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INVOLVEMENT OF CASPASE-8, -9 AND -3 IN P-PHENYLENEDIAMINE INDUCED APOPTOSIS IN MURINE MYELOMA P3 CELLS: REGULATION BY REACTIVE OXYGEN SPECIES

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ABSTRACT

P-phenylenediamine (PPD) is a monocyclic arylamine frequently used as a component in permanent hair dyes, inks and certain tattoos. It has been reported that a number of hair dye constituents including monocyclic PPD have genotoxic properties in experimental systems. In this study, the molecular mechanism of PPD, a suspected carcinogen, induced cell death in murine myeloma cells P3X63Ag8.653 (P3) was investigated. Apoptosis was evaluated by DAPI staining and DNA gel electrophoresis. The activity of caspase-8, -3 and -9 was assessed by spectrophotometry using colorimetric tests. Glutathione (GSH) was used in this study as antioxidant. Results have demonstrated that PPD decreased cell viability in a dose- and a time-dependent manner. In addition, cell death via apoptosis was confirmed by chromatin condensation and enhanced DNA fragmentation. Furthermore, caspase-8, -9 and -3 activities in PPD treated cells were higher than those of untreated cells. Moreover, pre-treatment of P3 cells with glutathione GSH as antioxidant inhibited PPD induced cytotoxicity and DNA damage and decreased the activity of caspases. Based on these results, we suggested that PPD induced apoptosis in murine myeloma P3 cells was mediated by caspase-8, -9 and caspase-3 activation and with the involvement of reactive oxygen species (ROS).

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INTRODUCTION

Arylamines include chemicals that are widely used in the pharmaceutical, photographic or cosmetic industries and represent a large chemical family with a wide spectrum of toxicological properties (Garrigue *et al*., 2006). As an arylamine, p-phenylenediamine (PPD) is the most widely used primary intermediate in hair dye formulations (Corbett and Menkart, 1973) and as an intermediate in the manufacture of azo dyes and antioxidants (Hansen *et al*., 1993). Some epidemiologic studies have suggested that the use of PPD based hair dyes might be related to the increased risk of various human malignancies including bladder cancer, non-Hodgkin's lymphoma and hematopoietic cancers (Gago-Dominguez *et al*., 2001; Rauscher *et al*., 2004; Thun *et al*., 1994). However, the carcinogenic risk associated with personal use of hair dyes remains uncertain since several studies did not find an association between hair dye use and cancer development (NTP, 1978; NTP, 1979; Sontag, 1981).

On the other hand, in vitro studies have reported that PPD is cytotoxic and may induce apoptosis (Coulter *et al*., 2007; Huang *et al*., 2007). Furthermore, Chen *et al*. (2006) have suggested that PPD could induce apoptosis in Mardin-Darby canine kidney (MDCK) cells via the p53 pathway. In addition, PPD was found to increase expression of p53 and cyclooxgenase 2 in SV-40 immortalized human uroepithelial cells (Huang *et al*. 2007). Moreover, our team has previously reported that PPD induced apoptosis in P3X63Ag8.653 cells (P3) through reactive oxygen species (ROS) production (Elyoussoufi *et al*., 2012). It is known that ROS can lead to protein, lipid, and DNA oxidation and to cell death (Apel and Hirt, 2004; Mittler *et al*., 2004). Multiple signals of cell death can be triggered based on various inducers. It has been reported that cytotoxic stress either from DNA damage or mitochondrial impairment leads to apoptosis via the intrinsic pathway which involves the release of proapoptotic proteins from the inner membrane of mitochondria to the cytosol

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leading to activation of caspase-9 (Chan *et al*., 2006; Riedl and Salvesen, 2007; Kang and Reynolds, 2009). The involvement of caspases in PPD induced apoptosis has been recently proposed (Chen *et al*., 2010; Bai *et al*., 2012). However, the role of these proteases as mediators of PPD induced apoptosis has not been completely investigated. We have previously reported that PPD could induce P3 cells apoptosis, but so far little is known about the intracellular signaling pathways involved in regulating PPD apoptosis in these cells. The present study was therefore undertaken to investigate the role of caspases in PPD induced apoptosis of P3 cells. Collective studies have established the importance of glutathione (GSH) redox in cell apoptosis in a variety of cell types (Okouchi *et al*., 2005; Circu *et al*., 2008). The implication of ROS in the PPD induced apoptosis was further determined in this study using GSH as antioxidant.

MATERIALS AND METHODS

Materials

PPD (1,4-diaminobenzene; purity ≥ 99%), glutathione (GSH, purity >99%), RPMI 1640 medium, fetal bovine serum (FBS) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (USA).

Cell culture

The murine myeloma cell line P3X63Ag8.653 (P3; European collection of cell cultures, grande bretagne) was cultured in RPMI 1640 medium supplemented with 10% FBS, 100 UI/ml penicillin and 100 µg/ml streptomycin in 75-cm^2 culture flasks. Cells were maintained under standard culture conditions at a temperature of 37°C and an atmosphere of 5% CO_2. Subculture was routinely performed three times a week.

Cell viability

Cell viability was assessed by the trypan blue exclusion assay.

