



Xenobiotica

the fate of foreign compounds in biological systems

ISSN: 0049-8254 (Print) 1366-5928 (Online) Journal homepage: <http://www.tandfonline.com/loi/ixen20>

The effects of Para-phenylenediamine (PPD) on the skin fibroblast cells

Enayatollah Seydi, Mohsen Fatahi, Parvaneh Naserzadeh & Jalal Pourahmad

To cite this article: Enayatollah Seydi, Mohsen Fatahi, Parvaneh Naserzadeh & Jalal Pourahmad (2018): The effects of Para-phenylenediamine (PPD) on the skin fibroblast cells, Xenobiotica, DOI: [10.1080/00498254.2018.1541264](https://doi.org/10.1080/00498254.2018.1541264)

To link to this article: <https://doi.org/10.1080/00498254.2018.1541264>



Accepted author version posted online: 25 Nov 2018.



Submit your article to this journal [↗](#)



View Crossmark data [↗](#)

The effects of Para-phenylenediamine (PPD) on the skin fibroblast cells

Running Title: The effects of PPD on the skin fibroblast cells

Enayatollah Seydi¹, Mohsen Fatahi², Parvaneh Naserzadeh³, Jalal Pourahmad^{2*}

¹ Department of Occupational Health and Safety Engineering, School of Health, Alborz University of Medical Sciences, Karaj, Iran.

² Department of Pharmacology and Toxicology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

³ Pharmaceutical Sciences Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Corresponding Authors:

* Jalal Pourahmad , E-mail: j.pourahmadjaktaji@utoronto.ca , P.O. Box :14155-6153, Tehran, Iran. Phone: +98(21)2255-8786 Fax: +98(21)8820-9620.

The effects of Para-phenylenediamine (PPD) on the skin fibroblast cells

Abstract:

1. Para-phenylenediamine (PPD) is the commonest and most well-known component of hair dyes. PPD is found in more than 1000 hair dye formulations and is the most frequently used permanent hair dye component in Europe, North America, and East Asia. PPD containing hair dyes have been associated with cancer and mutagenicity. Apart from that, PPD has potential toxicity which includes acute toxicity such as allergic contact dermatitis and subacute toxicity. 2. In this study, we examined the effects of the PPD composition on the skin-isolated fibroblast cells. Fibroblast cells were isolated from the skin and cell viability, reactive oxygen species (ROS) production, the collapse of mitochondrial membrane potential

(MMP), lipid peroxidation, damage to the lysosome and finally release of cytochrome *c* were examined following the exposure to various concentrations of PPD.

3. Our results showed that exposure to PPD increased reactive oxygen species (ROS) generation, lipid peroxidation (LPO), the collapse of mitochondrial membrane potential (MMP), and cytochrome *c* release. Our results suggest that PPD can induce damage to the lysosomal membrane.

4. These results showed that PPD composition has a selective toxicity on skin fibroblasts cell and mitochondria are considered one of the goals of its toxicity.

Keywords: Para-phenylenediamine; Fibroblast; Reactive Oxygen Species; Mitochondria; Lysosome.

Introduction:

The use of hair dye has roots in the past times and in Egypt. Furthermore, the hair dye has been used to enhance the attractiveness, health, femininity, and beauty (Al-Shaikh and others 2018). One of the most important components of the hair dye is p-phenylenediamine (PPD), which is a derivative of para-nitroaniline (Abd-ElZaher and others 2012; Al-Shaikh and others 2018; Blömeke and others 2009; Chye and others 2014; Hooff and others 2011; Singla and others 2005). Research has shown that about 30% of American women use PPD containing hair dyes (Abd-ElZaher and others 2012). Documents show that the toxicological properties of aromatic amines such as PPD are very widespread. One of these important properties is the induction of apoptosis through increased reactive oxygen species (ROS) (Chye and others 2014; Nohynek and others 2015). The most important route of exposing humans to PPD through the skin and inhalation during the dyeing process (Abd-ElZaher and others 2012). PPD can cause skin irritation, contact dermatitis, rhabdomyolysis, renal impairment and severe edema. These complications indicate that this compound is very toxic (Al-Shaikh and others 2018; Singla and others 2005).

Previous studies have demonstrated that PPD induced apoptosis in the Mardin-Darby canine kidney (MDCK) cells and Chang Liver Cells through the mitochondrial pathway (Chen and others 2006; Chye and others 2014). Furthermore, reports indicate that the PPD induce apoptosis signaling through the increase of ROS level (Chen and others 2010; Chye and others 2014). Despite its widespread use, it has the ability to induce oxidative stress in different cells (Brans and others 2005; Chen and others 2010; PICAUDO and others 1996; Rioux and Castonguay 2000). Observations show that changes in mitochondrial function lead

to the generation of harmful ROS (Demers-Lamarche and others 2016). Additionally, mitochondria are considered as an important source of ROS generation (often through Complexes I and III, and start induction of apoptosis signaling (Dunn and others 2015; Murphy 2013; Zorov and others 2014). Also, it is proven that impairment of mitochondrial function leads to disruption of the lysosomal function and structure (Demers-Lamarche and others 2016). Both mitochondria and lysosomes are sensitive to changes in the level of ROS, and these two intracellular organelles play a role in inducing apoptosis signaling (Ghosh and others 2011; Zhang and others 2017; Zhao and others 2003).

