

Cytotoxicity and Apoptosis Induction in Human HepG2 Hepatoma Cells by Decabromodiphenyl Ethane

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Abstract

Objective To investigate the toxic effects of decabromodiphenyl ethane (DBDPE), used as an alternative to decabromodiphenyl ether *in vitro*.

Methods HepG2 cells were cultured in the presence of DBDPE at various concentrations (3.125-100.0 mg/L) for 24, 48, and 72 h respectively and the toxic effect of DBDPE was studied.

Results As evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and lactate dehydrogenase assays and nuclear morphological changes, DBDPE inhibited HepG2 viability in a time- and dose-dependent manner within a range of 12.5 mg/L to 100 mg/L and for 48 h and 72 h. Induction of apoptosis was detected at 12.5-100 mg/L at 48 h and 72 h by propidium iodide staining, accompanied with overproduction of reactive oxygen species (ROS). Furthermore, N-acetyl-L-cysteine, a widely used ROS scavenger, significantly reduced DBDPE-induced ROS levels and increased HepG2 cells viability.

Conclusion DBDPE has cytotoxic and anti-proliferation effect and can induce apoptosis in which ROS plays an important role

Key words: Apoptosis; Cytotoxicity; Decabromodiphenyl ethane; Flame retardants; Reactive oxygen species

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INTRODUCTION

Over the past 20 years, polybrominated dipentyl ethers (PBDEs) have been ubiquitous in the environment^[1]. PBDEs have been voluntarily phased out or restricted by law in several countries due to concerns over human and wildlife health. A demand for alternative flame-retardants has thus naturally been created. Decabromodiphenyl ethane (DBDPE) was brought into the market in the early 1990s^[2] as an alternative to its polybrominated analogue, decabromodiphenyl ether (PBDE-209) due to its high molecular weight (971.22 D) and hence was assumed to have limited bioavailability.

DBDPE is manufactured in the United State of

America and China, and exported around the globe. It is used to flame retard high-impact polystyrene and other styrenic resins, thermoplastic polyolefins, wire and cable insulation, and other electronic applications^[3]. DBDPE has been found in sewage sludge, sediment, and was detected for the first time in interior air in Europe in 2004^[4], which demonstrated that DBDPE, like PBDE-209, was leaking out of the technosphere and accumulating in the environment. In recent years, there have been more reports, suggesting that DBDPE is widely distributed in aquatic and indoor environments. Ricklund et al. conducted an international survey of DBDPE levels in sewage sludge and found that it was present in 12 different countries. The highest levels

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of DBDPE in sludge was 216 ng/g dwt. It was estimated that the DBDPE flux out of wastewater treatment plants from the technosphere to the environment was 1.7 ± 0.34 mg annually per person within the European Union countries^[5].

Although the DBDPE concentrations found in the environment, like other brominated flame retardants, may be below the levels to cause complications in laboratory tests, it can accumulate in tissues and thereby presents a potential and long-term risk to the health of humans and the environment^[6]. In recent years, concerns have been raised over the toxic effect of DBDPE as it becomes more widely distributed. Recent evidence from animal studies suggests that high-dose DBDPE may induce an increase in the mean absolute or relative liver weight, leading to slight hepatocellular vacuolation and centrilobular hepatocytomegaly (as determined by histomorphological evaluation), although these changes disappear after 28 days^[7]. Toxicity studies indicated that DBDPE was acutely toxic to water fleas and could accumulate in freshly separated hepatocytes. Moreover, it reduced the hatching rate of zebra-fish eggs and significantly increased larvae mortality^[8].

Animal toxicity studies of PBDEs, which are similar to DBDPE in structure, indicate that the liver is an important target organ^[9-10]. PBDEs induce apoptosis of cerebellar granule^[11] and astrocytoma cells, and a human promyelocytic leukemia line (HL-60), via different mechanisms^[12-13]. Given the increased contamination of DBDPE in the environment, its potential risk to human health, and its structural similarity to PBDEs, this study is aimed to investigate the toxic effects of DBDPE in vitro and, HepG2 cells were selected as a model system for the study because it exhibit a variety of cellular responses to different drugs^[14].