Detection of apoptosis by DAPI staining

P3 cells were cultured in 24-well plates at a density of $5x10^5$cells/well for 24h. At this time, 10mM PPD was freshly dissolved in RPMI 1640 medium and added to cells. The final concentrations for PPD were 23 and 46µM. Cells were then incubated for 48h. Each condition was run in three replicates in a three independent manner. Cells treated with 25µM of dexamethasone (Dex) were used as positive control of apoptosis estimation. To identify apoptotic nuclei, DAPI (4,6-diamidino-2-phenylindole dihydrochloride) staining was performed (Streiblova, 1988). $2x10^5$ Cells were collected, washed with PBS, fixed and permeabilized with 70% (v/v) ethanol for 15 min. The cells were then stained with DAPI solution (0.01mg/ml), washed with PBS and examined by fluorescence microscopy (Axio Scope A1).

DNA fragmentation assay

DNA was extracted using phenol-chloroform method (Sambrook *et al*., 1989). Briefly, $1x10^6$ cells were collected, washed with PBS and lysed in 100 µl of lysis buffer (20 mM Tris-HCl pH 8, 5 mM EDTA pH 8, 200 mM NaCl, 1% sodium dodecyl sulfate (SDS) and 2 mg/ ml proteinase K). The mixture was incubated for 3h at 55°C. Then, DNA was extracted with equal volume of phenol-chloroform-isoamylalcohool (25/24/1), precipitated with absolute ethanol and suspended in Tris-EDTA buffer. The samples (0.25 µg of DNA) were analyzed by gel electrophoresis (2 % agarose).

Measurement of caspase-8, -9 and -3 activities

P3 cells were cultured in 25-cm^2 culture flasks (3.10^5cells/ml) for 24h. The cells were pretreated with or without 1mM GSH for 5min and then were incubated with 23 or 46µM PPD for 12, 24 and 48h. Cells treated with 25µM of Dex were used as control. $3x10^6$ cells were collected, washed with PBS, centrifuged at 1000×g for 5min and the pellets were frozen at -20 °C. Activities of caspase-8, -9 and -3 were measured using colorimetric assays (ApoTarget^{TM}, Invitrogen, France). Briefly, the sample pellets were resuspended in 50 µl of lysis buffer, incubated at 4°C for 10 min and centrifuged at 10000xg for 1min. 50 µl of the supernatant were removed and placed in a 96-well microplate containing 50 µl 2X reaction buffer and 10mM DTT. Then 5 µl of IETD-pNA, LEHD-pNA or DEVD-pNA (for caspase-8, -9 or -3 assays, respectively) were added and the microplate was incubated in the dark at 37◦C for 2h. The absorbance was measured at 405 nm using an Automated Microplate Reader Biochrom Anthos 2020. Results were expressed as the quantity of colored pNA (µmol pNA/min/ml).

Statistical analysis

All experiments were performed at least three times. Values are expressed as means ± SEM. Statistical analyses were carried out using the Student's t-test. Statistical significance was accepted at the level of $p<0.05$.

RESULTS

Inhibitory effect of PPD on the growth of P3 cells

P3 cells were cultured with two concentrations of PPD (23 or 46µM) for 12, 24 and 48h. Cells treated with 25µM of Dex were used as control. Cell viability was assessed by trypan blue dye exclusion method. Results revealed that PPD induced a decrease in the viability of cells in a dose- and time-dependent manner (Figure 1). The dose of 23µM reduced cell viability to 72%±2, 67%±2 and 56%±4 ($p\leq0.002$) respectively at 12h, 24h and 48h, while at the same times, 46µM reduced cell viability to 67%±3, 54%±4 and 31%±3 ($p<0.003$) (Figure 1). In comparison, 25µM Dex reduced cell number to 87%±2, 78%±2 and 61%±2 ($p\leq0.02$). In addition, pre-treatment with 1mM GSH significantly inhibited the toxic effect of PPD on P3 cells viability ($p<0.05$) (Figure1).

PPD altered P3 cells morphology

Apoptosis was detected by the DAPI staining method after 48h of continuous exposure to 23 or 46 µM of PPD followed by fluorescence microscopy. As shown in figure 2, PPD induced apoptosis in a concentration-dependent manner. Approximately 50%±4% of PPD treated cells showed brightly stained condensed nuclei indicating chromatin fragmentation similar to that seen when apoptosis is induced by 25µM of Dex (Figure 2).

