Full Length Research Paper

The role of paraphenylenediamine in triggering apoptosis in murine myeloma cells through reactive oxygen species generation

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Paraphenylenediamine (p-PD) is the main aromatic amine used in the formulation of hair dyes. Epidemiologic studies have suggested that the use of p-PD based hair dyes might be related to increase risk of human malignant tumors including multiple myeloma and hematopoietic cancers. However, the toxicity of p-PD on myeloma cells has not been well elucidated yet. Therefore, the association between the cytotoxicity of p-PD and the reactive oxygen species (ROS) generation on murine myeloma cells P3X63Ag8.653 (P3) was evaluated. Treatment with p-PD decreases cell viability in a dose and time dependent manner. In addition, p-PD markedly enhanced lipid peroxidation. This increase was accompanied with a decrease in both glutathione reductase and superoxide dismutase activities. Furthermore, Pre-treatment of P3 cells with antioxidants, reduced glutathione or manganese II chloride, significantly inhibited p-PD induced cytotoxicity and ROS generation. Based on these results, p-PD might induce apoptosis via the involvement of ROS.

Key words: Murine myeloma cells, p-Phenylenediamine, apoptosis, oxidative stress, ROS, antioxidants.

INTRODUCTION

Paraphenylenediamine (p-PD) a monocyclic arylamine, is frequently used as an ingredient of oxidative hair coloring products (Corbett and Menkart, 1973) and black henna dyes (Kang and Lee, 2006). Currently, p-PD is present in more than 1000 hair dye formulations marketed all over the world (Stanley et al., 2005). Epidemiologic studies demonstrated that workers in the textile dye and rubber industries, hair dye users and barbers incurred a high risk of bladder cancer, non-Hodgkin's lymphoma, multiple myeloma and hematopoietic cancers (Thun et al., 1994; Yu et al., 1998; Gago-Dominguez et al., 2001; Rauscher et al., 2004). Carcinogens usually cause genomic damage to expose cells which may either undergo apoptosis or proliferation with genomic damage and potentially leading to transformation in cancerous cells (Steller, 1995; Thompson, 1995).

The apoptotic regulatory process involves activation of a cascade of molecular events that lead to cell death. Oxidants including reactive oxygen species (ROS), lipid hydroperoxides and nitrogen monoxide (NO) are believed to be widely involved in oxidative stress leading to the induction of apoptosis (Forrest et al., 1994). It was found that p-PD is able to induce oxidative stress in keratinocytes and other cells (Picardo et al., 1996; Rioux and Castonguay, 2000; Brans et al., 2007; Chen et al.,

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2010). This prompted us to investigate the impact of p-PD on the ROS pathway in murine myeloma cells P3X63Ag8.653. In this study we investigate more the role of ROS in p-PD induced apoptosis. Reduced glutathione (GSH) and manganese II chloride ($MnCl_2$) were used as antioxidant and Hydrogen peroxide (H_2O_2) was used as an oxidant to confirm the role of ROS in apoptosis.

MATERIALS AND METHODS

Cell culture

The murine myeloma cells P3X63Ag8.653 (P3) (European collection of cell cultures, grande bretagne) was maintained in RPMI 1640 medium (Sigma Aldrich, USA) supplemented with 5 or 10% fetal bovine serum (FBS), 100 UI/ml penicillin, 100 µg/ml streptomycin 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. For all experiments, P3 cells were seeded in 24 well plates (6.10^5cells/well) and grown for 36 h which correspond to the exponential phase. At this time, a series of diluted concentrations of p-PD (5, 2.5 or 1.25 µg/ml) were added to the culture medium. Cells were then incubated for 12, 24, 36 or 60 h, respectively. Each concentration was run in three replicates in a three independent manner.

In other experiments, P3 cells were pretreated with 0.014, 1.4 or 14 µM of $MnCl_2$ or with 1 mM of GSH (Sigma Aldrich, USA) and incubated with 2.5 or 5 µg/ml of p-PD for 12, 24 or 36 h, then the cell viability was determined. The results were reported as the tested groups versus the untreated group. Cells treated with 50 µM of H_2O_2 were used as positive control.

Cell viability

The viability of P3 was assessed from the intactness of the plasma membrane as determined by the trypan blue exclusion test.

Morphologic assessment of apoptosis

Cellular morphology was ascertained by light microscopy following May-Grünwald-Giemsa (MGG) staining of cytocentrifuged cells. Cytospins were prepared using a Shandon cytospin 2. At least, 300cells/slide were counted at 1000 x final magnification.

Evaluation of lipid peroxidation

Lipid peroxidation was estimated by thiobarbituric acid reactive substances (TBARS) reaction with malondialdehyde (MDA) as described by Samokyszyn and Marnett (1990). Briefly, 250 µl of supernatant were added to 10% trichloroacetic (TCA) and 0.375% TBA. The MDA level was expressed as µmol/min/mg protein.

