

Toxicity of paraphenylenediamine in human neutrophils: apoptosis and oxidative stress

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Abstract

The purpose of the present study was to evaluate the effect of paraphenylenediamine (PPD), a derivative of paranitroaniline widely used as an oxidisable hair dye, on human neutrophil viability and on ROS production in vitro. Neutrophils from healthy volunteers were incubated with three concentrations of PPD (1.25, 2.5 and 5 µg/ml). Apoptosis was evaluated by light microscopy and DNA gel electrophoresis. After 5 hours of incubation, PPD accelerated neutrophil apoptosis in a dose dependant manner. Treatment with 2.5µg/ml of PPD for 5 h significantly enhanced the activity of catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) and also markedly increased lipid peroxidation. These results suggest that high doses of PPD accelerate neutrophils apoptosis and induce an oxidative stress.

Key words: apoptosis; lipid peroxidation; neutrophils; oxidative stress; paraphenylenediamine (PPD).

Introduction

Paraphenylenediamine (PPD) is a synthetic compound that is widely used as hair dye (Corbett and Menkart, 1973). PPD studies have found that women using dye with a formulation that included PPD suffered acute sensitive responses such as headaches, dizziness and systemic anaphylaxis for the long time after using the product (Goldberg *et al.*, 1987).

In Morocco, intoxication with PPD is a major health problem. A reported series of 315 cases of PPD poisoning admitted to the medical resuscitation service in Ibn Roshd hospital between 1999 and 2004. In these series, 47% of the patients have died (Motaouakkil *et al.*, 2006).

Although these data suggest that PPD is immunogenic, the potential

effect of this arylamine on immune cells has not been fully elucidated and needs further investigations. The aims of the present work were to evaluate the effect of PPD on human cultured neutrophils and to investigate the implication of oxidative stress in the cytotoxicity of PPD.

Materials and methods

Isolation of human neutrophils

Human neutrophils were isolated from peripheral blood of healthy donors using a method of density centrifugation and dextran sedimentation. Briefly, human peripheral blood (10 ml) was layered on Ficoll-Hypaque (Eurobio, France) and centrifuged at 400g for 25 min. The supernatant and mononuclear

cells were carefully moved. The pellet was mixed with 4ml of Hanks buffer and 3 ml of 5 % dextran in 0.9 % NaCl solution. The mixture was incubated at 4°C for 1h. The supernatant was centrifuged at 300 g for 10 min. Residual erythrocytes in the polymorphonuclear leukocytes (PMN) pellet were lysed by hypotonic lysis. The cells were pelleted at 300g for 10 min, washed with Hanks buffer and resuspended in RPMI 1640 medium supplemented with 100 UI/ml penicillin, 100 µg/ml streptomycin and 10 % fetal calf serum. Neutrophils were counted using a Malassez hemocytometer slide. In all cases purity was greater than 97 %, with viability of neutrophils as assessed by trypan blue exclusion greater than 95 % immediately after purification.

Culture conditions

Neutrophils were cultured in RPMI 1640 medium supplemented with 100 UI/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum. Neutrophils were incubated with or without 1.25, 2.5 and 5 µg/ml of PPD for 1 to 24 hours at 37°C in a 5 % CO₂ atmosphere in 96 well plates at a concentration of 0.15×10^6 cell/ml. Freshly isolated neutrophils were used as a negative control, while neutrophils cultured for 24 h at 37°C in medium alone or cultured with 200nM of dexamethasone (Dex) for 2 to 24 h were used as positive control of apoptosis.

Morphological assessment of apoptosis

Cytospins slides were prepared using a Shandon cytospin 2 and stained with May-Grünwald-Giemsa (MGG). Cells were assessed for morphological changes characteristic of apoptosis with the use of x 40 objective. At least 300 cells per slide were counted.