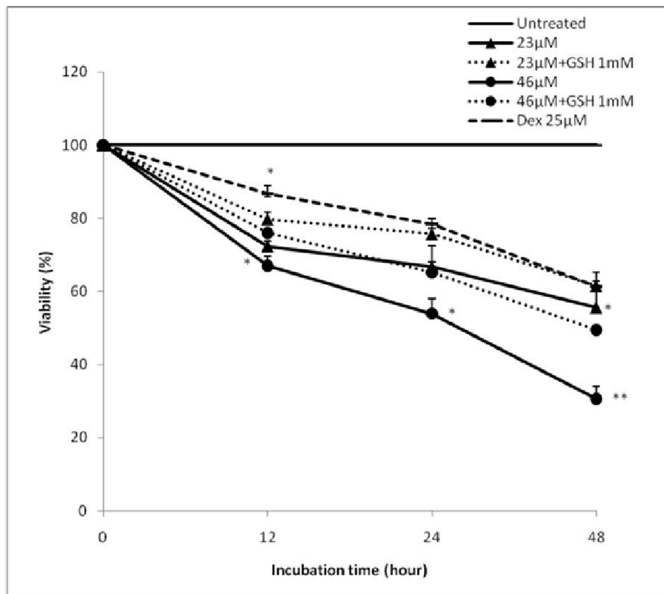

Fig. 1. Effect of PPD on the viability of P3 cells

Cells were pre-incubated with 1mM GSH 5 min before treatment with 23µM or 46µM of PPD for 12, 24 and 48h. Cells treated with 25µM Dex were used as control. Results are shown as mean ± SEM from three independent experiments. *p≤0.01. **p≤0.001.

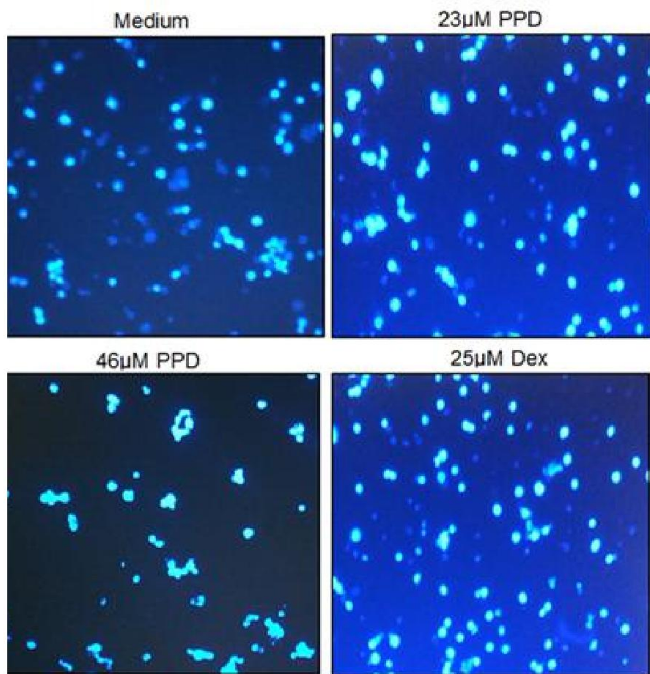

Fig. 2. DAPI staining of P3 cells

Cells were cultured with or without 23 or 46µM PPD for 48h and the whole cells were collected and stained by DAPI and viewed under the fluorescence microscope. Cells treated with 25µM Dex were used as control.

PPD induced DNA fragmentation

The effect on DNA fragmentation was visualized qualitatively on agarose gel. DNA from cells treated or not with 23 or 46µM of PPD or 25µM Dex for 48h was extracted and subjected to agarose gel electrophoresis. PPD induced DNA fragmentation was evidenced by DNA laddering (Figure 3). The intensity of the laddering increased dose-dependently by PPD (Figure 3, lanes 2 and 4) compared to untreated cells (Figure 3, lanes 1). Also, Dex used as a positive control causes DNA fragmentation in a stepladder manner similar to that induced by 23µM PPD (Figure 3, lane 6). Furthermore, pre-treatment with 1mM GSH slightly decreased DNA fragmentation (Figure 3, lanes 3 and 5).

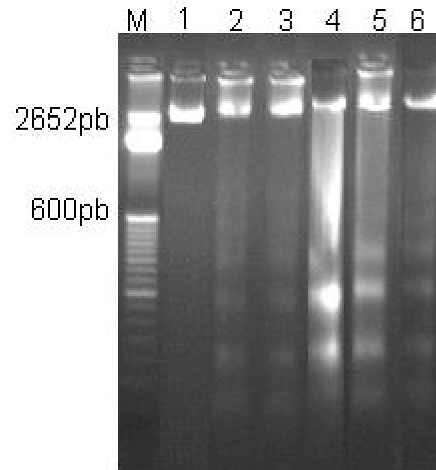

Fig. 3. Effect of PPD in DNA fragmentation in P3 cells

Cells were cultured for 48h: lane 1: in medium alone, lane 2: in 23µM PPD, lane 3: in 1mM GSH and 23µM PPD, lane 4: in 46µM PPD, lane 5: in 1mM GSH and 46µM PPD and lane 6: in 25µM Dex. The molecular size markers are indicated on the left, lane M.

PPD increased activities of caspase-8 and-9 in P3 cells

A common feature of cells undergoing apoptosis is activation of caspases. To determine the apoptotic pathway induced by PPD, caspase-8 and -9 activities were examined in untreated and PPD treated P3 cells. Results showed that PPD induced an increase in the activities of the two caspases in a dose and time dependent manner (Figures 4 and 5).