In this study, we studied the mechanism of PPD toxicity on mitochondria and lysosomes of fibroblastic cells.

Material and methods

Chemicals

All chemicals (such as, PPD, Dichloro-dihydro-fluorescein diacetate (DCFH-DA), Rhodamine 123 (Rh 123), and Acridine Orange) were purchased from Sigma-Aldrich (St. Louis, MO) in the best pharmaceutical grade.

Animals

Male Wistar rats (200-250 g) were purchased from Pasteur Institute and placed in an air-conditioned room with the controlled temperature of 25 ± 2 °C and maintained on a 12:12 hr light/dark cycle, humidity of 50-60 % with free access to water and food. All experimental methods were conducted according to the ethical standards and protocols approved by the Animal Experimentation Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Cell isolation

At first, all the necessary equipment were sterilized via autoclaving (under conditions 121 °C and high pressure). Briefly, the small pieces (using sterile tools) of Rat skin was removed and then moved to petri dishes including Phosphate Buffered Saline (PBS) on ice. In the next step, animal skin pieces were fragmented into smaller pieces, and then transferred to Falcon tube together with the PBS. Samples were centrifuged twice (First, 168 x g for 2 min and second, 203 x g for 2 min). In the first step, the PBS was removed and the trypsin 0.1% was added, and in the second step, the added trypsin was removed. The trypsin was neutralized using culture medium including FBS. After centrifugation (203 x g for 2 min), the medium

containing trypsin was removed from the tubes and once again added to the cells and the cells were transferred to culture medium (Chashmpoosh and others 2016).

Cell viability assay

Cell viability was assayed by MTT assay. Fibroblast cell were placed in 96-well plates. The cells were treated with 5–200 μM concentration of PPD for 24 hr (cells were maintained in DMEM, supplemented with 10% FBS and antibiotics (50 U/ml of penicillin and 50 $\mu\text{g/ml}$ streptomycin)). In the next step, after incubation for a specified time at 37°C in a humidified incubator, MTT (5 mg/ml in DMEM) was added to each well and incubated for 4 hour after which the plate was centrifuged at 1800 x g for 5 min. at 4°C. The buffer solution (containing PPD) was then removed from the wells by aspiration. After careful removal of the medium, 100 μl of buffered dimethyl sulfoxide (DMSO) (to dissolve the resulting formazan crystals) was added to each well, and plates were shaken. The absorbance was measured at 570 nm using ELISA reader (Tecan, Rainbow Thermo, Austria).

Evaluation of reactive oxygen species (ROS) level

Fibroblast cells (1×10^6 cells) was treated with PPD (50,100 and 150 μM) for 6, 12 and 18 hours. After treatment with PPD (50,100 and 150 μM), fibroblast cells was washed with PBS and medium containing PPD (50,100 and 150 μM) was removed. In the next step, cells were incubated with Dichloro-dihydro-fluorescein diacetate (DCFH-DA) at the concentration of 1.6 μM for 30 minutes. DCFH-DA penetrates fibroblast and becomes hydrolyzed to non-fluorescent dichlorofluorescein (DCFH). The latter then reacts with ROS to form the highly fluorescent dichlorofluorescein (DCF), which effluxes the cell. In the following, the fluorescence intensity of DCF at 6, 12 and 18 hours after the exposure was assayed using a Shimadzu RF5000U fluorescence spectrophotometer ($\text{EX}\lambda = 500 \text{ nm}$ and $\text{EM}\lambda = 520 \text{ nm}$) (Arast and others 2017; Shen and others 1996).

Evaluation of mitochondrial membrane (MMP) collapse

In this study, rhodamine 123 (Rh 123) as a fluorescent dye was used for the evaluation of MMP via PPD (50, 100 and 150 μM) in the fibroblast cells (10^6 cells). Finally, the fluorescence intensity of Rh 123 at 6, 12 and 18 hours after the exposure was assayed using a Shimadzu RF5000U fluorescence spectrophotometer ($\text{EX}\lambda = 490 \text{ nm}$ and $\text{EM}\lambda = 520 \text{ nm}$). The capacity of mitochondria to take up Rh 123 was measured as the difference between treated and control fibroblast cells in Rh 123 fluorescence (at concentration of 1.5 μM). In fact, an increase in fluorescence intensity due to redistribution of Rh 123 represents a

collapse in the MMP (Andersson and others 1987; Fakharnia and others 2017; Kwong and Molkentin 2015; Vaseva and others 2012).