DNA fragmentation assay

DNA was extracted from washed PMN. Briefly, 0.15×10^6 cells were lysed in 100 µl of 20 mM Tris buffer pH 7.5 containing 5 mM EDTA, 200 mM NaCl and 1% sodium dodecyl sulfate (SDS). 20 µl of 10 mg/ml proteinase K was added to the mixture which was then incubated at 55°C for 3h. DNA was extracted from the Supernatant with equal volume of phenol-chloroform-isoamylalcohol (25/24/1) and precipitated with absolute ethanol. The precipitate was rinsed with 70 % ethanol dried and suspended in 10mM Tris buffer pH 8 containing 1 mM EDTA. The DNA samples (0.25 µg of DNA per lane) were analyzed by gel electrophoresis (2 % agarose) and Ethidium bromide staining.

Evaluation of lipid peroxidation

Lipid peroxidation was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA). To 100 µl of supernatant, 900 of 15% trichloroacetic (TCA) and 0.375 % TBA on 0.25 M HCl were added. The mixture was incubated at 100°C for 15 min and kept in ice cold water for 10 min. The absorbance of the supernatant was read at 535 nm. The MDA levels were expressed as micromoles of MDA per minute per milligram protein (µmol/min/mg protein).

The catalase (CAT) activity

CAT activity was determined as follows. The assay mixture contained 50 µl supernatant and 950 µl of 50 mM phosphate buffer pH 7 and 7.5 mM H₂O₂. The specific activity is given as micromoles of consumed H₂O₂ per minute per milligram protein using molar extinction coefficient of 40 M⁻¹ cm⁻¹.

Glutathione reductase (GR) activity.

To estimate the GR activity, the reaction mixture consisted of 950 μ l of 50 mM phosphate buffer pH 7.4 containing 1 mM EDTA, 25 μ M oxidized glutathione, 1.6 μ M NADPH and 50 μ l supernatant in total volume of 1 ml. the disappearance of NADPH at 340 nm was recorded at 25°C. The enzyme activity was calculated as micromoles of NADPH oxidized per minute per milligram protein using molar extinction coefficient of 6220 $M^{-1} cm^{-1}$.

Superoxide dismutase (SOD) activity

SOD activity was measured as follows. The assay mixture in 1 ml cuvette contained 50 μ l supernatant and 950 μ l of 50mM phosphate buffer pH 7 containing 5 mM EDTA, 2.5 mM $MnCl_2$, 3.9 mM 2-mercaptoethanol and 0.27 mM NADH. The change in absorbance at 340 nm was followed for 1min. The enzyme activity was expressed as μ mol/min/mg protein using molar extinction coefficient of 6220 $M^{-1} cm^{-1}$.

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

Statistical analysis

All the values were expressed as the mean $\pm$ SEM. The statistical evaluation of the data was carried out by applying the student's t-test. Statistical significance was defined as $p < 0.05$.

Results**PPD induces human neutrophils death in a time and concentration dependant manner**

To determine the effect of PPD on human neutrophils viability, the cells

were cultured with 1.25, 2.5 and 5 μ g/ml of PPD for 1h to 24 h. After 2 h, an acceleration of neutrophils death was observed in cell cultured with PPD which was significant at 5 h (Figure 1). As assessed by trypan blue exclusion, at 5h, cell death was 27 % in control population compared with 36 %, 53 % and 67 % in cells treated respectively with 1.25, 2.5 and 5 μ g/ml of PPD ($p = 0.012$, $p = 0.011$ and $p = 0.01$, respectively) (Figure 1).

PPD induces human neutrophils apoptosis

To determine whether PPD induces apoptosis of human neutrophils, cells were incubated with 2.5 μ g/ml of PPD for 1 to 24 h and their morphologies were examined using MGG staining. As shown in figure 2D untreated neutrophils underwent spontaneous apoptosis after 24 h, showing typical apoptotic morphological changes such as chromatin condensation accompanied with loss of multilobular nuclear structure.

However, when incubated with 2.5 μ g/ml of PPD, the apoptotic process was markedly accelerated ($p < 0.05$) (Figure 2B). Apoptosis of neutrophils was further confirmed by a DNA fragmentation as shown in Figure 3.