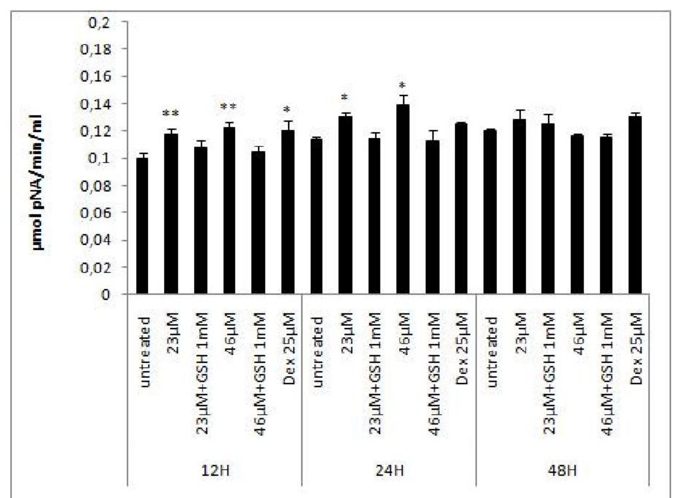

Fig. 4. Caspase-8 activity in P3 cells

Cells were pre-treated with 1mM GSH for 5min followed by treatment with 23µM or 46µM PPD for 12, 24 and 48h. Cells treated with 25µM Dex were used as control. Results are shown as mean ± SEM from three independent experiments.*p≤0.01. **p≤0.001.

As shown in figure 4, compared to untreated cells, a dose of 23µM PPD increased the caspase-8 activity by 7.9, 6.8 and 3.2% ($p \leq 0.01$) respectively at 12, 24 and 48 h, while a dose of 46µM increased this activity by 9.8 and 10% ($p \leq 0.04$) respectively at 12 and 24h (Figure 4). However, at 48h, 46µM of PPD seems to reduce the activity of caspase-8 by 2% ($p=0.005$) (Figure 4). Furthermore, Dex used as a control of apoptosis induction, enhanced this activity by 9.1, 5.1 and 3.9% ($p \leq 0.04$) respectively at 12, 24 and 48h (Figure 4). In addition, pre-treatment with 1mM GSH reduced the effect of PPD on caspase-8 activity by 4.6 and 7.9% ($p \leq 0.04$) at 12h, respectively in 23µM and 46µM treated cells (Figure 4). This effect was important at 24h where the caspase-8 activity decreased by 6.3 and 10.1% ($p<0.04$) respectively in 23µM and 46µM treated cells (Figure 4). No significant changes were observed at 48h. Caspase-9 activity was significantly increased when P3 cells were exposed to 23µM or 46µM of PPD for 12 and 24h (Figure 5) ($p<0.05$). The highest activity of caspase-9 was noted when cells were treated with 46µM of PPD for 24h (0.141 µmol pNA/min/ml) compared to untreated cells (0.109 µmol pNA/min/ml) ($p=0.04$) (Figure 5). At 48h, 23µM of PPD did not affect the caspase-9 activity while 46µM increased this activity by 7.2% ($p=0.005$) (Figure 5). In comparison, the caspase-9 activity was significantly increased in Dex treated cells only after 24h of treatment (0.126 µmol pNA/min/ml) ($p=0.03$) (Figure 5). Furthermore, pre-treatment with 1mM GSH significantly reduced the effect of PPD on caspase-9 activity by 7% at 12 and 24h ($p \leq 0.03$) (Figure 5). No significant changes were noted at 48h.

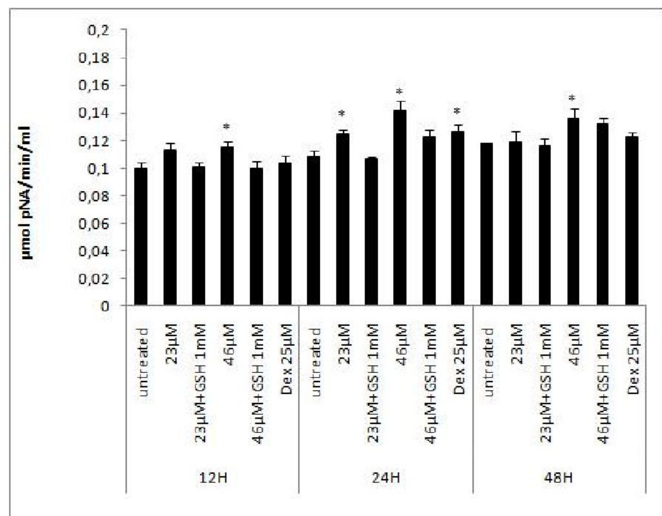

Fig. 5. Caspase-9 activity in P3 cells

Cells were pre-treated with 1mM GSH for 5min followed by treatment with 23µM or 46µM PPD for 12, 24 and 48h. Cells treated with 25µM Dex were used as control. Results are shown as mean ± SEM from three independent experiments. *$p \leq 0.03$.