MATERIALS AND METHODS

Materials

Hep G2 cells were purchased from the Cell Storage Center of Wuhan University (Wuhan, China). Dulbecco's Modified Eagle Medium (DMEM) and heat-inactivated fetal calf serum were purchased from Gibco (USA). DNase-free RNase A was purchased from Tiangen Biotech (Beijing, China) Co., LTD. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33 258, propidium iodide (PI), 2,7-Dichlorofluorescein-diacetate (DCFH-DA), Dimethyl sulfoxide (DMSO) and N-acetylcyteine (NAC)

were purchased from Sigma-Aldrich (St. Louis, MO, USA). DBDPE was purchased from AccuStandard, Inc. (New Haven, USA), and all other chemicals were obtained from Sigma-Aldrich. All reagents were of analytic grade or higher purity.

Cell Culture and Treatment with DBDPE

HepG2 cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal calf serum at 37 °C, 5% CO₂ for 24 h prior to dosing. Cells were used for experiments within eight passages to ensure stability of cell line. DBDPE (50 mg) was dissolved in 2.5 mL DMSO and sonicated for 30 min. Solutions were then sonicated for 15 min prior to use. The DMSO concentration was held constant at 0.5% (v/v). HepG2 cells were seeded in culturing plates and divided into three groups: (1) blank control group; (2) DMSO control group; (3) DBDPE supplemented at 3.125, 6.25, 12.5, 25.0, 50.0, and 100.0 mg/L. Each sample had at least three replicates. Cells were then cultured for 24, 48, or 72 h separately. Experiments were performed in duplicate afterwards.

Determination of Cytotoxicity

Mitochondrial capacity to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan was used as a measure of cell viability^[15]. Cell were seeded in 96-well plates at 1×10^4 cells/well in 0.1 mL of culture medium and incubated with different concentrations of DBDPE for a set time frame, followed by incubation with serum-free medium containing 5 mg MTT/mL for 4 h at 37 °C. After the removal of MTT, cells were washed with phosphate buffer saline (PBS) warmed to 37 °C. Formazan was then extracted from cells with 150 μ L DMSO and incubated for 10 min at room temperature afterwards. Formazan concentration was determined on a microplate reader (Multiskan MS352, Labsystems, Helsinki, Finland), at 540 nm. Cell viability was calculated using DMSO treated cells as the 100% viable control and was calculated as $(A_{540} \text{ of drug-treated sample} / A_{540} \text{ of control}) \times 100$.

HepG2 plasma membrane integrity was assayed by measuring lactate dehydrogenase (LDH) leakage into the culture medium^[16]. To a cuvet, 100 μ L media, 1 mL PBS containing 66 mg/L pyruvate, and 20 μ L NADH were added and absorbance measured was at 340 nm. Total LDH release, corresponding to complete HepG2 death, was determined at the end of each experiment following freezing at -70 °C and rapid thawing. LDH release (%) = (LDH activity in

media)/ (LDH activity in media + LDH activity in total death cells) $\times 100$. Test concentrations were selected after determination of cytotoxicity by MTT assays, which were performed at four concentrations: 12.5, 25.0, 50.0, and 100.0 mg/L respectively.

Hoechst 33258 Nuclear Staining Assay

Nuclear staining with Hoechst 33 258 was performed as described elsewhere^[17-20]. HepG2 cells were seeded in 24-well plates at a density of 5×10^4 cells/well in 1 mL of culture medium. After exposure for 72 h, cells were collected and washed twice with PBS. The cells were then fixed with 4% paraformaldehyde in PBS for 30 min at 4 °C. After washing, the cells were incubated in Hoechst 33 258 at a final concentration of 30 $\mu\text{g}/\text{mL}$ at room temperature for 30 min. Nuclear morphology was then observed under an inverted fluorescence microscope and recorded using an imaging system at 400 \times (DMI3000B, Leica, Germany).

Detection of Apoptosis

Flow cytometry was used to detect apoptotic cells with diminished DNA content. HepG2 cells were seeded into 6-well plates at 1×10^5 cells/well. After the treatment with DBDPE, cells were fixed in ice-cold 70% ethanol at -20 °C overnight. After centrifugation and washing one time with PBS, low-molecular-weight DNA was extracted using 0.2 mol/L phosphate-citrate buffer and stained with 200 μL of 1 mg/mL propidium iodide (PI)/10 mL, and 0.1% Triton X-100/2 mg DNase-free RNase A. The solution was then incubated for 30 min at room temperature in the dark, followed by flow cytometric analysis at 488 nm (BD, Franklin Lakes, NJ USA).

