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Primary neuronal-astrocytic co-culture platform for neurotoxicity assessment of di-(2-ethylhexyl) phthalate

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A B S T R A C T
Plastics such as polyvinyl chlorides (PVC) are widely used in many indoor constructed environments; however, their unbound chemicals, such as di-(2-ethylhexyl) phthalates (DEHP), can leach into the surrounding environment. This study focused on DEHP’s effect on the central nervous system by determining the precise DEHP content in mice brain tissue after exposure to the chemical, to evaluate the specific exposure range. Primary neuronal-astrocyte co-culture systems were used as in vitro models for chemical hazard identification of DEHP. Oxidative stress was hypothesized as a probable mechanism involved, and therefore the total reactive oxygen species (ROS) concentration was determined as a biomarker of oxidative stress. In addition, NeuriteTracer, a neurite tracing plugin with ImageJ, was used to develop an assay for neurotoxicity to provide quantitative measurements of neurological parameters, such as neuronal number, neuron count and neurite length, all of which could indicate neurotoxic effects. The results showed that with 1 nmol/L DEHP exposure, there was a significant increase in ROS concentrations, indicating that the neuronal-astrocyte cultures were injured due to exposure to DEHP. In response, astrocyte proliferation (gliosis) was initiated, serving as a mechanism to maintain a homeostatic environment for neurons and protect neurons from toxic chemicals. There is a need to assess the cumulative effects of DEHP in animals to evaluate the possible uptake and effects on the human neuronal system from exposure to DEHP in the indoor environment.

Introduction

The industrial plasticizer di-(2-ethylhexyl) phthalate (DEHP) has recently attracted the attention of the scientific community due to its neural toxic effects as well as possible reductive/developmental effects on human beings. DEHP could also be a probable carcinogen (US EPA Air Toxics Website, 2013). DEHP is widely used as plasticizer for the production of a wide variety of polyvinyl chloride (PVC) plastics widely used in indoor environments in a broad variety of applications (Silva et al., 2004; Meeker, 2010; Weuve et al., 2010; Banker and Goslin, 1998). Bornehag et al. (2005) reported an association between DEHP in dust and PVC used as flooring and walling material in homes. There is, however, limited information concerning the neurotoxic effects of DEHP in animals following DEHP exposure by any route. There is therefore a need to investigate these effects and to explore the potential risks to humans due to exposure in indoor environments to minimize neurological health problems in people.

This study focuses on the effect of DEHP on the central nervous system (CNS), an area very sensitive to foreign toxic substances. DEHP is a lipophilic compound with the greatest absorption after oral exposure. It has been
estimated that human absorption of DEHP could be as high as 25% (Schmid and Schlatter, 1985), but rodents could absorb more than 55% of an oral dose (Rhodes et al., 1986). There is therefore an un-answered question, regarding the possible crossover of DEHP through the blood-brain barrier into the CNS tissues, which could be vital for evaluation of neural toxic effects. An *in vitro* model involving a primary neuronal-astrocyte co-culture system derived from nervous system tissue has proven to be a powerful tool for showing cellular and molecular mechanisms of nervous system development and function (Hsieh et al., 2006). This model can show axons and dendrites that have the characteristics of those from neurons. Meanwhile, astrocytes have been shown to protect neurons against several types of toxic insult and to critically influence neuronal survival. These studies have also suggested that the use of astrocytes in this system might prove more relevant to human CNS structure and function and be more sensitive to chemical effects than neuronal cells alone (Andersen et al., 2000).

An often-mentioned mechanism of chemical-induced neuronal damage is oxidative stress (Swan, 2008; Hauser et al., 2007; Ferguson et al., 2011; Hauser et al., 2006). Astrocytes have also played an important role in controlling oxidative stress in the CNS as a result of antioxidants activity; vitamin C might serve as a useful biomarker of decreases in oxidative stress effects. The dye 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) is a cell-permeable indicator for the presence of reactive oxygen species (ROS) (Testa et al., 2011).