The PPD effect on lipid peroxidation in human neutrophils

Neutrophils were treated with 2.5 μ g/ml of PPD for 1 to 24 h. Lipid peroxidation was assessed by spectrophotometry. PPD did not appear to alter the MDA levels in earlier hours of incubation. However, at 5 h, MDA level increases dramatically in treated neutrophils (209 μ mol/min/mg protein) compared with untreated cells (83 μ mol/min/mg protein) where the increase of MDA level was observed at

Figure 1. Time course of the viability of neutrophils after treatment with PPD. Cells were treated with 1.25, 2.5 and 5 $\mu\text{g/ml}$ of PPD for 1 to 24 h. Results are shown as mean $\pm$ SEM from three independent experiments.

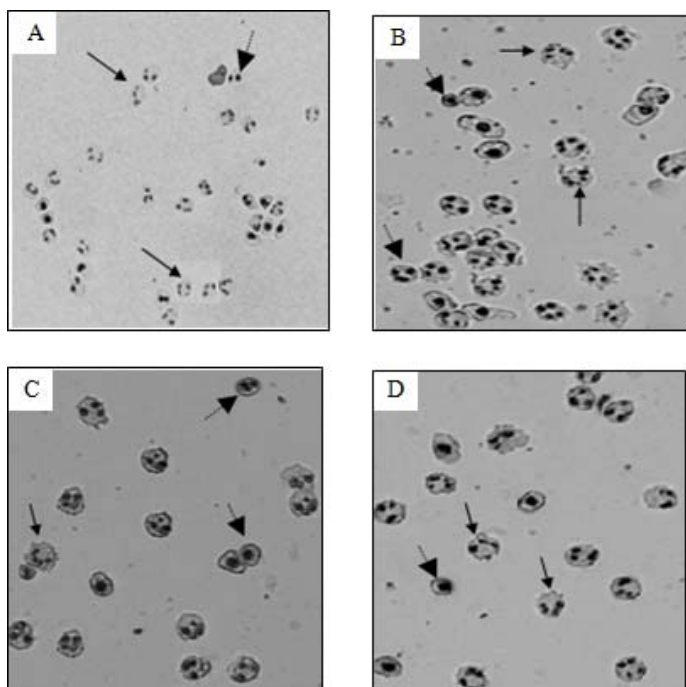
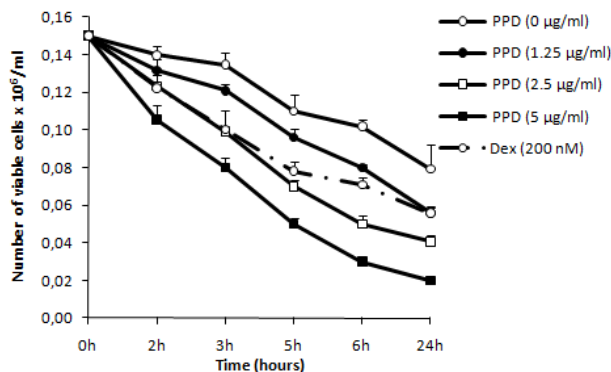


Figure 2. Light microscopy of cultured neutrophils. Cytospin slides preparations of neutrophils were stained with MGG and apoptotic cells (.....► arrows) were evaluated morphologically. The micrographs show neutrophils treated as follows: A) incubated in medium alone for 5h; B) treated with 2.5 $\mu\text{g/ml}$ of PPD for 5h; C) treated with 200nM of Dexamethasone for 5h and D) incubated in medium alone for 24h.

24 h (104 $\mu\text{mol/min/mg}$ protein) ($p < 0.05$) (Figure 4).

The effect of PPD in ROS scavenging enzymes

Enzyme activities of CAT, GR and SOD in human neutrophils treated with 2.5 $\mu\text{g/ml}$ of PPD for 1 to 24 h are shown in Figure 5A, 5B and 5C. The CAT, GR and SOD activities were significantly elevated by PPD at 5h of incubation compared with untreated neutrophils whereas these activities

were increased at 24h ($p = 0.043$) (Figure 5).