Measurement of Reactive Oxygen Species (ROS)

ROS levels were determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA)^[21], which is cleaved by cellular esterases to form DCFH, and oxidized to the fluorescent compound DCF by intracellular ROS. After treatment with DBDPE, cells were washed with PBS and incubated with 10 $\mu\text{mol}/\text{L}$ DCFH-DA in DMEM for 30 min at 37 °C under an inverted fluorescence microscope. Images were recorded using an imaging system (Leica) at 400 \times magnification. The cellular fluorescence intensity was then measured by a fluorescence spectrophotometer using 485 nm excitation and 525 nm emission filter settings (Hitachi High-Tech Company, Japan).

Confirmation of ROS Formation

HepG2 cells were separately seeded in 96-well and 6-well plates. NAC was then added to culture medium with the concentration maintained at 5 mmol/L. After 10 min, cells were exposed to different concentrations of DBDPE. As referred to aforementioned methods, ROS formation was measured after 24 h, the same as cell viability and apoptosis after 72 h.

Data Analysis

Data were expressed as mean \pm standard deviation. One-way analysis of variance, followed by a Dunnet's significant difference test was used to determine statistically significant ($P < 0.05$) differences. All statistical analyses were carried out using PASW Statistics 18 (Armonk, NY USA).

RESULTS

Cytotoxic Effect of DBDPE on HepG2

The MTT cell viability assay yielded no significant difference in viability between the blank groups and DMSO controls. Low concentrations of DBDPE (3.125-6.25 mg/L) did not show a significant difference in viability compared to untreated controls. In addition, the viability of HepG2 cells was significantly inhibited by DBDPE in high concentration groups (12.5-100.0 mg/L), which was presented in a time and dose-dependent manner at 48 h and 72 h (Figure 1).

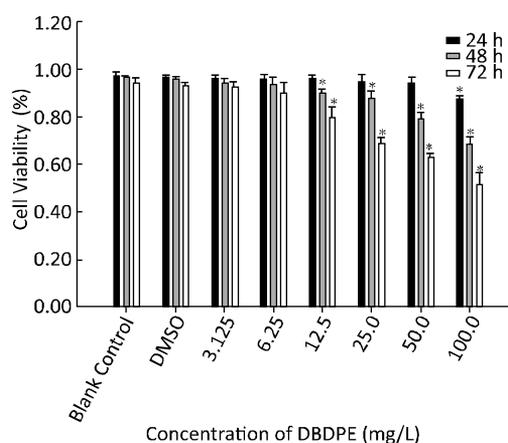


Figure 1. Effects of DBDPE on HepG2 viability. Viable HepG2 cells are expressed as a percentage of untreated control cell samples. The viability of the control was set as 100%. All data are expressed as mean \pm SD ($n=5$). Significantly different from control, $P < 0.05$.

Findings from the cell plasma membrane integrity assay showed that the increase in LDH release increased with both time and concentration from 12.5 to 100.0 mg/L at 48 h and 72 h, and from 50 to 100 mg/L at 24 h (Figure 2).

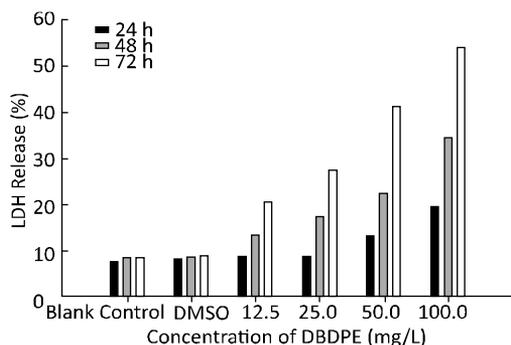


Figure 2. DBDPE-induced LDH release in HepG2 cells. All data was expressed as mean \pm SD ($n=5$). Significantly different from control, * $P<0.05$.