Neurotoxicity markers, such as neuronal number, neurite length and astrocyte reactive gliosis, can be assessed based on immunostaining with selective fluorescence probes (Meijering et al., 2004). Quantification assays (Neurite Tracer plugin with Image J software) are commonly used to examine chemical effects on neurite outgrowth, changes in neuron numbers, and the expression level of glial fibrillary acidic protein (GFAP, an intermediate filament protein expressed by numerous CNS cell types, including astrocytes) (Pool et al., 2008), along with applications for quantifying neurodevelopmental processes (Radio and Mundy, 2008). In this investigation, the neurotoxicity and adverse effects of DEHP exposure were comprehensively evaluated in mice.

Our research group has previously carried out some *in vivo* toxicological studies on the toxicities of DEHP, for example immunotoxicity and pulmonary toxicity (Yang et al., 2008; Guo et al., 2012), and there is now a growing interest in DEHP-induced neurotoxicity in China due to the prevalence of the chemical in the environment and its use in medical applications.

### 1 Materials and methods

#### 1.1 Ethics statement

The experimental procedures used here were approved by the Office of Scientific Research Management of Central China Normal University, with certification on the Application for the Use of Animals dated March 26, 2010 (approval ID: CCNU-SKY-2010-005). According to the ethics requirements, only six mice were used in each treatment group to minimize the number of experimental animals while ensuring the statistical validity of the results. Ten female mice were used as source material in preparing primary neuronal-astrocyte co-cultures.

#### 1.2 Animals

Twenty-four, 4-week-old, specific pathogen-free, Balb/c mice were obtained from the Experimental Animal Centre of Hubei Province (Wuhan, China). The mice were randomly grouped into 4 groups of 24 and housed in separate cages under standard conditions, with a 12 hr light/12 hr dark cycle. Three groups were exposed to DEHP at 0.1, 1, or 10 mg/(kg·day) and a fourth control group given saline via daily gavage at a fixed time for 2 weeks. Another group of mice (10 in all) were used for preparing primary neuronal-astrocyte co-cultures.

#### 1.3 Gas chromatography-mass spectrometry assay (GC-MS)

An Agilent 7890A 5975C gas chromatography-mass spectrometry system (GC-MS) (Agilent Technologies, Inc. USA) was used for the analysis of DEHP, and a HP-5ms quartz capillary column (30 m × 0.25 mm × 0.25 μm) was used. GC-MS operating conditions: helium (purity ≥ 99.999) at 1.0 mL/min, inlet temperature of 250°C, injection volume of 1 μL, and a programmed temperature regime with an initial column temperature of 60°C, for 1 min, temperature raised to 220°C, held for 1 min, then raised to 280°C, and held for 1 min. GC-MS interface temperature was 280°C, ionization was by an electron impact source, monitored using selected ion monitoring mode, ion source temperature was at 230°C, quadrupole temperature was at 160°C.

The regression equation for a standard DEHP (Sigma-Aldrich, Inc. St. Louis, MO, USA) solution was \( y = 4.0 \times 10^{-7} x + 0.1049, r = 0.9992 \). This method produced a good linear relationship within the range of 0.201–10 μg/mL.

Sample solutions were prepared by placing around 0.4 g mouse brain in centrifuge tubes, and precisely adding hexyl hydride to obtain 5 mL of final volume. The samples were weighed and homogenized, then sonicated for 30 min. Finally, the sample solutions were centrifuged (2000 r/min, 20°C, and 20 min) to retrieve supernatant for injection. DEHP content \( (M_{\text{DEHP}}, \mu \text{mol/L}) \) was calculated by Eqs. (1)–(3):
1.4 Cell culture

Astrocyte feeder layers preparation: dissection of cortex from newborn mouse brains which was temporarily stored in DMEM on ice. The cortices were dissociated into cell suspension using mechanical digestion. Then the media was removed, trypsin (0.25%) was added, and the sample incubated at 37°C for 5 min. Next, astrocyte culture media was added. The supernatant containing single cells was then filtered through a 70 μm mesh into a fresh tube. The single cells were then sedimented and resuspended in astrocyte culture media plus 10% FBS media. After 1 to 3 passages, the media was added. The supernatant containing single cells was then filtered through a 70 μm mesh into a fresh tube.