Assay of catalase (CAT) activity

The catalase activity was measured according to Aebi, (1984). The specific activity is given as micromoles of consumed H_2O_2/min/mg protein.

Assay of glutathione reductase (GR) activity

To estimate the GR activity, the method of DI HIO et al. (1983) was used. The enzyme activity was calculated as micromoles of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidized /min/mg protein.

Assay of superoxide dismutase (SOD) activity

The SOD activity was measured according to Paoletti et al. (1986). The enzyme activity was expressed as µmol/min/mg protein.

Determination of protein concentrations:

Protein concentrations were determined by the Bradford (1976) method using bovine serum albumin as a standard.

Statistical analysis

Results were expressed as the mean ± SEM of three to six independent experiments. The statistical evaluation of the data was carried out by applying the student's t-test (paired). Significant difference was taken as $p < 0.05$.

RESULTS

Effects of p-PD on P_3 cells viability

P3 cells, in their exponential phase, were cultured with increasing dose of p-PD (1.25, 2.5 and 5 µg/ml) for 12, 36 and 60 h. Cell viability was evaluated by trypan blue dye exclusion method. p-PD treatment reduced cell viability in a dose dependent manner. After 36 h of treatment, doses of 1.25, 2.5 and 5 µg/ml reduced cell viability to 31, 40 and 51%, respectively (Figure 1). Our results showed also that p-PD reduced cell viability in a time dependent manner. In addition, p-PD inhibited the growth of P3 cells effectively (p=0.001) with a DL_{50} value of 5µg/ml at 36 h (Figure 1). Thus 5 µg/ml of p-PD was selected for some experiments.

p-PD effected cell morphology of P3 cells

To determine whether p-PD induces apoptosis of P3 cells, cells were incubated with 5 µg/ml of p-PD for 12, 24 and 36 h and their morphologies were examined using MGG staining. As shown in Figure 2a, untreated P3 cells retained their normal size and shape. Morphological alterations were appreciated after incubation for 12 h with 5 µg/ml of p-PD. At this time, toxic effect of p-PD was
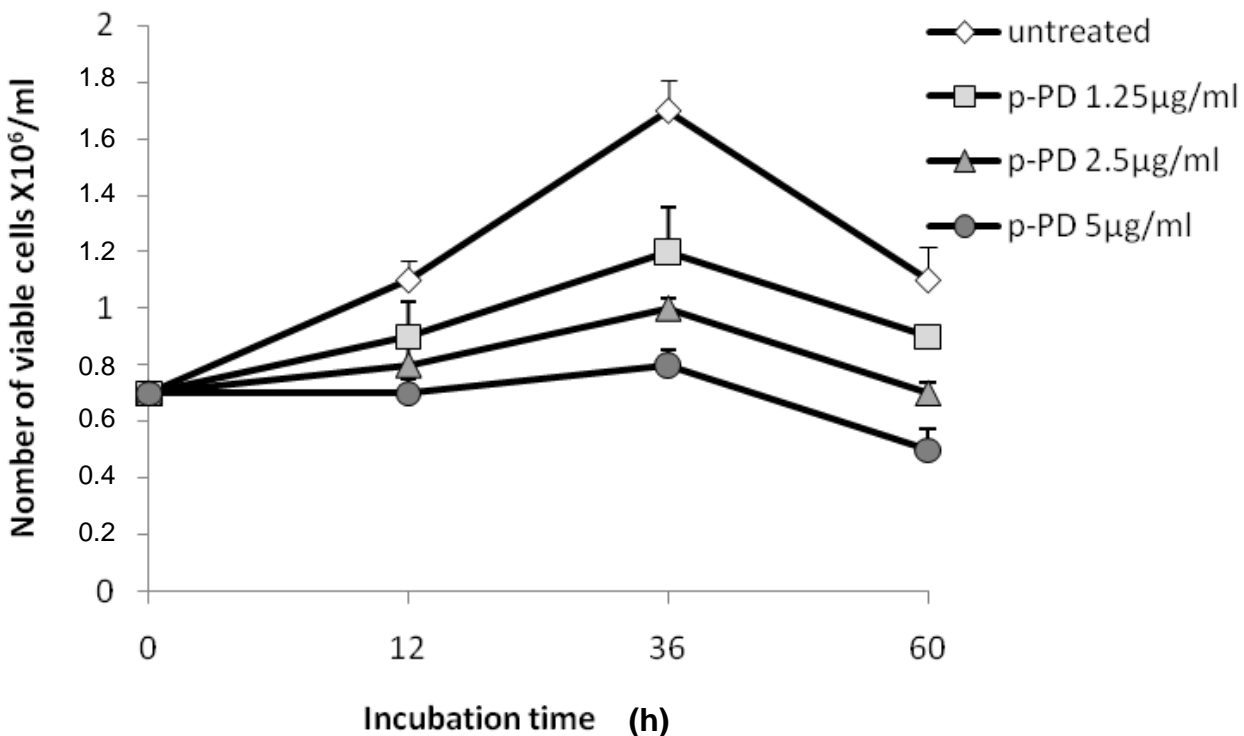
Figure 1. Time course of the viability of P3 cells after treatment with p-PD. Cells in exponential phase were treated with 1.25, 2.5 or 5 µg/ml of p-PD for 12, 36 and 60 h. Results are shown as mean ± SEM from three independent experiments ($p<0.05$).

characterized by loss of the typical morphology and cell enlargement and by nuclear condensation (Figure 2b).