Nuclear morphological changes induced by DBDPE were also observed, demonstrating a tendency over the concentration range from 12.5 mg/L to 100.0 mg/L at 72 h (Figure 3). After exposure for 48 h, cells began to shrink and became retracted and ill-defined, followed by floating cells

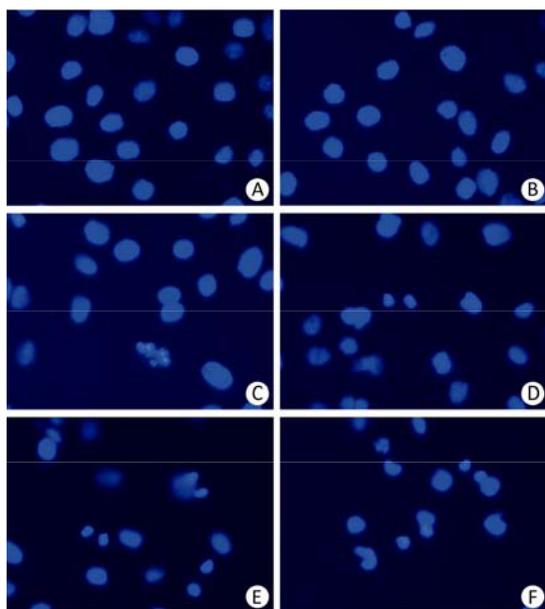


Figure 3. Effects of DBDPE on HepG2 morphology. (A): blank control group; (B): DMSO control group; (C): 12.5 mg/L group; (D): 25.0 mg/L group; (E): 50.0 mg/L group; (F): 100.0 mg/L group.

and a decrease in cell adhesion at high concentration groups (12.5-100.0 mg/L) (data not shown). Fluorescence microscopy also revealed an increased level of chromatin condensation or fragmented nuclei, which increased with concentration, especially at 100 mg/L DBDPE. Relative to the control group, the number of live cells decreased when in the concentration increased.

Induction of Apoptotic Cell Death by DBDPE in HepG2 Cells

HepG2 cells were treated with DBDPE with varying concentrations for 48 h and 72 h respectively. The amount of cell death (apoptosis and necrosis) was determined afterwards by PI staining and flow cytometry.

Owing to the reduced DNA content, apoptotic cells could be separated from normal cells by flow cytometry. Since nuclear fragmentation is a hallmark of apoptosis, the nuclear DNA staining is a measure of cells treated with DBDPE and serves as an indicator of cell apoptosis. Analysis of DNA content revealed that DBDPE could induce apoptosis of HepG2 cells, and showed a time- and dose-dependent response (Figure 4). Compared to the control and DMSO groups, the difference in apoptotic rates were significant.

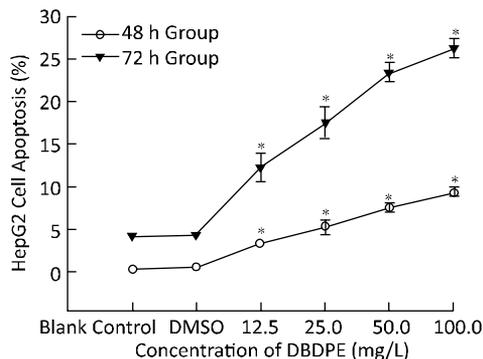


Figure 4. Flow cytometry analysis of apoptosis of Hep G2 cells treated with DBDPE for 48 h and 72 h. Significantly different from the control, * $P<0.05$.

ROS Generation Inducted by DBDPE

The generation of intracellular ROS induced by DBDPE was measured after culturing cells with different concentrations of DBDPE for 5, 15, and 24 h. At 5 h, no significant change of intracellular ROS was observed between control and experimental groups of 12.5 mg/L and 25.0 mg/L (Figure 5). A significant difference of ROS generation was observed between

the blank and DMSO controls in the range of 12.5-100.0 mg/L at 15 h and 24 h, similar to what was observed for 50 mg/L and 100 mg/L at 5 h. ROS formation was dependent on DBDPE concentration, and ROS generation peaked at 24 h. To confirm that ROS formation was an important factor in DBDPE induced apoptosis, NAC (5 mmol/L) was added to culture medium 10min before exposure. As it was shown, DBDPE could increase intracellular ROS level, whereas NAC decreased ROS formation (Figure 6). Meanwhile, cell viability was improved and cell apoptosis was inhibited by the addition of NAC (Figures 7-8). It was therefore suggested that the decrease in viability and induction of apoptosis by DBDPE were ROS dependent.