Discussion

The present study shows that PPD rapidly induced high levels of cell death in human cultured neutrophils. We found that this cell death was apoptotic. This finding was evidenced by characteristic light microscopic features of apoptosis (Savill *et al.*, 1989) and by DNA fragmentation.

Figure 3. Effect of PPD in DNA fragmentation in neutrophils. Cells were cultured with or without 2.5 $\mu\text{g/ml}$ of PPD for 1 to 24h. Lane 1 and 2: cells cultured in medium alone for 5h and 24h, lane 3 and 4: cells cultured with 2.5 $\mu\text{g/ml}$ of PPD for 5 and 24h, lane 5 and 6 cells cultured with 200 nM of Dexamethasone for 5h and 24h. The molecular size markers are indicated on the left, lane M.

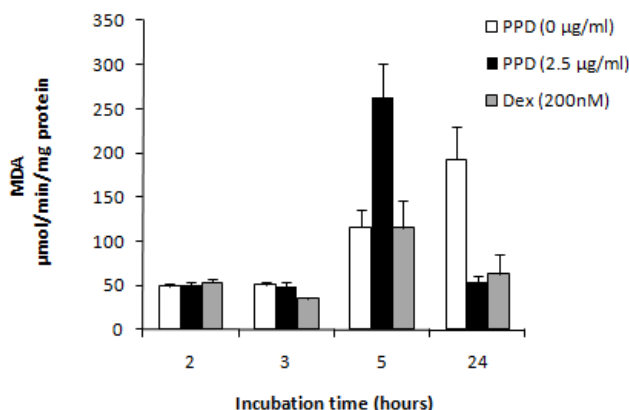
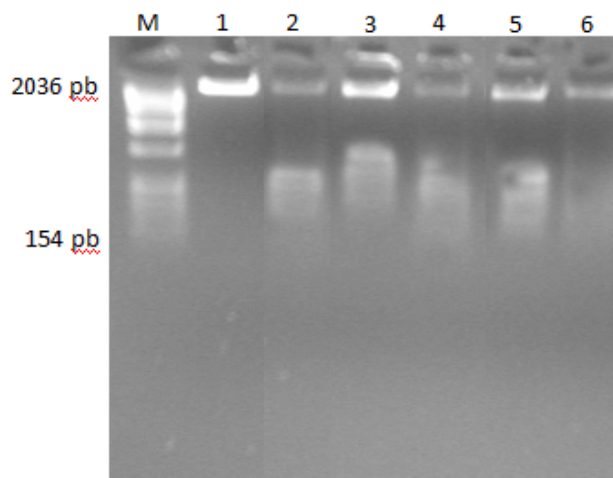


Figure 4. Lipid peroxidation in human neutrophils. Cells were treated with 2.5 $\mu\text{g/ml}$ of PPD for 2 to 24 h. Results are shown as mean $\pm$ SEM from three independent experiments.

Studies by Chen *et al.*, (2006) demonstrated that PPD increases the protein p53 expression and induces apoptosis of Mardin Darby canine Kidney cells.

Neutrophils apoptosis is classically regarded as a form of cell death that will promote resolution of inflammation (Haslett, 1997). Since apoptotic death is not proinflammatory, induction of death by apoptosis rather than necrosis could confer further advantage to intoxication with PPD, limiting further host responses. There is however, evidence of extensive tissue injury in the context of PPD poisoning (Yagi *et al.*, 1991; Motaouakkil *et al.*, 2006). This might arise because of proinflammatory effect of PPD itself

(e.g., generation of ROS) (Mathur *et al.*, 1990) or of its metabolites such as Bandrowski's base (Krasteva *et al.*, 1993).

The enhanced activity of CAT, GR and SOD enzymes observed in this work shows that defense mechanism is increased to counter the damaging effects of PPD, whereas the observed increased in MDA suggest that increased free radical formation is responsible for the damaging effects of PPD.