Effects of p-PD in lipid peroxidation and ROS scavenging enzymes

Since p-PD has been shown to trigger production of ROS, we next assayed for p-PD induced production of ROS in P3 cell. Cells were treated with 1.25, 2.5 and 5 µg/ml p-PD for 12 and 36 h, MDA level and activities of the main antioxidant enzymes (SOD, GR and CAT) were determined (Figures 3 and 4, respectively).

p-PD clearly increase the MDA level dose dependently between 12 and 36 h ($p<0.05$), reaching 27, 34 and 41 µmol/min/mg protein after treatment for 36 h with 1.25, 2.5 and 5 µg/ml of p-PD, respectively, compared to untreated cells (20 µmol/min/mg protein) (Figure 3).

p-PD significantly reduces SOD activity ($p<0.05$). This reduction was important after treatment with 5µg/ml for 36 h ($p = 0.002$) (Figure 4a). Although p-PD induced a decrease in GR activity, a minimal activity of this enzyme was observed when cells were treated with 1.25 µg/ml for 36 h ($p = 0.006$) (Figure 4b). However, at the same

time, treatment with 5µg/ml increased this activity ($p<0.05$). The CAT activity increased only when cells were treated with 1.25 and 2.5 µg/ml for 12 h ($p=0.006$ and $p<0.001$, respectively). While treatment with 5 µg/ml clearly decreas this activity ($p = 0.02$). After 36 h no significant effect was observed (Figure 4c).

The antioxidative effect of $MnCl_2$ on p-PD treated P3 cells viability

To elucidate more on the involvement of oxidative stress in the mechanism of cell death, the effect of $MnCl_2$ on the p-PD induced apoptosis was examined. P3 cells were pretreated with three concentrations (0.014, 1.14 and 14 µM) of manganese II chloride ($MnCl_2$) 5 min before treatment with 2.5 or 5 µg/ml of p-PD. Cells then were incubated for 12, 24 and 36 h. H_2O_2 50 µM served as positive control. The three doses of $MnCl_2$ used in this experiment increased significantly the viability of p-PD treated cells only after 12 h of treatment ($p<0.05$). They did not affect the H_2O_2 treated cells (Figure 5). After 24 and 36 h of treatment, $MnCl_2$ had no effect on cell viability (data not shown).

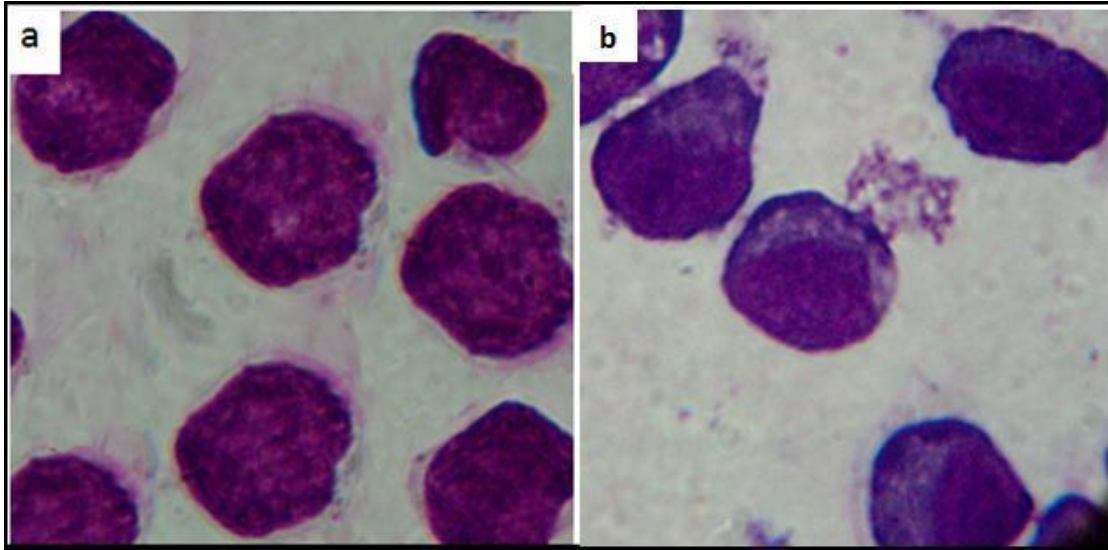We examined whether $MnCl_2$ would be more protective