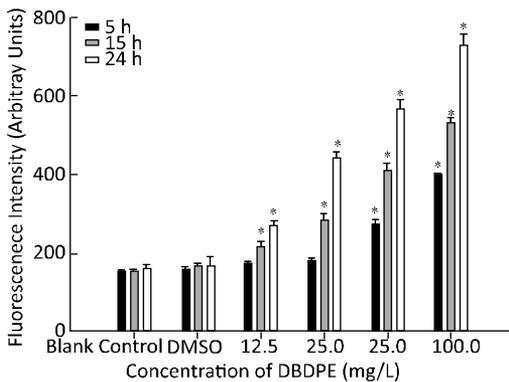


Figure 5. Effects of DBDPE on ROS generation in HepG2 cells. Results are expressed as mean±SD ($n=5$). Significantly different from control, * $P<0.05$.

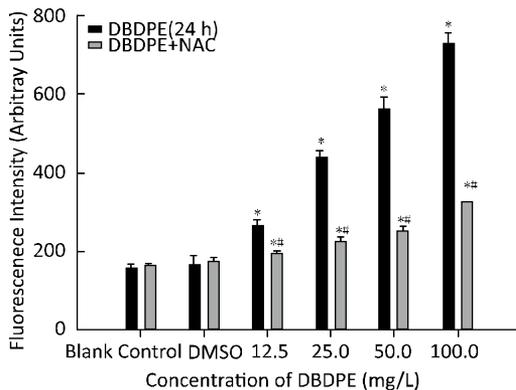


Figure 6. The inhibitory effects of NAC on intracellular ROS generation by DBDPE in HepG2 cells. Values were given as mean±SD ($n=5$). Significantly different from control, * $P<0.05$. Statistically significant difference before and after treatment, # $P<0.05$.

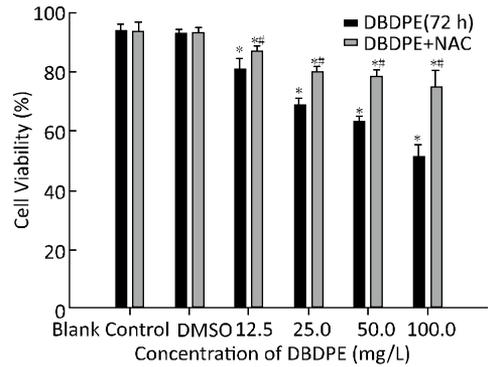


Figure 7. Reversion of NAC on cell viability induced by DBDPE. Viable Hep G2 cells were expressed as a percentage of untreated control cell samples. The viability of the control was set as 100%. All data were expressed as mean±SD ($n=5$). Significantly different from control, * $P<0.05$. Statistically different before and after treatment, # $P<0.05$.

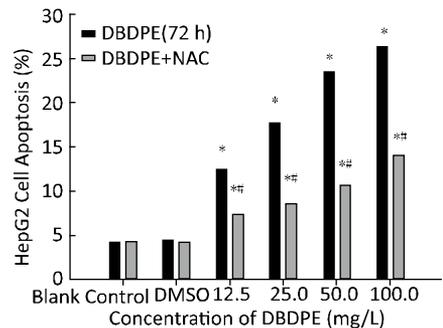


Figure 8. Reversion of NAC on HepG2 cell apoptosis induced by DBDPE. All data were expressed as mean±SD. ($n=3$). Significantly different from control, * $P<0.05$. Statistically different before and after treatment, # $P<0.05$.

DISCUSSION

Liver is the major target organ for brominated flame retardant accumulation. HepG2 is a highly differentiated human hepatoma cell line that retains many of the cellular functions often lost by cells in culture^[14], including some of the xenobiotic metabolizing capacity of normal hepatocytes^[22]. A number of PBDEs, like 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47), 2,2',4,4',5-pentabromodiphenyl ether (PBDE-99), and decabromodiphenyl ether (PBDE-209) are metabolized to form hydroxylated or

debrominated metabolites, which are considered to cause more adverse effects than PBDEs themselves, and represent the main sources of toxicity in some cases^[23-24].