Evaluation of lipid peroxidation (LPO) content

In this study, fibroblast lipid peroxidation (LPO) level at time interval 6, 12 and 18 hours was assayed via measuring the level of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides using a Beckman DU-7 spectrophotometer (at an absorbance of 532 nm) after treating 1.0 ml aliquots of fibroblast suspension (10^6 cells/ml) with trichloroacetic acid [70% (w/v)] and oiling the suspension with thiobarbituric acid [0.8% (w/v)]. Malondialdehyde (MDA) content was expressed as $\mu\text{g}/\text{mg}$ protein (Gasparovic and others 2013; Zhang and others 2008).

Cytochrome c release assay

Briefly, the cytochrome c release was evaluated through the Quantikine Rat/Mouse cytochrome c Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn.). Cytochrome c specific antibodies have been pre-coated onto 96-well plates. Then, 50 μl of control and test samples are added to the wells along with 75 μl of biotinylated cytochrome c detection antibody and the microplate is then incubated at room temperature. Finally, the optical density of each well was determined through the aforementioned micro-plate spectrophotometer set to 450 nm (Faizi and others 2016; Kamada and others 1998).

Lysosomal membrane integrity assay

The level of damage to lysosomal membrane in fibroblast cells exposed to PPD (50, 100 and 150 μM) was evaluated through the redistribution of the acridine orange at concentration of 5 μM (as a fluorescent dye). 6, 12 and 18 after exposure, aliquots of the cell suspension (0.5 ml) stained with acridine orange (5 μM) were separated using centrifugation at $168 \times g$ for 1 min. The cell pellet was then re-suspended in 2 ml of fresh incubation medium. This washing process was carried out twice to remove the fluorescent dye from the media. Finally, acridine orange redistribution in the fibroblast cell suspension was assayed using a Shimadzu RF5000U fluorescence spectrophotometer ($\text{EX}\lambda = 495 \text{ nm}$ and $\text{EM}\lambda = 530 \text{ nm}$) (Andersson and others 1987; Brunk and others 1995).

Lactate dehydrogenase (LDH) release assay

LDH release is used to evaluate the toxicity and survival of the cell. LDH activity was measured by specific commercial kit from Sigma-Aldrich Co. LDH released during the experiment was expressed as a percentage of total cellular LDH (Everse and others 1975; HO and others 2001; Wallace and others 2014).

Statistical analysis

Data are reported as mean $\pm$ SD. All statistical analyses were performed using Graph Pad Prism, version 6. The assays were performed 3 times. Statistical significance was determined using the one-way ANOVA test, followed by the post hoc Tukey test, and the two-way ANOVA test, followed by the post hoc Bonferroni test. Statistical significance was set at $P < 0.05$. Furthermore, the one-way ANOVA test was used as a specific statistical analysis for the determinations of cell viability, and cytochrome c release. The two-way ANOVA test was used for the determinations of mitochondrial ROS level, collapse of MMP, lipid peroxidation level and lysosomal membrane damage.

Results:

Cell viability:

The effect of PPD at concentrations 0-200 μ M on the viability of fibroblast cells isolated from the skin was examined for 24 hr. As shown in Figure 1, PPD at all concentrations except 5 μ M were able to significantly ($p < 0.05$) decrease cell viability in a dose dependent pattern. The results of the cytotoxicity showed that the IC_{50} concentration of this compound is 100 μ M. Subsequently, we used concentrations of 50 ($1/2 IC_{50}$), 100 (IC_{50}) and 150 ($1/5 IC_{50}$) for subsequent experiments.

Evaluation of ROS level:

In Figure 2, our results showed that PPD at all applied concentrations (50, 100 and 150 μ M) and at all times (6, 12 and 18 hours) have been able to significantly increase the level of ROS in the fibroblast cells. DCFH-DA penetrates fibroblast and becomes hydrolyzed to non-fluorescent dichlorofluorescein (DCFH). The latter then reacts with ROS to form the highly fluorescent dichlorofluorescein (DCF), which effluxes the cell. Finally, the measurement of fluorescence intensity of DCF indicates the level of ROS. Also, cyclosporine A (2 μ M) decrease the generation of ROS from PPD (100 μ M) (Figure 2).

Evaluation of MMP collapse:

The results shown in Figure 3 indicate that PPD in concentrations of 50, 100 and 150 μM significantly ($p < 0.05$) decreased the MMP in a time- and concentration-dependent manner in the rat fibroblast compared with control group. MMP collapse and fluorescence intensity (Rh123) reported in our experiments are inversely proportional. Our results showed that cyclosporine A (2 μM) decrease the collapse of MMP from PPD (100 μM) (Figure 3).