In the present study, PPD was found to induce lipid peroxidation as well as in the studies of Mathur *et al.*, (2005). Increased lipid peroxidation may support the concept that ROS may lead to the oxidative damage in PPD

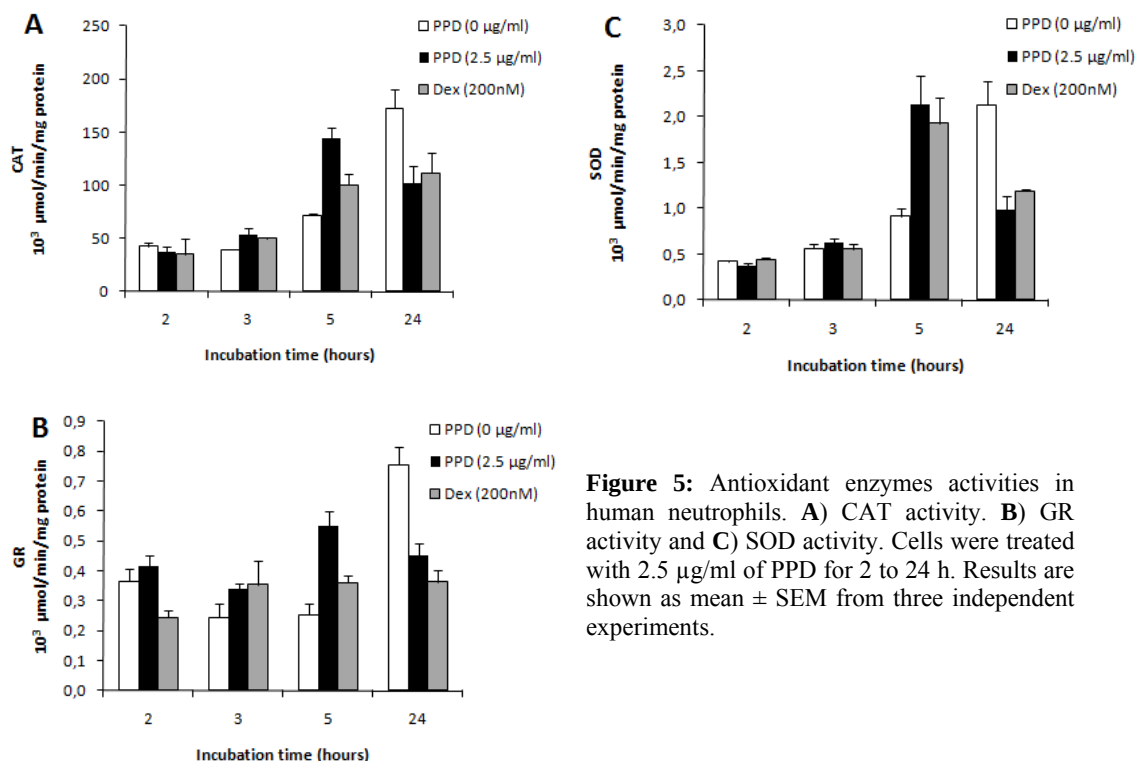


Figure 5: Antioxidant enzymes activities in human neutrophils. **A)** CAT activity. **B)** GR activity and **C)** SOD activity. Cells were treated with 2.5 μ g/ml of PPD for 2 to 24 h. Results are shown as mean $\pm$ SEM from three independent experiments.

treated neutrophils. In addition, it might be a relationship between this oxidative stress and cell death induced by PPD.

In conclusion, our findings suggest that PPD induces apoptosis of human cultured neutrophils via generation of ROS. It may be suggested that increased production of ROS, as reflected by higher level of MDA, may impair neutrophils membrane integrity and may lead to the alterations in neutrophils antioxidant defense system. Further work is needed to elucidate the cellular mechanism of PPD induced death and to determine in the PPD induced apoptosis in ROS dependent.

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