PPD stimulated caspase-3 activity in P3 cells

To further gain insight into the mechanism of PPD-induced apoptosis of P3 cells, we also examined the possible involvement of caspase-3 activation. Our results showed that caspase-3 activity slightly increased after 12h and significantly increased after 24 and 48h of treatment with PPD ($p<0.05$) (Figure 6). The highest activity was observed after 48h, it was 0.150, 0.155 and 0.147 µmol pNA/min/ml ($p \leq 0.01$) respectively in 23µM and 46µM PPD treated cells and Dex treated cells compared to untreated cells 0.111 µmol pNA/min/ml (Figure 6). Pre-treating cells with 1mM GSH significantly reduced the effect of PPD on caspase-3 activity only after 24h ($p<0.05$) (Figure 6).

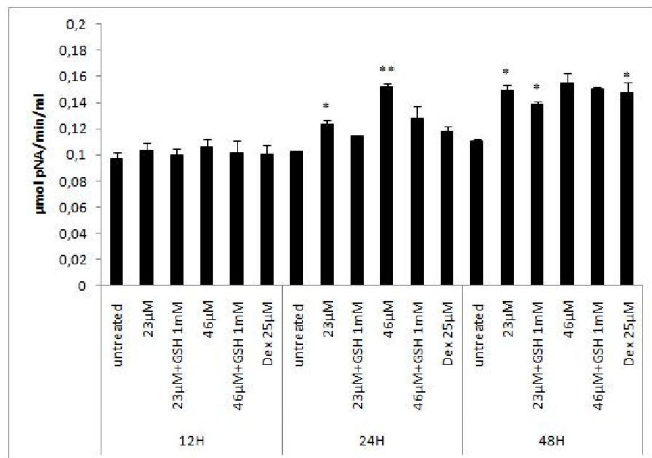

Fig. 6. Caspase-3 activity in P3 cells

Cells were pre-treated with 1mM GSH for 5min followed by treatment with 23µM or 46µM PPD for 12, 24 and 48h. Cells treated with 25µM Dex were used as control. Results are shown as mean ± SEM from three independent experiments. *$p \leq 0.01$. **$p \leq 0.001$.

DISCUSSION

PPD is present in more than 1000 hair dye formulations marketed all over the world (Stanley *et al*., 2005). It was reported that a number of hair dye constituents have carcinogenic properties in experimental system (Van Duuren, 1980). In fact, carcinogens usually cause genomic damage to expose cells which may either undergo proliferation with genomic damage or apoptosis (Steller, 1995; Thompson, 1995). In our previous work we have found that PPD, a suspected carcinogen (Sontag, 1981), induced apoptosis in P3 cells in a dose- and time -dependent manner (Elyoussoufi *et al*., 2012). For a better understanding of the molecular mechanism of this apoptosis we investigated in this paper the role of the caspase cascade in the signaling pathways of PPD-induced P3 cells apoptosis. In this study, the data indicated that PPD decreases cell viability of P3 cells in a dose- and time -dependent manner, which confirm our previous results (Elyoussoufi *et al*., 2012) and correlate with results found by Chen *et al*. 2010 in MDCK cells and Coulter *et al*. 2007 in human dendritic cells. In order to investigate the mechanism of this cytotoxicity, we next used two complementary approaches, DAPI staining and DNA fragmentation assay. We have found that PPD induced a chromatin condensation and DNA fragmentation in a dose-and time-dependent manner. Similarly, Huang *et al*. (2007) showed that PPD was able to induce DNA fragmentation dose dependently. It is known that DNA fragmentation is a biological hallmark of apoptosis (Schwartzman and Cidlowski, 1993). This may point to the possibility of apoptosis induction by PPD. In addition, previous studies have reported that PPD induced apoptosis in lymphocytes, MDCK cells and neutrophils (Chye *et al*., 2008; Chen *et al*., 2010; Elyoussoufi *et al*., 2013). Cell transformations associated with apoptosis result from the biochemical action of an execution program, whose main

characteristic is activation of caspases (Wyllie, 2010). This is associated with proteolysis of key cellular components like poly (ADP-ribose) polymerase (Lazebnik *et al*., 1994). Thus, detection of caspases activities may serve as a marker for PPD induced apoptosis in P3 cells. In the present study we have found that PPD increased the caspase-8 and -9 activities in a dose- and time -dependent manner. Previous report has shown similar results in MDCK cells (Chen *et al*., 2010). These data may suggest that PPD induced apoptosis through extrinsic (caspase-8) and intrinsic (caspase-9) pathways. It is known that in mammals, mitochondria function as central checkpoints for many forms of apoptosis. The mitochondria pathway is thought to be the principal target of the survival signaling system (Evan and Vousden, 2001; Rego and Oliveira, 2003; Dlamini *et al*., 2004). Furthermore, our results showed also that PPD activate the caspase-3 dose and time dependently. Chen *et al*. 2010 have found an induction of the caspase 3/7 activity in MDCK cells after PPD treatment. Moreover, Bai *et al*. 2012 have found that PPD induced an increase in mRNA levels of caspase-3 in human HK-2 proximal tubular epithelial cells. Next, we were interested by investigating the role of GSH in the control of PPD-induced apoptosis in P3 cells. This objective has been guided by our previous work indicating that ROS are involved in PPD-induced apoptosis (Elyoussoufi *et al*., 2012). In fact, GSH has a ubiquitous distribution within most tissue and plays a major role in protecting cells from oxidative stress, mostly by scavenging intracellular ROS (Reed, 1990). It was interesting to note that pretreatment of P3 cells with 1mM GSH reduced PPD cytotoxicity. This finding confirms our last results about the protective effect of GSH against ROS production (Elyoussoufi *et al*., 2012).