Lipid peroxidation (LPO) content assay

The effect of PPD at all applied concentration (50, 100 and 150 μM) on the LPO content of fibroblast cells was examined for 6, 12 and 18 hours. As shown in Figure 4, PPD significantly ($p < 0.05$) increase the malondialdehyde (MDA) content as an indicator of LPO (in a dose and time-dependent manner) in fibroblast cells. Also, cyclosporine A at concentration of 2 μM decrease the content of LPO caused by PPD (100 μM) (Figure 4).

Cytochrome c release assay:

As shown in Figure 5, PPD at the concentrations of 50, 100 and 150 μM raised cytochrome c release from rat fibroblast cells mitochondria. Furthermore, our results showed that Butylated hydroxytoluene (BHT; as an antioxidant) and cyclosporine A (as a pore sealing agent) inhibit the release of cytochrome c from PPD (100 μM) (Figure 5).

Lysosomal membrane damage assay

When fibroblast lysosomes were loaded with acridine orange (a lysosomotropic agent), a significant release of acridine orange ensued into the cytosolic fraction within 6, 12 and 18 hours of incubation with PPD (50, 100 and 150 μM) showing damage to the lysosomal membrane (Figure 6). The redistribution of acridine orange from lysosomes to cytosol indicates damage to lysosomes.

LDH release assay:

The effect of PPD (0-200 μM) on the LDH release was examined for 24 hr. LDH released into the medium was used as measures of cell death. As shown in Figure 7, PPD at all concentrations were able to significantly ($p < 0.05$) increase the release of LDH compare to the control group.

Discussion:

In recent years, the use of PPD has increased. Therefore, assessing the effects and consequences of using it on human health is very necessary (Goyal and others 2015). In this study, we examined PPD toxicity parameters, including oxidative stress, mitochondrial and lysosomal damage, and apoptosis in the fibroblast cells. Cytotoxicity in fibroblast cells was measured by MTT assay which shows a significant reduction in fibroblast cells viability treated with PPD (50, 100 and 150 μ M) compared to control cells.

Furthermore, DCFH-DA assay displays the level of intracellular ROS in fibroblast cells. Our results showed that PPD in a dose and time-dependent manner increased the generation of ROS in the fibroblastic cell. Our results are in agreement with the previous study that showed PPD induce ROS generation in a dose and time-dependent manner in Mardin–Darby canine kidney cells and Murine myeloma cells (Chen and others 2010; Elyoussoufi and others 2012). It is well known that ROS are considered to cause damage to cells through different mechanisms. Furthermore, oxidants including ROS (As the initial signals) and lipid hydroperoxides play a role in the induction of apoptosis through the oxidative stress process (Elyoussoufi and others 2012).

In our study, LPO was found to be raised through PPD. Our result is in agreement with the previous study (Elyoussoufi and others 2012). The documentation shows that ROS increase the content of LPO. Furthermore, it has been shown that PPD raised LPO to support the significance that ROS are involved in PPD induced apoptosis signaling (Elyoussoufi and others 2012). It has been shown that the mitochondrial pathway leads to the ROS attack of phospholipids on the membrane and decline in the MMP. Eventually, it leads to the activation of apoptotic factors and apoptosis signaling (Goyal and others 2015; Zhou and others 2009). Using an Rh 123 reagent, we showed that the PPD in a concentration and time-dependent manner leads to a decline in the MMP of the fibroblast cells. This result is in agreement with previous studies (Goyal and others 2015).

In our study, we found the release of cytochrome *c* into the cytosol in fibroblast cells treated to PPD. The level of cytochrome *c* was increased in fibroblast cells after the treatment with PPD. These results suggest that PPD (100 μ M) affects mitochondrial permeability transition (MPT) pores through oxidative stress leading to raised cytochrome *c* release. The MPT is defined as the abrupt rise in the permeability of the inner mitochondrial membrane (IMM) to solutes material (1500 Da), mitochondrial swelling, and the collapse of MMP (Juhaszova and others 2008). Furthermore, the opening of the MPT pores initiates the start of the MPT that is an element important in apoptosis signaling (Kim and others 2003). Studies have shown that cytochrome *c* plays a vital role in beginning the process of apoptosis. The release of this

protein from mitochondria to cytosol activates caspase cascade. Caspase 3 is one of the most important caspases that can induce apoptosis. Thus, cytochrome c release can induce apoptosis (Faizi and others 2016; Ko and others 2005).

In addition, our results showed that the PPD caused instability and damage to the lysosomal membrane in fibroblast cells. Studies have shown that free radicals (especially H_2O_2) have the potential to cross the lysosomal lipid membrane and can produce radical hydroxyl ($OH\bullet$); according to Haber- Weiss reaction. Furthermore, lysosomal is involved in the apoptosis process (Pourahmad and others 2009; Pourahmad and others 2011). In addition, reports show that lysosomes play an important role in the induction of apoptosis with the release of protease enzyme, such as cathepsins. The lysosomal pathway and mitochondrial pathway are connected together through Bcl₂ family proteins (Cirman and others 2003; Goyal and others 2015; Zhang and others 2009).

