS and/or increased GSH level in the proliferation-inhibiting activity of TCDD was investigated. If decreased production of basal ROS may mediate inhibition of neuronal proliferation by TCDD, exogenous application of H_2O_2 would prevent the cells from inhibited proliferation. In fact, treatment with H_2O_2 (50 μM) significantly blocked decrease in proliferation as shown in Fig. 4. When the cells were treated with BSO, a GSH synthesis inhibitor, lipid peroxidation was significantly higher than that of control cells as shown in Fig. 5A, representing tilting up the balance toward enhanced ROS level. The level of lipid peroxidation in the cells pretreated with BSO prior to TCDD addition was similar to that treated with BSO alone. Moreover, pretreatment with BSO (50 μM) significantly prevented the TCDD-induced decreased cell proliferation as depicted in Fig. 5B. These results imply that decreased ROS production and increased GSH level may play an important role in the TCDD-induced inhibition of neuronal cell proliferation.

DISCUSSION

Dioxins, especially TCDD is a widespread environmental contaminant which accumulates in food chains and consequently in humans due to its high lipophilicity and resistance to chemical and biological degradation.[1] Men have no way of getting rid of TCDD from the body, whereas women have two ways, one is through placenta and another is via lactation.[2] Thus, the human fetus and breast-fed infant are regarded as the primary risk group due to a high exposure and high sensitivity. TCDD has been known to cause profound alterations in various aspects such as development, reproduction, immune system and neurobehavior. Perinatal exposure to TCDD has been shown to affect brain development resulting in brain asymmetry,[3] abnormal NMDA receptor expression[34] and learning impairment.[35] However, the mechanism and neurotoxic effects of TCDD have not been fully elucidated.

The present study clearly demonstrates that TCDD inhibits proliferation of SK-N-SH human neuronal

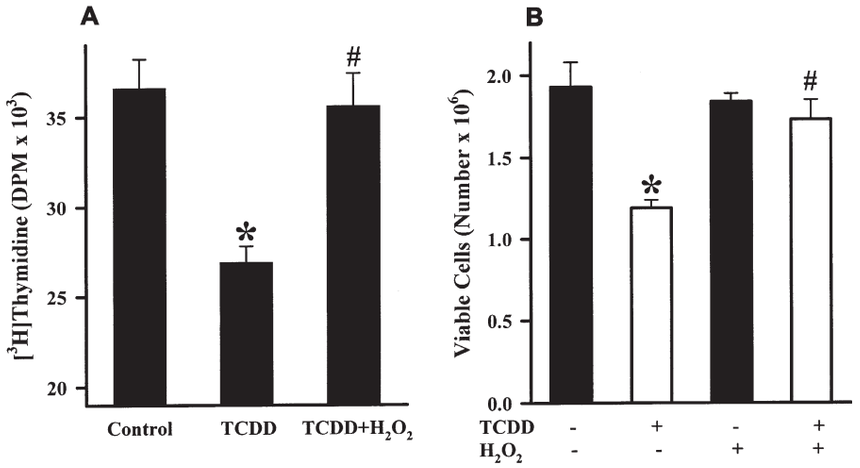

FIGURE 4 Exogenous application of H_2O_2 prevents the TCDD-induced inhibition of cellular proliferation. Cells treated for 48 h with or without TCDD (10 nM) or H_2O_2 (50 μM) were analyzed for proliferation by [^{3}H]thymidine incorporation assay (A) and for cell viability by counting the number of trypan blue-negative cells (B). The data represent the mean values of four replications with bars indicating SEM. *$P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to TCDD alone.

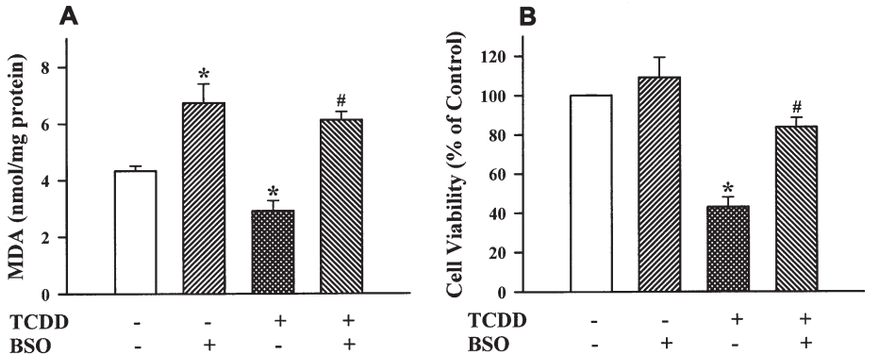

FIGURE 5 Effects of BSO on the TCDD-induced decrease in lipid peroxidation and cell proliferation in human neuronal cells. Cells treated for 48 h with or without TCDD (10 nM) or BSO (50 μM) were analyzed for lipid peroxidation by measuring MDA concentration (A) and for cell viability by MTT assay (B). In these experiments, BSO was added 60 min before TCDD application. The data represent the mean values of four replications with bars indicating SEM. *$P < 0.05$ compared to control. $P < 0.05$ compared to TCDD alone.

cells (Fig. 1). Furthermore, our results demonstrate that TCDD decreased basal production of ROS and lipid peroxidation (Figs. 2 and 3). These findings seem to be contradictory to previous other reports showing increased oxidative stress with enhanced ROS production and lipid peroxidation.[15] However, most of these studies were conducted in animals and were performed with high dose of TCDD ranging 50–100 μg/kg. Recent reports performed in cultured cells with relatively low concentration of TCDD (10 nM) have shown that TCDD increased GSH level and SOD activity in hepatocytes[16,17] and adipocytes.[36] In addition, the present study showed that in human neuronal cells TCDD enhanced activities of SOD and catalase, while decreasing GSH-Px and GSH-Red (Fig. 3), resulting in reduced ROS level and saving of GSH efficiently.

Normally, aerobic mammalian cells produce ROS in the course of energy metabolism, which is counterbalanced by the free radical scavenging system. Cellular hydrogen peroxide and superoxide anions are increasingly reported as an intracellular signaling molecules playing an important role in cell proliferation as well as cell death[37,38] depending upon their concentration. It has been shown that low concentration of exogenous H_2O_2 application stimulated growth of fibroblasts in culture.[39] In contrast, addition of catalase and SOD has been shown to induce decrease in cell growth.[40] In conjunction with these reports, our data showing that suppression of neuronal cell proliferation by TCDD was restored by either exogenous application of H_2O_2 (Fig. 4) or pretreatment with BSO, an inhibitor of GSH synthesis (Fig. 5), implicate that reduced ROS level and increased GSH level may be responsible for the anti-proliferative action of TCDD.

In conclusion, TCDD inhibited proliferation of human neuronal cells. Reduced production of ROS and increased GSH level may mediate this action of TCDD. These results may have an important implication for impaired brain maturation resulting from developmental exposure to TCDD.

Acknowledgements

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