It is known that ROS play a crucial role in cell growth and apoptosis. An appropriate level of intracellular ROS promotes cellular proliferation (Liou and Storz, 2010), whereas excessive production of ROS leads to oxidative stress, loss of cell function, and ultimately apoptosis (Circu and Aw, 2010; Liou and Storz, 2010). Moreover, depletion of GSH has been associated with the initiation of apoptosis (Lash *et al*., 2002; Laube *et al*., 2006), probably because the lowering of GSH increases ROS production. In fact, several studies have suggested that PPD induced apoptosis could be mediated by ROS production (Mathur *et al*., 2005; Chen *et al*., 2006; Chye *et al*., 2012; Elyoussoufi *et al*., 2012). ROS has been shown to cause DNA damage through strand breaks and base modification (Liu *et al*., 2001). The involvement of ROS in DNA damage was visualized here qualitatively on agarose gel. Using GSH, we have found that this antioxidant reduced DNA damage. Moreover, recent results showed that the use of vitamins E and C inhibited the ROS generation and DNA damage in MDCK cells after treatment with PPD (Chen *et al*., 2010). It is becoming increasingly apparent that caspases are redox-sensitive enzymes. They possess an active site cysteine, which is predicted to be susceptible to oxidation (Fadeel *et al*., 1998). Thus, evaluating the role of ROS in the regulation of these cysteine proteases in P3 cells was important. In this work, we have noted that pre-treatment with GSH reduced the caspase-8, -9 and -3 activities in PPD treated cells. Nobel *et al*. 1997 have demonstrated that dithiocarbamate disulfides inhibited caspase-1 and caspase-3 activities and that the inhibitory effect was accomplished most likely through thiol-disulfide exchange. In addition, it has been proposed that thiol antioxidants could inhibit apoptosis in T-lymphocytes by altering the redox status of thiol enzymes, such as the caspases, in the pathway leading to apoptosis (Fadeel *et al*., 1998). Questions remain as to how caspases are activated by PPD in P3 cells to lead into apoptosis. This raises the possibility of ROS involvement in this activation. It was shown that ROS production leads to disruption of mitochondrial membrane potential ($\Delta\Psi m$) and releases pro-apoptotic molecules from mitochondria into the cytosol (Garrido *et al*., 2006). Chen *et al*. 2006 have shown that the decrease of $\Delta\Psi m$ played an important role in PPD induced apoptosis in MDCK cells. According to the literature data, the release of pro-apoptotic molecules such as cytochrome c may activate caspase-9, which, in turn, triggers the effector caspase-3 (Garrido *et al*., 2006).

In fact, activated caspase-3 orchestrate DNA fragmentation (Enari *et al*., 1998), nuclear condensation (Sahara *et al*., 1999), and membrane blebbing (Coleman *et al*., 2001; Sebbagh *et al*., 2001) through cleavage of specific substrates. These data may explain the caspases activation observed here. However further studies must be done to confirm it. Another point to be discussed and demonstrated is the involvement of other molecular effectors, like p53, in PPD induced P3 cells apoptosis. It is known that p53 is stimulated by cellular stresses such as DNA damage and hypoxia (Ohiro *et al*., 2002). Furthermore, Chen *et al*. 2006 have demonstrated that the p53 protein level was elevated after treatment with PPD. Moreover, the process of p53-dependent apoptosis requires mitochondria-dependent apoptotic machinery, including caspase-9, as well as the release of cytochrome c from the mitochondria (Soengas *et al*., 1999), and is therefore a caspase-dependent mechanism (Ohiro *et al*., 2002). In conclusion, our results have provided experimental evidence supporting that PPD can induce apoptosis in P3 cells. We have found that PPD can initiate two major pro-apoptotic signaling pathways involving either the mediation of death receptors leading to the activation of initiator caspase-8 and the activation of initiator caspase-9 via the intrinsic or mitochondria pathways following the activation of caspase-3 leading to apoptosis. Furthermore, the increase of ROS levels may be the key mediator in PPD induced apoptosis in P3 cells.

Conflict of interest

We, the undersigned authors, warrant that there are no conflicts of interest.

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REFERENCES

Apel, K. and Hirt, H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant. Biol., 55: 373-399.

Bai, Y.H., Peng, Y.M., Yin, W.Q., Liu, H., Liu, F.Y., Duan, S.B. and Xiao, P. 2012. p-Aminophenol and p-

paraphenylenediamine induce injury and apopt osis of human HK-2 proximal tubular epithelial cells. J. Nephrol., 25(4): 481-489.

Chen, S.C., Chen, C.H., Chern, C.L., Hsu, L.S., Huang, Y.C., Chung, K.T. and Chye, S.M. 2006. p-Phenylenediamine induces p53-mediated apoptosis in Mardin Darby canine Kidney cells. Toxicol. in Vitro, 20: 801-207.