In conclusion, all the results of this study indicate that both mitochondria and lysosomes are targeted by PPD. Furthermore, PPD with changes in mitochondria and instability in lysosomes can induce apoptosis signaling.

Conflict of interest

The authors declare that there is no conflict of interest.

References:

- Abd-ElZaher MA, Fawzy IA, Ahmed HM, Abd-Allah AM, Gayyed MF. (2012). Some toxicological health hazards associated with subchronic dermal exposure to paraphenylenediamine (PPD): An experimental study. *Egyptian Journal of Forensic Sciences*, 2, 105-11.
- Al-Shaikh TM, Alkaseb KS, Mudawi MM, Yassin AY, Habeballa RS, Alzain SD. (2018). Toxicological studies on commercial hair dye brand commonly used in Saudi Arabia: histological study of the liver, kidney, heart, and testis. *The Journal of Basic and Applied Zoology*, 79, 1-10.
- Andersson B, Aw T, Jones DP. (1987). Mitochondrial transmembrane potential and pH gradient during anoxia. *American Journal of Physiology-Cell Physiology*, 252, C349-C55.
- Arast Y, Razi NS, Seydi E, Naserzadeh P, Nazemi M, Pourahmad J. (2017). Selective Toxicity of Non Polar Bioactive Compounds of Persian Gulf Sea Squirt *Phallusia Nigra* on Skin Mitochondria Isolated from Rat Model of Melanoma. *Asian Pacific journal of cancer prevention: APJCP*, 18, 811.
- Blömeke B, Brans R, Coenraads PJ, Dickel H, Bruckner T, Hein D, Heesen M, Merk HF, Kawakubo Y. (2009). Para-phenylenediamine and allergic sensitization: risk modification by N-acetyltransferase 1 and 2 genotypes. *British Journal of Dermatology*, 161, 1130-5.
- Brans R, Dickel H, Bruckner T, Coenraads P-J, Heesen M, Merk HF, Blömeke B. (2005). MnSOD polymorphisms in sensitized patients with delayed-type hypersensitivity reactions to the chemical allergen para-phenylene diamine: a case-control study. *Toxicology*, 212, 148-54.
- Brunk U, Zhang H, Roberg K, Öllinger K. (1995). Lethal hydrogen peroxide toxicity involves lysosomal iron-catalyzed reactions with membrane damage. *Redox Report*, 1, 267-77.
- Chashmpoosh M, Mohammadi A, Abdovis N, Ghasempoor H, Kheirollah A. (2016). Isolation and culture of mouse newborn skin fibroblast cells in a simple and inexpensive method.
- Chen S-C, Chen C-H, Chern C-L, Hsu L-S, Huang Y-C, Chung K-T, Chye S-M. (2006). p-Phenylenediamine induces p53-mediated apoptosis in Mardin-Darby canine kidney cells. *Toxicology in vitro*, 20, 801-7.
- Chen SC, Chen CH, Tioh YL, Zhong PY, Lin YS, Chye SM. (2010). Para-phenylenediamine induced DNA damage and apoptosis through oxidative stress and enhanced caspase-8 and -9 activities in Mardin-Darby canine kidney cells. *Toxicology in Vitro*, 24, 1197-202.
- Chye SM, Tiong YL, Yip WK, Koh RY, Len YW, Seow HF, Ng KY, Ranjit DA, Chen SC. (2014). Apoptosis induced by para-phenylenediamine involves formation of ROS and activation of p38 and JNK in Chang liver cells. *Environmental toxicology*, 29, 981-90.
- Cirman T, Oresic K, Mazovec GD, Turk V, Reed JC, Myers RM, Salvesen GS, Turk B. (2003). Selective disruption of lysosomes in HeLa cells triggers apoptosis, mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. *Journal of Biological Chemistry*.
- Demers-Lamarche J, Guillebaud G, Tlili M, Todkar K, Bélanger N, Grondin M, Nguyen AP, Michel J, Germain M. (2016). Loss of mitochondrial function impairs lysosomes. *Journal of Biological Chemistry, jbc*. M115. 695825.
- Dunn JD, Alvarez LA, Zhang X, Soldati T. (2015). Reactive oxygen species and mitochondria: a nexus of cellular homeostasis. *Redox biology*, 6, 472-85.
- Elyoussoufi Z, Habti N, Motaouakkil S, Cadi R. (2012). Paraphenylenediamine induces apoptosis of Murine myeloma cells in a reactive oxygen species dependant mechanism. *Journal of Toxicology and Environmental Health Sciences*, 4, 76-84.
- Everse J, Reich RM, Kaplan NO, Finn WD. (1975). New instrument for rapid determination of activities of lactate dehydrogenase isoenzymes. *Clinical chemistry*, 21, 1277-81.
- Faizi M, Salimi A, Seydi E, Naserzadeh P, Kouhnavard M, Rahimi A, Pourahmad J. (2016). Toxicity of cuprizone a Cu²⁺ chelating agent on isolated mouse brain mitochondria: a justification for demyelination and subsequent behavioral dysfunction. *Toxicology mechanisms and methods*, 26, 276-83.
- Fakharnia F, Khodagholi F, Dargahi L, Ahmadiani A. (2017). Prevention of cyclophilin D-mediated mPTP opening using cyclosporine-A alleviates the elevation of necroptosis, autophagy and apoptosis-related markers following global cerebral ischemia-reperfusion. *Journal of Molecular Neuroscience*, 61, 52-60.