DBDPE toxicology is well known in the literature. Hardy et al did not observe toxicologically significant differences in body weight, weight gain, or food consumption in rats challenged with DBDPE. However, significant differences were found between controls and high-dose animals with respect to mean absolute, or relative liver weights^[7]. No evidence of maternal toxicity, developmental toxicity, or teratogenicity was observed in rats or rabbits treated with DBDPE at dosage levels up to 1250 mg/kg per day^[3]. Together, these studies suggest that low level toxicity of DBDPE is most likely related to its high molecular weight and low solubility, which likely restricts bioavailability of DBDPE. However, Nakari et al have recently reported that DBDPE is acutely toxic to *Daphnia magna* and is proven to have ill effects on hepatocyte detoxification metabolism, and its estrogenicity, which indicates that DBDPE is metabolized in the fish liver^[8].

In this paper, the DBDPE concentration ranged 3.125-100.0 mg/L was chosen since the literature has reported toxicity at these levels for PBDEs, such as PBDE209, and the two other kinds of compounds have similar structures^[25].

The cell viability and morphological data observed here demonstrate that DBDPE is toxic to HepG2 cells. However, the mechanism of cytotoxicity of DBDPE is unclear and therefore requires further study.

We observed significant apoptosis in HepG2 cells after exposure to 12.5-100 mg/L DBDPE at 48 h and 72 h. These changes were accompanied with a decrease in HepG2 cell viability. Taken together into account, these results suggested that apoptosis might be the main cause of the reduction in cell viability. Considering the current results and the facts that some PBDEs, including PBDE-47 and PBDE-209, can induce apoptosis in other cell lines, it is suggested that DBDPE may induce a similar apoptotic response.

In order to examine the mechanism of DBDPE-induced apoptosis, we investigated the effects of DBDPE on ROS formation. A significant overproduction of ROS was detected in DBDPE-mediated cytotoxicity in a dose-dependent manner at 15 h and 24 h. At all time points, ROS production increased as the exposure time was

extended. Moreover, the significant difference in ROS production between the control group and the high concentration groups appeared earlier than that of cell viability and apoptosis. Especially, the significant difference between the high concentration group (50 mg/L and 100 mg/L) and the control group appeared at 5 h. Therefore, we can infer that ROS may be an important initiation factor of cell apoptosis and death. In addition, NAC, which is used as an ROS scavenger in apoptotic studies^[26], can protect cells against oxidative damage by reacting with H₂O₂ extracellularly as a direct antioxidant, or by increasing the cytoplasmic reserve of glutathione. Our results show that NAC has a significant suppressive effect on DBDPE-induced apoptosis. Furthermore, the presence of NAC increases cell viability. Previous studies have shown that ROS plays an important role in apoptosis induction under both physiologic and pathologic conditions^[27]. Mitochondria may be main targets of ROS attack since they are a major source of ROS production. Fleury et al.^[28] reported that changes in mitochondrial permeability were important events leading to the collapse of the mitochondrial transmembrane electrochemical gradient. Subsequently, cytochrome C and other proapoptotic molecules are released into the cytoplasm, activating apoptosis afterwards. Chuan Yan et al. reported that PBDE-47 induced apoptosis in Jurkat cells, and that ROS played an important role in the apoptotic process^[29] which was mediated by PBDE-209 in HepG2 cells^[25], and by PCBs in human T47D and MDA-MB-231 breast cancer cells^[30]. Since DBDPEs are structurally similar to PBDEs and PCBs, especially PBDE-209, it is reasonable to suggest that toxicity of DBDPE in HepG2 cells is mediated through ROS generation.

In conclusion, DBDPE can significantly inhibit cell viability and induce apoptosis in HepG2 cells. The ROS results, together with the protective effect of NAC, suggest that the overproduction of ROS may play a key role in the apoptotic process induced by DBDPE. Additional research on the molecular mechanisms of DBDPE induced apoptosis in HepG2 cells will be therefore required to further understand toxicity of DBDPE at the cellular and molecular level.

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