Chen, S.C., Chen, C.H., Tioh, Y.L., Zhong, P.Y., Lin, Y.S. and Chye, S.M. 2010. Para-phenylenediamine induced DNA damage and apoptosis through oxidative stress and enhanced caspase-8 and -9 activities in Mardin–Darby canine kidney cells. Toxicol. in Vitro, 24: 1197-1202.

Chye, S.M., Hseu, Y.C., Liang, S.H., Chen, C.H. and Chen, S.C. 2008. Single strand DNA breaks in human lymphocytes exposed to para-phenylenediamine and its derivatives. Bull. Environ. Contam. Toxicol., 80: 58-62.

Chye, S.M., Tiong, Y.L., Yip, W.K., Koh, R.Y., Len, Y.W., Seow, H.F., Ng, K.Y., Ranjit, D.A. and Chen, S.C. 2012. Apoptosis induced by para-phenylenediamine involves formation of ROS and activation of p38 and JNK in chang liver cells. *Environ. Toxicol.*, doi: 10.1002/tox.21828.

Circu, M.L., Rodriguez, C., Maloney, R., Moyer, M.P. and Aw, T.Y. 2008. Contribution of mitochondrial GSH transport to matrix GSH status and colonic epithelial cell apoptosis. *Free Radic. Biol. Med.,* 44: 768-778.

Circu, M.L. and Aw, T.Y. 2010. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med*., 48: 749-762.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A. and Olson, M.F. 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat. Cell Biol*., 3: 339-345.

Corbett, J.F. and Menkart, J. 1973. Hair colouring. Cutis, 12: 190-193.

Coulter, E.M., Farrell, J., Mathews, K.L., Maggs, J.L., Pease, C.K., Lockley, D.J., David, A., Basketter, B., Park, K. and Naisbitt, D.J. 2007. Activation of human dendritic cells by p-phenylenediamine. J. Pharmacol. *Exp. Ther*., 320: 885-892.

Dlamini, Z., Mbita, Z. and Zungu, M. 2004. Genealogy, expression and molecular mechanisms in apoptosis. *Pharmacol. Ther*., 101: 1-15.

Elyoussoufi, Z., Habti, N., Motaouakkil, S. and Cadi, R. 2012. Paraphenylenediamine induces apoptosis of Murine myeloma cells in a reactive oxygen species dependant mechanism. *J. Toxicol. Environ. Health Sci*., 4(4): 76-84.

Elyoussoufi, Z., Habti, N., MOUNAJI, K., Motaouakkil, S. and Cadi, R. 2013. Induction of oxidative stress and apoptosis in human neutrophils by p-phenylenediamine. *J. Toxicol. Environ. Health Sci*., 5(8): 142-149.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*, 391: 43-50.

Evan, G.I. and Vousden, K.H. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature*, 411: 342-348.

Fadeel, B., Åhlin, A., Henter, J.I., Orrenius, S. and Hampton, M.B. 1998. Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. *Blood*, 12(92): 4808-4818.

Gago-Dominguez, M., Castelao, J.E., Yuan, J.M., Yu, M.C. and Ross, R.K. 2001. Use of permanent hair dyes and bladder-cancer risk. *Int. J. Cancer*, 91: 575-579.

Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C. and Kroemer, G. 2006. Mechanisms of cytochrome c release from mitochondria. *Cell Death. Differ*., 13: 1423-1433.

Garrigue, J.L., Ballantyne M., Kumaravel, T., Lloyd, M., Nohynek, G., Kirkland, D. and Toutain, H. 2006. In vitro genotoxicity of para-phenylenediamine and its N-monoacetyl or N, N′-diacetyl metabolites. *Mutat. Res*., 608: 58-71.

Hansen, J., Mollgaard, B., Avnstorp, C. and Menne, T. 1993. Paraben contact allergy: Patch testing and in vitro absorption/metabolism. *Am. J. Contact. Dermat*., 4: 78-86.

Huang, Y.C., Hung, W.C., Kang, W.Y., Chen, W.T. and Chai, C.Y. 2007. pPhenylenediamine induced DNA damage in SV-40 immortalized human uroepithelial cells and expression of mutant p53 and COX-2 proteins. *Toxicology Lett*., 170: 116-123.

Kang M.H. and Reynolds C.P. 2009. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin. Cancer Res*., 15: 1126-1132.

Lash, L.H., Putt, D.A. and Matherly, L.H. 2002. Protection of NRK-52E cells, a rat renal proximal tubular cell line, from chemical-induced apoptosis by overexpression of a mitochondrial glutathione transporter. *J. Pharmacol. Exp. Ther*., 303: 476-486.

Laube, G.F., Shah, V., Stewart, V.C., Hargreaves, I.P., Haq, M.R., Heales, S.J. and van't Hoff, W.G. 2006. Glutathione depletion and increased apoptosis rate in human cystinotic proximal tubular cells. Pediatr. *Nephrol*., 21: 503-509.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*, 371: 346-347.

Liou, G.Y. and Storz, P. 2010. Reactive oxygen species in cancer. *Free. Radic. Res*., 44: 479-496.