- Gasparovic AC, Jaganjac M, Mihaljevic B, Sunjic SB, Zarkovic N, (2013). Assays for the measurement of lipid peroxidation. *Cell Senescence*: Springer. 283-96.
- Ghosh M, Carlsson F, Laskar A, Yuan X-M, Li W. (2011). Lysosomal membrane permeabilization causes oxidative stress and ferritin induction in macrophages. *FEBS letters*, 585, 623-9.
- Goyal S, Amar SK, Dubey D, Pal MK, Singh J, Verma A, Kushwaha HN, Ray RS. (2015). Involvement of cathepsin B in mitochondrial apoptosis by p-phenylenediamine under ambient UV radiation. *Journal of hazardous materials*, 300, 415-25.
- HO MC, LO AC, KURIHARA H, YU AC, CHUNG SS, CHUNG SK. (2001). Endothelin-1 protects astrocytes from hypoxic/ischemic injury. *The FASEB Journal*, 15, 618-26.
- Hooff GP, van Huizen NA, Meesters RJ, Zijlstra EE, Abdelraheem M, Abdelraheem W, Hamdouk M, Lindemans J, Luidier TM. (2011). Analytical investigations of toxic p-phenylenediamine (PPD) levels in clinical urine samples with special focus on MALDI-MS/MS. *PLoS One*, 6, e22191.
- Juhaszova M, Wang S, Zorov DB, Bradley Nuss H, Gleichmann M, Mattson MP, Sollott SJ. (2008). The identity and regulation of the mitochondrial permeability transition pore. *Annals of the New York Academy of Sciences*, 1123, 197-212.
- Kamada S, Kusano H, Fujita H, Ohtsu M, Koya RC, Kuzumaki N, Tsujimoto Y. (1998). A cloning method for caspase substrates that uses the yeast two-hybrid system: cloning of the antiapoptotic gene gelsolin. *Proceedings of the National Academy of Sciences*, 95, 8532-7.
- Kim J-S, He L, Lemasters JJ. (2003). Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. *Biochemical and biophysical research communications*, 304, 463-70.
- Ko CH, Shen S-C, Hsu C-S, Chen Y-C. (2005). Mitochondrial-dependent, reactive oxygen species-independent apoptosis by myricetin: roles of protein kinase C, cytochrome c, and caspase cascade. *Biochemical pharmacology*, 69, 913-27.
- Kwong JQ, Molkentin JD. (2015). Physiological and pathological roles of the mitochondrial permeability transition pore in the heart. *Cell metabolism*, 21, 206-14.
- Murphy MP. (2013). Mitochondrial dysfunction indirectly elevates ROS production by the endoplasmic reticulum. *Cell metabolism*, 18, 145-6.
- Nohynek GJ, Skare JA, Meuling WJ, Wehmeyer KR, de Bie ATH, Vaes WH, Dufour EK, Fautz R, Steiling W, Bramante M. (2015). Human systemic exposure to [14C]-paraphenylenediamine-containing oxidative hair dyes: Absorption, kinetics, metabolism, excretion and safety assessment. *Food and Chemical Toxicology*, 81, 71-80.
- PICAKDO M, Zompetta C, CRANDINETTI M, AMELIO F, Santucci B, Faggioni A, Passi S. (1996). Paraphenylene diamine, a contact allergen, induces oxidative stress in normal human keratinocytes in culture. *British Journal of Dermatology*, 134, 681-5.
- Pourahmad J, Amirmostofian M, Kobarfard F, Shahraki J. (2009). Biological reactive intermediates that mediate dacarbazine cytotoxicity. *Cancer chemotherapy and pharmacology*, 65, 89-96.
- Pourahmad J, Hosseini M-J, Bakan S, Ghazi-Khansari M. (2011). Hepatoprotective activity of angiotensin-converting enzyme (ACE) inhibitors, captopril and enalapril, against paraquat toxicity. *Pesticide biochemistry and physiology*, 99, 105-10.
- Rioux N, Castonguay A. (2000). The induction of cyclooxygenase-1 by a tobacco carcinogen in U937 human macrophages is correlated to the activation of NF- κ B. *Carcinogenesis*, 21, 1745-51.
- Shen H-M, Shi C-Y, Shen Y, Ong C-N. (1996). Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1. *Free Radical Biology and Medicine*, 21, 139-46.
- Singla S, Miglani S, Lal A, Gupta P, Agarwal A. (2005). Para-phenylenediamine (PPD) poisoning. *Journal of the Indian Academy of Clinical Medicine*, 6, 236-8.
- Vaseva AV, Marchenko ND, Ji K, Tsirka SE, Holzmans S, Moll UM. (2012). p53 opens the mitochondrial permeability transition pore to trigger necrosis. *Cell*, 149, 1536-48.
- Wallace CJ, Medina SH, ElSayed ME. (2014). Effect of rhamnolipids on permeability across Caco-2 cell monolayers. *Pharmaceutical research*, 31, 887-94.
- Zhang C, Lei J-l, Yu P, Xia Y-z, Zhang H, Yang L, Kong L-y. (2017). Walsuronoid b induces mitochondrial and lysosomal dysfunction leading to apoptotic rather than autophagic cell death via ROS/p53 signaling pathways in liver cancer. *Biochemical pharmacology*, 142, 71-86.