The neuronal-astrocyte co-culture system was prepared using trypsin and 10% FBS media. After neuronal-astrocyte co-culture systems had been incubated for 7 days, the media was removed. Then 1 mL of neuron culture media was mixed with DEHP added in five separate proportions, each having a final DEHP concentration of 1, 10, or 100 nmol/L and 1 or 10 μmol/L. The media solutions were incubated for 24 hr. Meanwhile, half of the DEHP-exposed wells in a plate column were also treated with 1 μmol/L vitamin C (Sigma-Aldrich, Inc. USA). Two wells of each column on a plate were held as controls (without added DEHP and vitamin C). Then the media was removed after 24 hr, the plates were washed twice with Hank’s balanced salt solution (Invitrogen Corp., USA), and DCFH-DA (Sigma-Aldrich, Inc. USA) was added to each well to a final 10 μmol/L concentration. DCFH-DA enters the cell as a non-fluorescent compound and becomes fluorescent after cellular esterases remove the acetate group and the compound is oxidized. The fluorescence intensity can be measured using a Wallac Victor2 1420 plate reader at the excitation and emission wavelengths of 485 and 535 nm, respectively. Green fluorescence is an indication of the number of cells in an oxidative state. Samples were next incubated at 37°C and in 5% CO₂ for 30 min in complete darkness and washed twice with HBSS to remove residual stain. The fluorescence intensity was finally measured using a Wallac Victor2 1420 plate reader at the excitation and emission wavelengths of 488 and 525 nm.

1.5 ROS determination

After neuronal-astrocyte co-culture systems had been incubated for 7 days, the media was removed. The neuronal-astrocyte co-culture systems were next incubated at 37°C and in 5% CO₂. As above, two wells in each column (of four) served as controls and each was given an equal volume of culture media. After 24 hr, the culture coverslips were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, USA) in phosphate buffered saline (PBS) solution, then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, Inc. USA) in PBS, and blocked with blocking buffer (1% normal donkey serum and 0.3% Triton X-100, with PBS) at room temperature for 30 min. Second, coverslips were incubated in primary monoclonal antibody mouse anti-TJU1 in 1:500 (V/V) and rabbit anti-GFAP in 1:500 (V/V) (Covance, Inc., Emeryville, USA) at 4°C in a bacteria shaker in order to de-attach any neurons and microglia. The single cells were then sedimented and resuspended in astrocyte culture media plus 10% FBS media. After 1 to 3 passages, the media was removed. Then 1 mL of neuron culture media was mixed with DEHP added in five separate proportions, each having a final DEHP concentration of 1, 10, or 100 nmol/L and 1 or 10 μmol/L. The media solutions were incubated for 24 hr. Meanwhile, half of the DEHP-exposed wells in a plate column were also treated with 1 μmol/L vitamin C (Sigma-Aldrich, Inc. USA). Two wells of each column on a plate were held as controls (without added DEHP and vitamin C). Then the media was removed after 24 hr, the plates were washed twice with Hank’s balanced salt solution (Invitrogen Corp., USA), and DCFH-DA (Sigma-Aldrich, Inc. USA) was added to each well to a final 10 μmol/L concentration.