Figure 2. Light microscopy of cultured P3 cells. Cells were incubated for 12 h without (2a) or with 5 µg/ml of p-PD (2b) then stained with MGG and apoptotic cells were evaluated morphologically. At least, 300 cells/slide were counted at 1000 x final magnification.
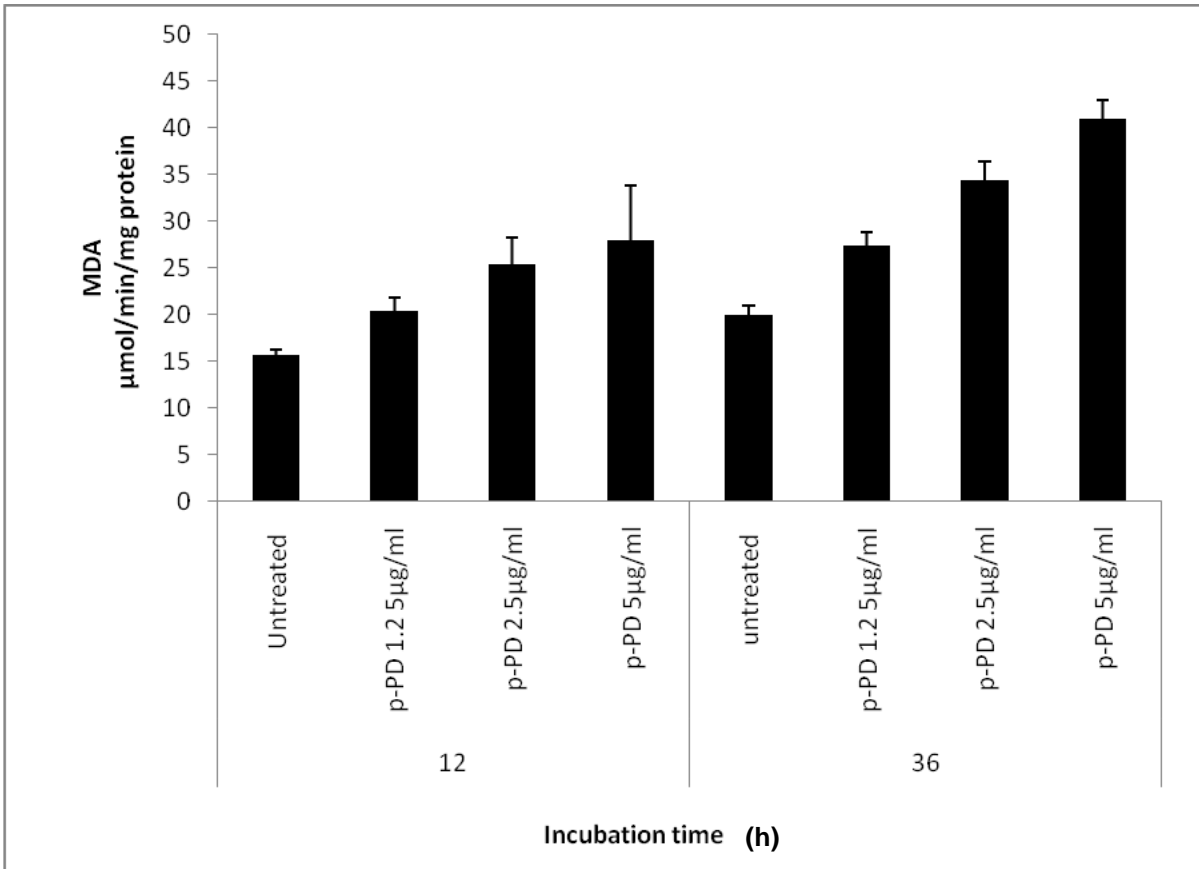