Liu, K., Husler, J., Ye, J., Leonard, S.S., Cutler, D., Chen, F., Wang, S., Zhang, Z., Ding, M., Wang, L. and Shi, X. 2001. On the mechanism of Cr (VI)-induced carcinogenesis: dose dependence of uptake and cellular responses. *Mol. Cell. Biochem*., 222: 221-229.

Mathur, A.K., Raizada, R.B., Srivastava, M.K. and Singh, A. 2005. Effect of Dermal Exposure to Paraphenylenediamine and Linear Alkylbenzene Sulphonate in Guinea Pigs. Biomed. *Environ. Sci*., 18: 238-240.

Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. 2004. Reactive oxygen gene network of plants. *Trends. Plant. Sci*., 9: 490-498.

Nobel, C.S.I., Burgess, D.H., Zhivotovsky, B., Burkitt, M.J., Orrenius S. and Slater, A.F.G. 1997. Mechanisms of dithiocarbamate inhibition of apoptosis: Thiol oxidation by dithiocarbamate disulfides directly inhibits processing of the caspase-3 proenzyme. *Chem. Res. Toxicol*., 10: 636-643.

N.T.P (National Toxicology Program). 1978. Bioassay of 2,5-toluenediamine sulphate for possible carcinogenicity, U.S. National Toxicology Program Technical Report Series no. 126.

N.T.P (National Toxicology Program). 1979. Bioassay of p-phenylenediamine dihydrochloride for possible

carcinogenicity, U.S. National Toxicology Program Technical Report Series no. 174.

Ohiro, Y., Garkavtsev, I., Kobayashi, S., Sreekumar, K.R., Nantz, R., Higashikubo, B.T., Duffy, S.L., Higashikubo, R., Usheva, A., Gius, D., Kley, N. and Horikoshi, N. 2002. A novel p53-inducible apoptogenic gene, PRG3, encodes a homologue of the apoptosis-inducing factor (AIF). FEBS. Lett., 524: 163-171.

Okouchi, M., Okayama, N. and Aw, T.Y. 2005. Hyperglycemia potentiates carbonyl stressinduced apoptosis in naive PC-12 cells: relationship to cellular redox and activator protease factor-1 expression. *Curr. Neurovasc. Res*., 2: 375-386.

Rauscher, G.H., Shore, D. and Sandler, D.P. 2004. Hair dye use and risk of adult acute leukemia. *Am. J. Epidemiol*., 160: 19-25.

Reed, D.J. 1990. Glutathione: toxicological implications. *Annu. Rev. Pharmacol. Toxicol*., 30: 603-631.

Rego, A.C. and Oliveira, C.R. 2003. Mitochondria dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem. Res*., 28: 1563-1574.

Riedl, S.J. and Salvesen, G.S. 2007. The apoptosome: signalling platform of cell death. *Nat. Rev. Mol. Cell. Biol*., 8: 405-413.

Sahara, S., Aoto, M., Eguchi, Y., Imamoto, N., Yoneda, Y. and Tsujimoto, Y. 1999. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. *Nature*, 401: 168-173.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. molecular cloning: a laboratory manual cold spring harbor. Cold spring harbor laboratory press, 3rd ed, New York, USA.

Schwartzman, R.A. and Cidlowski, J.A. 1993. Mechanism of tissue-speciWc induction of internucleosomal deoxyribonucleic acid cleavage activity and apoptosis by glucocorticoids. *Endocrinology*, 133: 591-599.

Sebbagh, M., Renvoize, C., Hamelin, J., Riche, N., Bertoglio, J. and Breard, J. 2001. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat. Cell. Biol*., 3: 346-352.

Soengas, M.S., Alarcon, R.M., Yoshida, H., Giaccia, A.J., Hakem, R., Mak, T.W. and Lowe, S.W. 1999. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science*, 284: 156-159.

Sontag, J.M. 1981. Carcinogenicity of substituted-benzenediamines (phenylenediamines) in rats and mice. *J. Natl. Cancer. Inst*., 66: 591-602.

Stanley, L.A., Skare, J.A., Doyle, E., Powrie, R., D'Angelo, D. and Elcombe, C.R. 2005. Lack of evidence for metabolism of p-phenylenediamine by human hepatic cytochrome P450 enzymes. *Toxicology*, 210: 147-157.

Steller, H. 1995. Mechanisms and genes of cellular suicide. *Science*, 267: 1445-1449.

Streiblova, E. 1988. Cytological methods. In Yeast-A Practical Approach, ed. J. Campbell and J. M. Buffers, Oxford, United Kingdom: IRL Press, pp. 9-49.

Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science*, 267: 1456-1462.

Thun, M.J., Altekruse, S.F., Namboodiri, M.M., Calle, E.E., Myers, D.G. and Heath, Jr C.W. 1994. Hair dye use and risk of fatal cancers in US women. *J. Natl. Cancer. Inst*., 86: 210-215.

Van Duuren, B.L. 1980. Carcinogenicity of hair dye components. J. Environ. Pathol. Toxicol., 3: 237-251.

Wyllie, A.H. 2010. Where o death, is thy sting? A brief review of apoptosis biology. *Mol. Neurobiol*., 42: 4-9.

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