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1.6 Quantitative morphometric analysis

Immunostaining was performed on neuronal and astrocyte cells cultured on coverslips in a 24-well plate after reaching 70% confluence at 7 days. Neuronal culture media was mixed with DEHP, as described above. The media was then applied to five groups of wells and incubated at 37°C and in 5% CO₂. As above, two wells in each column (of four) served as controls and each was given an equal volume of culture media. After 24 hr, the culture coverslips were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, USA) in phosphate buffered saline (PBS) solution, then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, Inc. USA) in PBS, and blocked with blocking buffer (1% normal donkey serum and 0.3% Triton X-100, with PBS) at room temperature for 30 min. Second, coverslips were incubated in primary monoclonal antibody mouse anti-TJU1 in 1:500 (V/V) and rabbit anti-GFAP in 1:500 (V/V) (Covance, Inc., Emeryville, USA) at 4°C in a bacteria shaker in order to de-attach any neurons and microglia. The single cells were then sedimented and resuspended in astrocyte culture media plus 10% FBS media. After 1 to 3 passages, the media was removed. Then 1 mL of neuron culture media was mixed with DEHP added in five separate proportions, each having a final DEHP concentration of 1, 10, or 100 nmol/L and 1 or 10 μmol/L. The media solutions were incubated for 24 hr. Meanwhile, half of the DEHP-exposed wells in a plate column were also treated with 1 μmol/L vitamin C (Sigma-Aldrich, Inc. USA). Two wells of each column on a plate were held as controls (without added DEHP and vitamin C). Then the media was removed after 24 hr, the plates were washed twice with Hank’s balanced salt solution (Invitrogen Corp., USA), and DCFH-DA (Sigma-Aldrich, Inc. USA) was added to each well to a final 10 μmol/L concentration.

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4°C overnight. Then the coverslips were rinsed in PBS 3 times for 10 min and incubated in fluorophore-conjugated secondary antibodies Cy2 goat anti-mouse IgG in 1:200 (V/V) and Cy3 goat anti-rabbit IgG 1:200 (V/V) (Jackson ImmunoResearch Laboratories, Inc., West Grove, USA) in darkness for 1 hr. After rinsing in PBS 3 times over a period of 10 min protected from light, the coverslips were incubated by adding 10 µg/mL Hoechst 33342 (Sigma-Aldrich, Inc., USA) at room temperature protected from light for 1 hr. Finally, the coverslips were mounted and observed under a fixed-stage upright fluorescence microscope (BX51WI, Olympus Corp., Japan) and laser confocal microscope (Leica TCS SP MP Inverted Confocal Microscope, Leica Microsystems GmbH, Wetzlar, Germany).

Image acquisition and analysis conditions are summarized in Table 1. The measurements were automated using the NeuriteTracer with ImageJ (National Institutes of Health, USA) to analyze the fluorescence microscopy images of neurite outgrowth, neuron numbers, and GFAP concentration. We took four pictures in the average distribution of lens vision from one coverslip, and there were 6 coverslips in a group. Data were obtained from three independent experiments (Abramo et al., 2004; Ahmed et al., 2006; Koprivica et al., 2005; Fournier et al., 2002, 2003).

1.7 Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical graphs were generated using Origin 7.0 software (OriginLab Corp., Northampton, USA).

<table>
<thead>
<tr>
<th>Detection reagent</th>
<th>Objective lens</th>
<th>Excitation filter range (nm)</th>
<th>Emission filter range (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoechst 33342</td>
<td>20x</td>
<td>360/40</td>
<td>460/40</td>
</tr>
<tr>
<td>Cy2 goat anti-mouse IgG</td>
<td>20x</td>
<td>480/40</td>
<td>535/50</td>
</tr>
<tr>
<td>Cy3 goat anti-rabbit IgG</td>
<td>20x</td>
<td>535/50</td>
<td>600/50</td>
</tr>
</tbody>
</table>