Figure 3. MDA level in P3 cells. Cells were treated with 1.25, 2.5 or 5 µg/ml p-PD for 12 and 36 h. Results are shown as mean ± SEM from three independent experiments ($p < 0.05$).
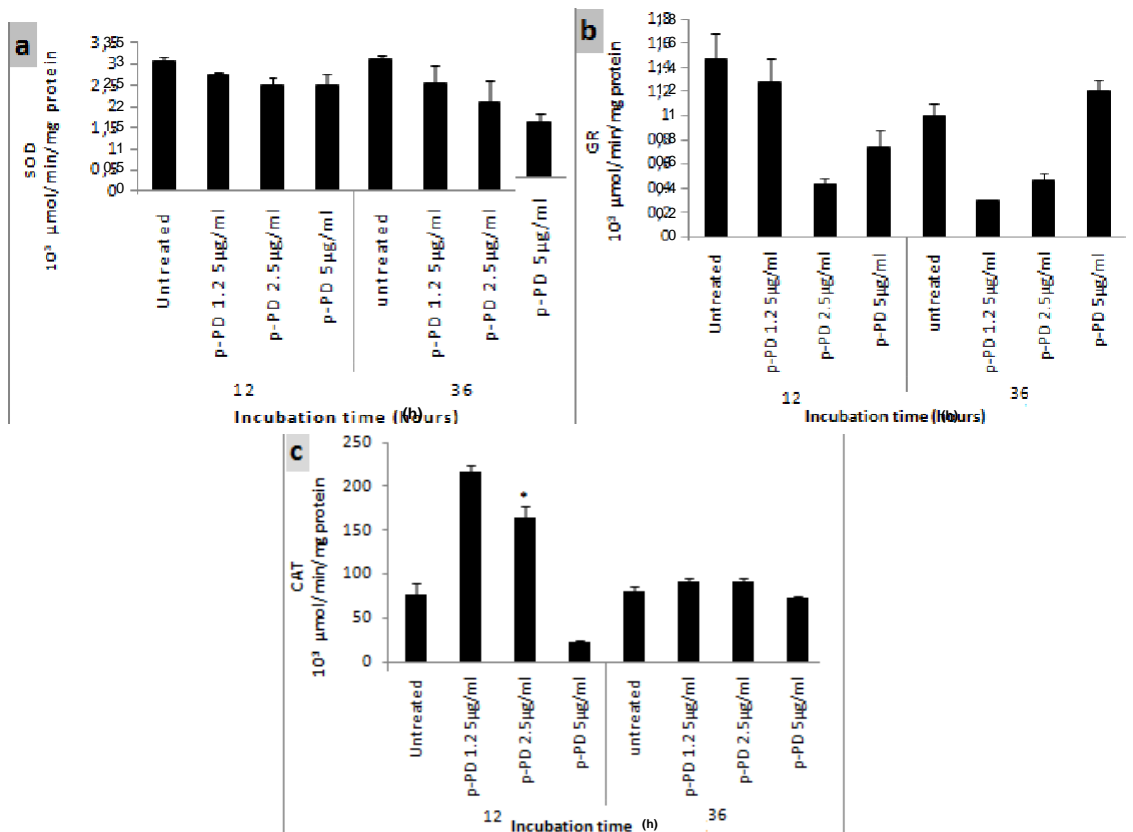
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Figure 4. Antioxidant enzymes activities in P3 cells. a) SOD activity. b) GR activity and c) CAT activity. Cells were treated with 1.25, 2.5 or 5 µg/ml p-PD for 12 and 36 h. Results are shown as mean ± SEM from three independent experiments (p<0.05). *p<0.001.
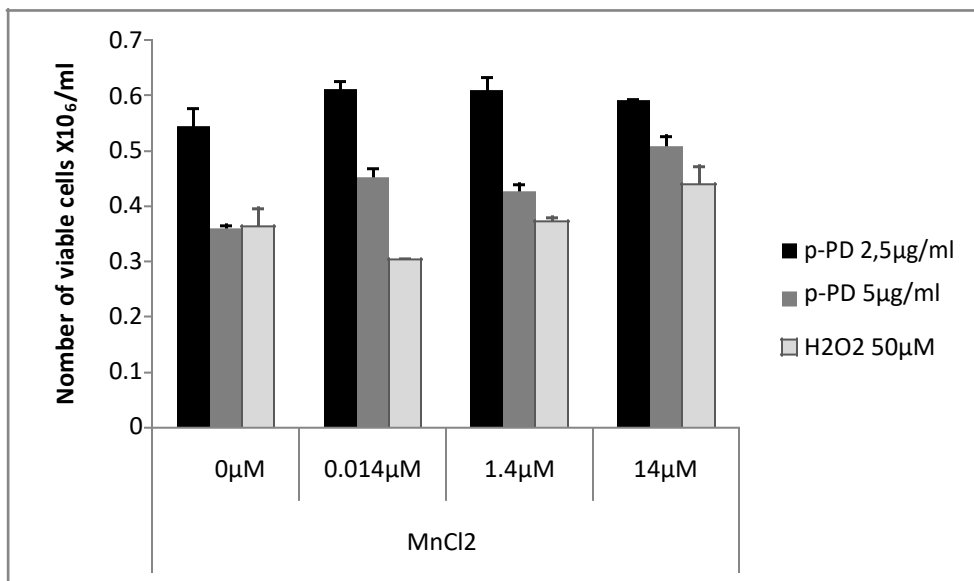

Figure 5. Effects of MnCl$_2$ on P3 cells viability. Cells were pretreated with 0.014, 1.14 or 14 µM MnCl$_2$ 5 min before treatment with 2.5 or 5 µg/ml p-PD. Cells then were incubated for 12 h. H$_2$O$_2$ 50 µM served as positive control. Results are shown as mean ± SEM from three independent experiments (p<0.05).