- Zhang F, Xu Z, Gao J, Xu B, Deng Y. (2008). In vitro effect of manganese chloride exposure on energy metabolism and oxidative damage of mitochondria isolated from rat brain. *Environmental toxicology and pharmacology*, 26, 232-6.
- Zhang H, Zhong C, Shi L, Guo Y, Fan Z. (2009). Granulysin induces cathepsin B release from lysosomes of target tumor cells to attack mitochondria through processing of bid leading to Necroptosis. *The Journal of Immunology*, 182, 6993-7000.
- Zhao M, Antunes F, Eaton JW, Brunk UT. (2003). Lysosomal enzymes promote mitochondrial oxidant production, cytochrome c release and apoptosis. *Eur J Biochem*, 270, 3778-86.
- Zhou Y-j, Zhang S-p, Liu C-w, Cai Y-q. (2009). The protection of selenium on ROS mediated-apoptosis by mitochondria dysfunction in cadmium-induced LLC-PK1 cells. *Toxicology in Vitro*, 23, 288-94.
- Zorov DB, Juhaszova M, Sollott SJ. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiological reviews*, 94, 909-50.

Accepted Manuscript

Figure Legends:

Figure 1: Effect of PPD (50, 100 and 150 μM) on fibroblast cells viability for 24 hr. All results were showed as the mean $\pm$ S.D (n=3). *** p < 0.001: significantly different from the vehicle-only group (PPD concentration = “0”).

Figure 1:

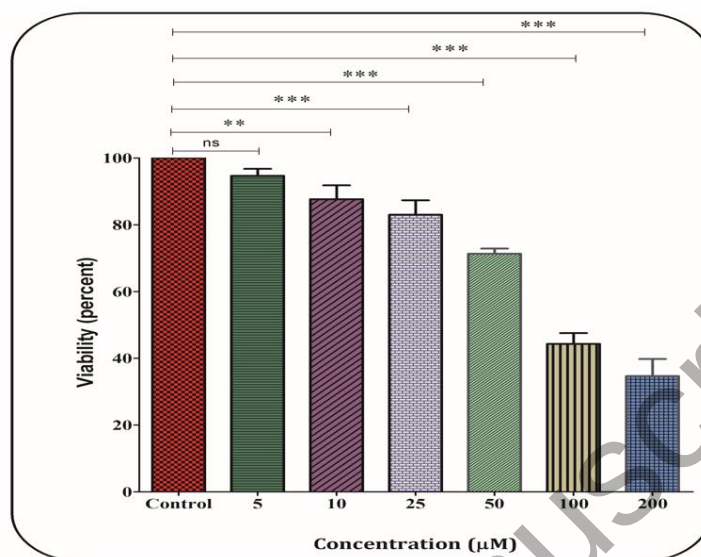


Figure 2: Effect of PPD (50, 100 and 150 μM) on fibroblast cells ROS generation for 6, 12 and 18 hr. All results were showed as the mean $\pm$ S.D (n=3). * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$: significantly different from the vehicle-only group (PPD concentration = “0”). #### $p < 0.0001$: significantly different between 100 μM and Cs.A (2 μM) + 100 μM .