2 Results

2.1 Distribution and quantification of DEHP

Based on the minimal risk levels (DEHP 0.1 mg/(kg-day)) derived by the Agency for Toxic Substances and Disease Registry (ATSDR), USA (Heudorf et al., 2007), three mice groups were exposed to DEHP at 0.1, 1, or 10 mg/kg, with an additional saline control group. Detectable DEHP in brain homogenate samples was analysed using GC-MS quantification, showing DEHP had crossed the blood-brain barrier. The DEHP concentration in the homogenate samples was shown to have significantly increased, correlating with an increase in DEHP exposure given to mice. In specificity tests, DEHP was consistently shown as a chromatographic peak at a retention time of 11.01 min (Fig. 1a, b) and a relative standard deviation (RSD) of 2.35%. An RSD of < 3.0% would indicate good instrument precision. DEHP contents of 2.97, 3.03, 4.69, and 8.72 µg/g, were shown in the brain homogenates corresponding to DEHP exposures of the following concentrations: control, 0.1, 1, and 10 mg/(kg/day) as shown in (Fig. 1c).

Phthalates can occur in our daily lives in sources such as medical supplies used in medical treatments. The lower limit for the present testing was based on DEHP leachate reported at 7.4 nmol/L from DEHP-plasticized PVC medical bags (Yang et al., 2007). Combined with the GC-MS results derived from the group of minimal risk level 0.1 mg/(kg·day), we calculated $\text{C}_{\text{DEHP}} = 3.03 \times 1000/390.56 = 7.8 \, \mu\text{mol/L}$. So the DEHP exposure concentrations in vitro were set at 1, 10,
and 100 nmol/L and 1 and 10 μmol/L.

### 2.2 Models of neurotoxicity assessment

Neurons were dissected from 17-day embryonic mice and introduced into astrocyte feeder wells prepared a few days earlier. These immature neural cells were not yet able to modulate DEHP activity and therefore more susceptible to neurotoxicity than mature cells (Fig. 2a). Recent studies have suggested that the use of astrocytes in an *in vitro* neurotoxicity test system might prove more relevant to human CNS structure and function than neuronal cells alone. After being cultured for seven days, neurons gradually matured, with tightening of connections between axons and dendrites that had significantly increased in neurite outgrowth. This was also accompanied by astrocyte proliferation in the background (Fig. 2b).

### 2.3 Total ROS determination

The observed ROS concentrations, indicated by fluorescence responses in the groups (Fig. 3a), showed that ROS concentrations in the co-culture system were significantly increased as DEHP exposure dosage given to the mice was also increased (*p* < 0.01). From the figure, a dose-response relationship between DEHP and total ROS concentrations was observed up to 10 μmol/L DEHP exposure (Fig. 3b). Vitamin C possesses potent antioxidant properties and thus acted here as an ROS scavenger in the vitamin C-treated groups. In accordance with the increase in ROS concentrations with DEHP exposure, ROS levels were significantly reduced with vitamin C treatment (Fig. 3c). Under such conditions as DEHP exposure, co-cultured cells could be potentially susceptible to oxidative stress. With the combined but opposing changes in ROS induced by DEHP and vitamin C, a relative oxidative-reductive balance was observed in these experimental groups.

### 2.4 Quantitative assessment

Seven days after astrocytes from 17-day embryonic mice were seeded into astrocyte feeder wells, the cells were treated with DEHP at various concentrations for 24 hr. Nuclei were labelled blue by Hoechst 33342 staining, cell bodies and processes labelled green using TJU1 with secondary antibodies Cy2 IgG, and GFAP on astrocytes labelled red with Cy3 goat anti-rabbit IgG secondary antibodies. Fused images of TJU1 (green) and GFAP (red) fluorescence taken by CLSM were used for differentiating between controls and the two high-dose DEHP exposure groups (1 and 10 μmol/L). In morphometric analysis, comparison with the control showed regional injury of neurite processes (Fig. 4a), with some damage in the central culture regions, and losses in neuron numbers in both high-dose exposure groups (Fig. 4b and 4c). With gradually deepening GFAP color, astrocytes produced active gliosis in the 1 μmol/L DEHP exposure group. Conversely, because of DEHP toxic effects which would increase with increased concentrations up to 10 μmol/L, large areas of astrocyte damage were observed.