Figure 6. Effects of pretreatment with MnCl₂ on the viability of P3 cells. Cells were pre-incubated with 0.014µM MnCl₂ for 8h then the same amount was added 5 min before treatment with 2.5 and 5 µg/ml p-PD then incubated for 12, 24 and 36 h. H₂O₂ 50 µM served as positive control. Results are shown as mean ± SEM from three independent experiments (p<0.05).
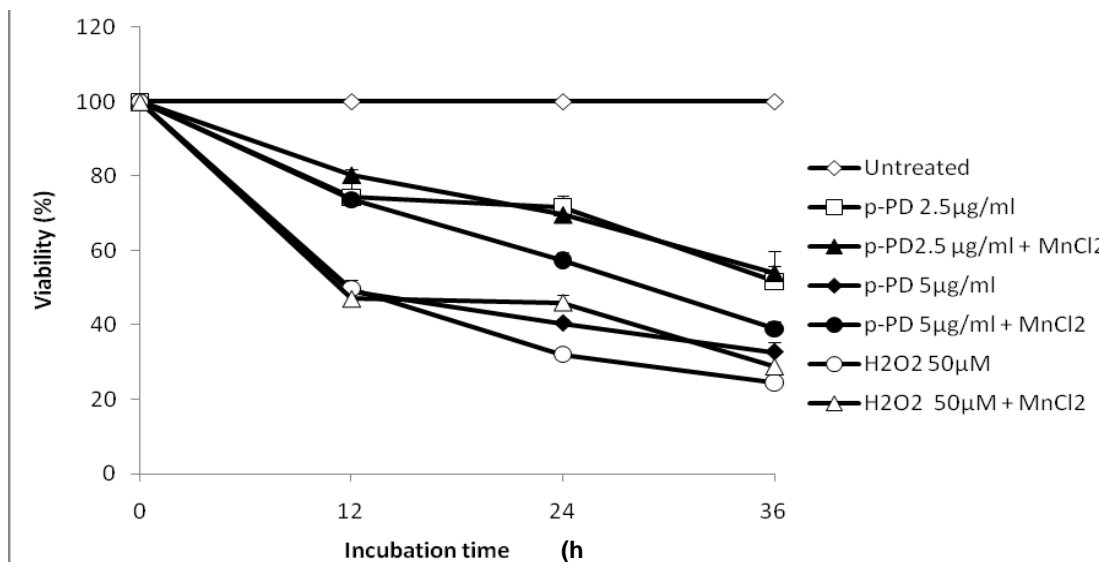
if added hours before treatment with p-PD. Cells were pre-incubated with 0.014µM MnCl₂ for 8 h then the same amount was added 5 min before treatment with 2.5 or 5 µg/ml p-PD and incubated for 12, 24 and 36 h. As shown in Figure 6, the MnCl₂ added was significantly able to prolong the viability of cells treated with 5 µg/ml of p-PD for 12 to 36 h (p = 0.05, p = 0.002 and p=0.026, respectively). However, MnCl₂ had few protective effects on cells treated with 2.5 µg/ml p-PD or 50 µM H₂O₂.

Effects of GSH on p-PD treated P3 cells viability

Reduced glutathione (GSH) and related thiols play important roles in the survival of various cells. In order to investigate the antioxidative effect of GSH on p-PD induced apoptosis we pre-incubated P3 cells for 5 min with 1 mM GSH followed by p-PD treatment (2.5 or 5 µg/ml p-PD). Cells then were incubated for 12, 24 and 36 h. As shown in Figure 7, GSH increased cell viability of treated cells. This increase was significant for cells treated with 5 µg/ml p-PD (p=0.001).

Effects of antioxidants on MDA level of p-PD treated cells

To confirm the involvement of oxidative stress on p-PD induced apoptosis, P3 cells were pretreated for 5 min

with 0.014 µM MnCl₂ or 1 mM GSH and treated with 2.5 or 5 µg/ml p-PD then incubated for 12 and 36 h. MDA level was determined. Treatment with MnCl₂ or GSH showed a clear protective effect against lipid peroxidation (p= 0.001 and p<0.001, respectively) (Figure 8a and b).

DISCUSSION

Mutagenic/carcinogenic compounds can induce apoptosis in cells. p-PD is a suspected carcinogen that can induce apoptosis (Sontag, 1981; Chen et al., 2006; Chen et al., 2010).

In the present work, p-PD induces cytotoxicity in a dose and time dependent manner and inhibited the growth of P3 cells effectively with DL_{50} value of 5µg/ml at 36 h. A similar effect was observed with SV-40 cells (Huang et al., 2007), human dendritic cells (Coulter et al., 2006) and previously with our team with P3 cells (Benzakour et al., 2011).

After observation of stained P3 cells microscopically, we found that p-PD induces P3 cells apoptosis. This finding was confirmed by DNA fragmentation (data no shown). We have found a similar effect on human neutrophils (Elyoussoufi et al., in press). Studies by Chen et al. (2006) demonstrated that p-PD induced apoptosis of MDCK cells in a dose and time dependent manner.

Also, Huang et al. (2007) revealed that p-PD was able to induce DNA damage dose dependently.
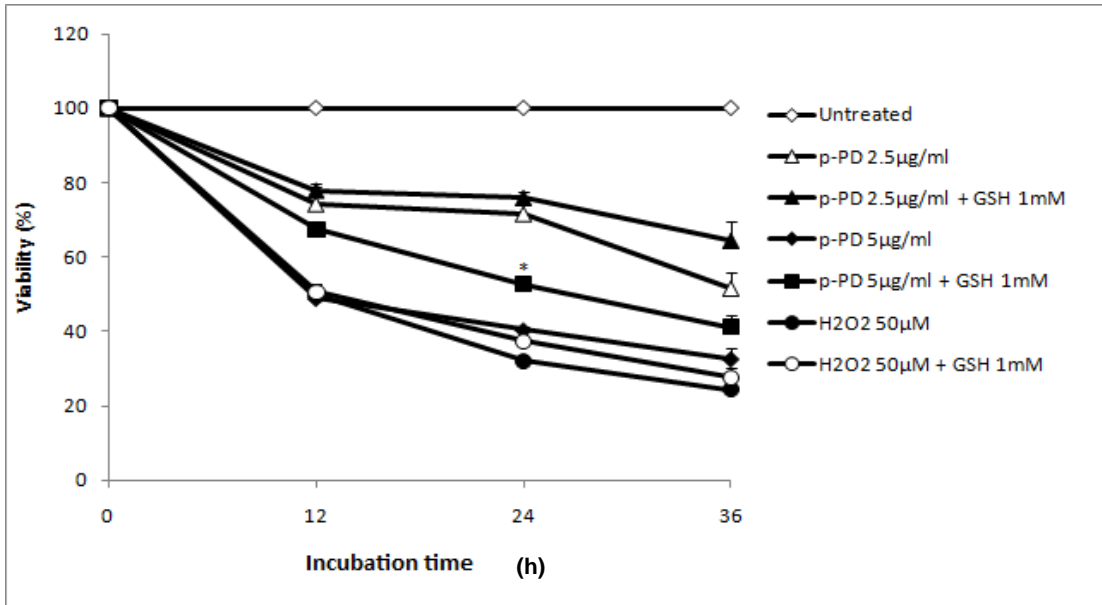
Figure 7. Effects of GSH on the viability of P3 cells. Cells were pre-incubated with 1 mM GSH 5 min before treatment with 2.5 and 5 µg/ml p-PD then incubated for 12, 24 and 36 h. H_2O_2 50 µM served as positive control. Results are shown as mean ± SEM from three independent experiments ($p < 0.05$). *$p < 0.01$.
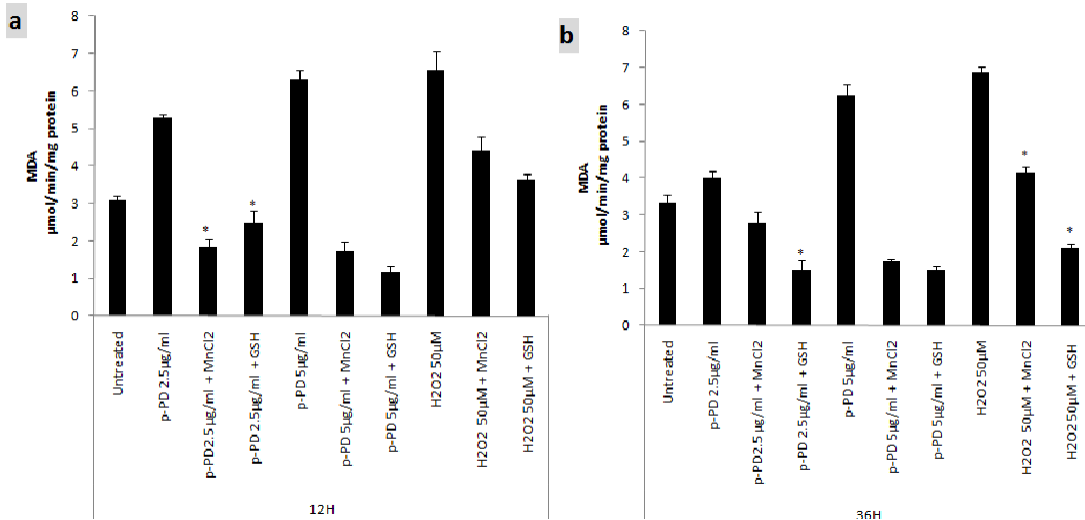

Figure 8. Effect of antioxidants on lipid peroxidation in P3 cells. Cells were pretreated for 5 min with 0.014 µM $MnCl_2$ or 1 mM GSH and treated with 2.5 and 5 µg/ml p-PD then incubated for 12 (a) and 36 h (b). H_2O_2 50 µM served as positive control. Results are shown as mean ± SEM from three independent experiments ($p < 0.05$). *$p < 0.01$.