Quantitative measurements of neurotoxicity markers including neuron number, neurite length, and astrocyte reactive gliosis level, were assessed using NeuriteTracer image-processing program ImageJ (Govek et al., 2005; Darenfed et al., 2007). Morphological picture images were captured by fixed stage upright fluorescence microscopy. Quantitative analysis showed that, with the increased DEHP concentration, the neuron number was gradually reduced above the DEHP 10 nmol/L group (*p* < 0.05). The 100 nmol/L, 1 and 10 μmol/L column of wells showed significant reductions in neuron number (*p* < 0.01, Fig. 5a1), with the same trend observed in the neurite lengths. Images revealed decreased outgrowth with increased DEHP treatment relative to the controls, with 100 nmol/L and 1 and 10 μmol/L groups exhibiting significant reductions in neurite outgrowth (*p* < 0.01, Fig. 5b1).

---

**Fig. 2** Early events in neurite outgrowth of neuronal-astrocytic co-culture system: (a) diagram of an early stage in neurite outgrowth development of minor processes (24 hr). Transformation of processes into an axon and dendrites; (b) co-culture system after 7 days, showing some astrocytes (arrow point) that had prosperously grown in background.
**Fig. 3** Effects of DEHP on oxidative stress represented by the reactive oxygen species (ROS) concentration in neuronal-astrocyte co-cultured cells after 24 hr exposure. (a) concentration-dependent increase of ROS concentrations by DEHP exposure in culture system. (b) dose-response relationship between DEHP and total fluorescence concentrations. Those groups from control (group 0) to DEHP exposure dose 10 μmol/L (group 5). With DEHP exposure, vitamin C served as an antioxidant to decrease the oxidative stress effect. (c) Data obtained from three independent experiments and presented as mean ± SEM. **, p < 0.01 compared with corresponding control.

**Fig. 4** Fused images with antibody TJU1 (green) and GFAP (red) fluorescence were used in differentiating between the control group (a) and two high-dose DEHP groups 1 nmol/L (b), 10 μmol/L (c). Neurite processing and neuron numbers showed much damage and loss in both high-dose exposure groups. In astrocytes, the reactive gliosis effect occurred in the 1 μmol/L DEHP group while, in contrast, it caused damage in the highest-dose (10 μmol/L) DEHP group (arrow point).

Furthermore, examination of the morphology in these three groups revealed that neurite connections were damaged in many areas. As gliosis is a symbol of nervous system injury, GFAP thus served as a biomarker for neurotoxicity and was quantified by immunoassay (Fig. 5c2).

Here, significant GFAP staining was observed, resulting from significantly up regulated gliosis accompanied by astrocyte proliferation with DEHP exposure of 100 nmol/L and 1 μmol/L (p < 0.01) given to the mice. Because of DEHP toxic effects at the high dose of 10 μmol/L, astrocyte...
Fig. 5 Cytotoxic phenotypes were indicated by actual cell number, neurite outgrowth length, and GFAP expression. (a1) morphology image of neuron number from control and DEHP exposure groups, (a2) effect of neuron numbers trend with DEHP exposure; (b1) morphology image of neurite outgrowth length with control and DEHP exposure groups, (b2) effect of neurite outgrowth length trend with DEHP exposure; (c1) morphology image of neurite GFAP expression with control and DEHP exposure groups, (c2) effect of GFAP level trend with DEHP exposure. Data obtained from three independent experiments and presented as mean ± SEM. ∗∗p < 0.01 compared with the corresponding control, ##p < 0.01 (Fig. c2) compared with the column with DEHP exposure at 1 μmol/L.

Fig. 6 Oxidative stress induced by DEHP and the protective system formed by astrocyte gliosis on primary neuronal-astrocyte co-culture system.

formation of a protective system was prevented and a large area of astrocytes thus damaged. The GFAP concentration dropped below that of the control and was significantly less when compared with the 1 μmol/L DEHP column of wells (p < 0.01).