Oxidation reactions by ROS are regarded as a trigger of the oxidative stress. Several enzymes like SOD, GR and CAT serve as protective antioxidants against oxidative stress (Kuwabara et al., 2008). In this work we detected an enhanced activity of CAT when cells were treated with 1.25 or 2.5 µg/ml p-PD, which shows that defense mechanism is increased to counter the damaging effect of p-PD. However, we noted that with a concentration of 5 µg/ml, p-PD induced a decrease in CAT activity. To explain this decrease two hypotheses can be proposed,

either it is only the action of p-PD on the mitochondrial respiratory chain which generates by itself an overproduction of ROS, leading to the inactivation of CAT; or p-PD is able to interact directly with CAT, decreasing its efficiency in detoxifying peroxides and H_2O_2. Our results show also that p-PD reduced SOD and GR activities. It is revealed that activities of ROS scavenging enzymes such SOD and GR are inactivated by ROS (Sampson et al., 2001). According to Chen et al. (2010), p-PD was found to generate ROS in a time dependent manner. However, we found that with a high concentration, p-PD provoked an increase in GR activity. This funding can be explained by the ability of this enzyme to regenerate GSH from GSSG. Moreover, the level of reducing agents, such as GSH, is important in regulating the oxidative intracellular state. This result correlate with those found with human neutrophils (Elyoussoufi et al., in press).

ROS are considered to cause damage to cells by oxidizing lipids in the cell membrane or by attacking DNA directly (Inoue et al., 1994). In the present study, lipid peroxidation was found to be increased by p-PD. It is widely accepted that lipid peroxidation is increased by ROS (Sander et al., 2003). Our finding that p-PD increased lipid peroxidation support the concept that ROS are involved in p-PD induced apoptosis. This finding is in concert with those of Mathur et al. (2005) and Chen et al. (2006) which demonstrate a role of ROS in p-PD induced apoptosis in keratinocytes and in SV-40 cells. It is well known that ROS production serves as an early signal to mediate programmed neuronal death (Greenlund et al., 1995). Furthermore, it has been demonstrated that coordinate regulation of ROS, caspases and p53 facilitates apoptosis induced by p-PD suggesting that production of ROS may be essential for apoptosis (Huang et al., 2007; Chen et al., 2010). The involvement of ROS in p-PD induced apoptosis in P3 cells was validating using $MnCl_2$ and GSH which inhibited ROS generation.

GSH is one of the most important chemical antioxidant agent in mammalian cells (Diaz Vivancos et al., 2010; Jones and Go, 2010). In our work, treatment with GSH enhanced cell viability of p-PD treated P3 cells. In addition, Ozawa et al., 2006 have revealed that addition of GSH to cell culture medium lowered the production of apoptotic cells in blastocysts. Furthermore, many studies have established the importance of GSH redox in cell apoptosis in variety of cell types (Pias et al., 2003; Ekshyyan and Aw, 2005; Circu et al., 2008).

We next hypothesized that manganese has some effect on p-PD treated P3 cells. $MnCl_2$ was then used to confirm the hypothesis; and as expected, $MnCl_2$ showed the inhibitable effect on p-PD induced apoptosis.

Furthermore, Oishi and Machida (1997) have found

that $MnCl_2$ inhibited neutrophil apoptosis. The mechanism of Mn^{2+} actions, however, is not clear from these experiments. One possibility is that manganese itself effectively scavenges ROS not only in culture medium, but also in the intracellular space such as nucleus, since Mn^{2+} has SOD-like activity (Archibald and Fridovich, 1982). In fact, ROS scavenging abilities of manganese have been indicated in the brain (Donaldson, 1987) and lactobacilli's spaces (Archibald and Fridovich, 1981).

Whether p-PD itself or oxidative degradation products of p-PD are responsible for the observed results needs further investigations. Coulter et al. (2006) showed inhibition of Bandrowski's base formation in the presence of glutathione in dendritic cells and further the non-enzymatic oxidative degradation of p-PD to different unstable intermediates was prevented by antioxidants like ascorbic acid (Moeller and al., 2008).

In conclusion, our findings show that p-PD induces apoptosis of P3 cells via generation of ROS. The induced apoptosis in P3 cells may be mediated by p-PD, a reactive p-PD intermediate during chemical conversion or by the related ROS itself. To confirm this hypothesis further work is needed to elucidate the cellular mechanism of p-PD induced apoptosis.

ACKNOWLEDGEMENTS

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