3 Discussion

Information regarding the neurotoxic effects of DEHP exposure via any route is very limited. With a lack of studies involving low DEHP exposure, the U.S. Department of Health and Human Services (Stephanie et al., 2002) concluded that there is insufficient information to reach a conclusion regarding DEHP’s potential for adversely affecting neural system development. Considering the minimal risk exposure (0.1 mg/(kg·day)) derived by the Agency for Toxic Substances and Disease Registry, combined with the present study GC-MS assay results and the minimum content standards for DEHP in medical supplies, the in vitro exposure concentrations used here were set at 1, 10, and 100 nmol/L and 1 and 10 μmol/L. To our knowledge, this is the lowest concentration yet studied, and a range close to DEHP exposure in people’s daily life. DEHP is known to cross the blood-brain barrier and accumulate in the CNS. Primary neuronal and astrocyte cultures consisted of cortex tissue-derived cells that retain many of the morphological, neurochemical, and electrophysiological properties of neurons in situ, serving...
as an important research model for brain development and function.

Oxidative stress is commonly defined as a condition in which cellular protective antioxidant systems are overwhelmed by ROS, leading to neurological injury, and has also been suggested as a major pathologic mechanism in neurotoxicity. In this study, vitamin C, with its potent antioxidant effect, was employed as an ROS scavenger. The results showed an accumulation of ROS leading to oxidative stress, evidenced by cells exposed to 1 nmol/L DEHP showing a significant increase in ROS concentrations. Thus, neuronal-astrocyte cultures would be injured both morphologically and physiologically (Gao et al., 1998; Sakurai et al., 1997; Schwab and Caroni, 1988). In subsequent experiments, some injury was observed with increasing DEHP exposure concentration; the parameters of nerve injury; both neuron number and neurite outgrowth, were decreased respectively. These results are not difficult to explain; the death of neurons increased, and also the outgrowth of the neurites was interfered with by DEHP. Astrocyte proliferation (gliosis) is important in maintaining a homeostatic environment for neurons as well as protecting neurons from harmful chemicals (Chao et al., 1996; Dringen et al., 1999). Astrocyte activation of gliosis could lead to GFAP up regulation and has also been proposed as an early marker of nervous system damage. Here, GFAP staining was significantly increased with gliosis and accompanied by astrocyte proliferation in cells exposed to 100 nmol/L and 1 µmol/L DEHP. At high concentrations (10 µmol/L), DEHP’s toxic effects resulted in harm to astrocytes’ ability to form a protective system, evidenced by large areas of astrocyte damage. In fact, with 10 µmol/L DEHP, all neuronal parameters in these cultures were down regulated, producing severe neurological damage to the co-culture system (Fig. 6).

Neurotoxicity assessment represents a significant focus for environmental pollution monitoring and is also a marker of development of neurodegenerative diseases, such as Alzheimer’s and Parkinson’s diseases (Anderl et al., 2009). There is a need to extrapolate the results obtained to correlate the exposure-uptake dosage in humans in realistic scenarios of constructed environments. The animal experiment presented in this paper used typical DEHP concentrations found in indoor constructed environments. The uptake in humans needs careful interpretation; however as the experiment with mice indicated, precaution should be used to regulate uses of plasticized plastics in constructed environments, especially in medical applications.

4 Conclusions

The results from this study indicated strong and significant associations between oxidative stress and neurotoxicity, in other words, oxidative stress was one of the main mechanisms of neurotoxic effects induced by DEHP. Especially in the high concentration exposure range (1 and 10 µmol/L), the weakened protective effect of the astrocytes allowed oxidative damage in this model to occur immediately. As DEHP is a widely used plasticizer in daily-life products and appears to have exerted high cumulative effects in animals, such as mice, DEHP usage should be under more stringent scrutiny and control through the description and determination of its range of neurotoxic effects.

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