Figure 2:

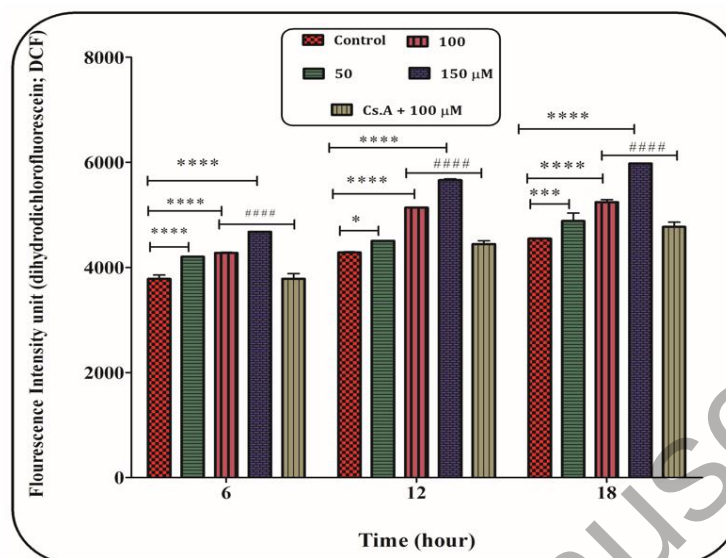


Figure 3: Effect of PPD (50, 100 and 150 μM) on fibroblast cells MMP collapse for 6, 12 and 18 hr. All results were showed as the mean $\pm$ S.D (n=3). *** $p < 0.0001$: significantly different from the vehicle-only group (PPD concentration = “0”). #### $p < 0.0001$: significantly different between 100 μM and Cs.A (2 μM) + 100 μM .

Figure 3:

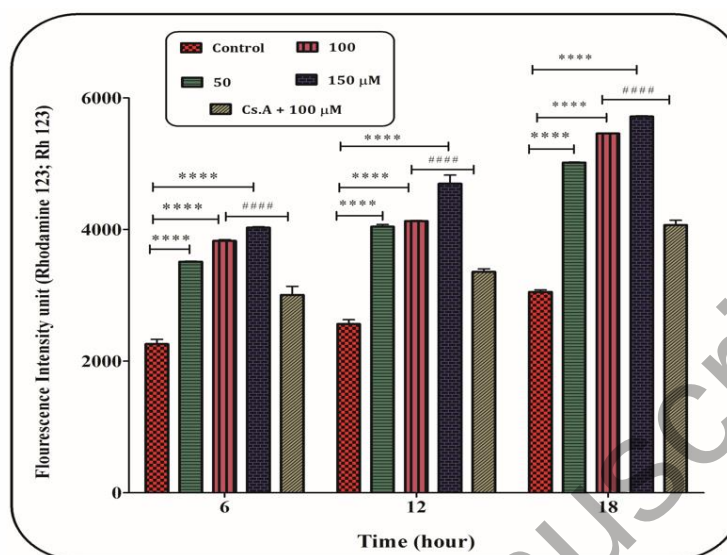


Figure 4: Effect of PPD (50, 100 and 150 μ M) on fibroblast cells lipid peroxidation for 6, 12 and 18 hr. All results were showed as the mean $\pm$ S.D (n=3). * $p < 0.05$ and **** $p < 0.0001$: significantly different from the vehicle-only group (PPD concentration = “0”). ### $p < 0.001$ and #### $p < 0.0001$: significantly different between 100 μ M and Cs.A (2 μ M) + 100 μ M.

Figure 4:

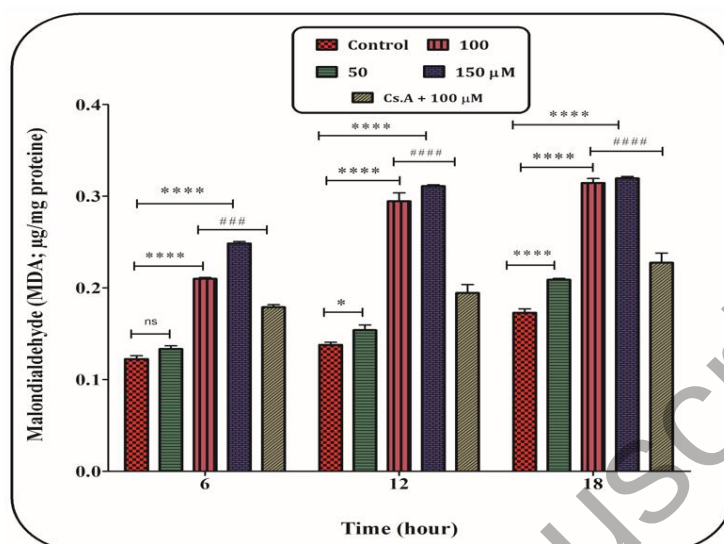


Figure 5: Effect of PPD (50, 100 and 150 μ M) on fibroblast cells cytochrome c release using Rat/Mouse Cytochrome c ELISA kit. All results were showed as the mean $\pm$ S.D (n=3). * $p < 0.05$ and *** $p < 0.001$: significantly different from the vehicle-only group (PPD concentration = “0”). ### $p < 0.001$ significant difference in comparison with 100 μ M PPD.

Figure 5:

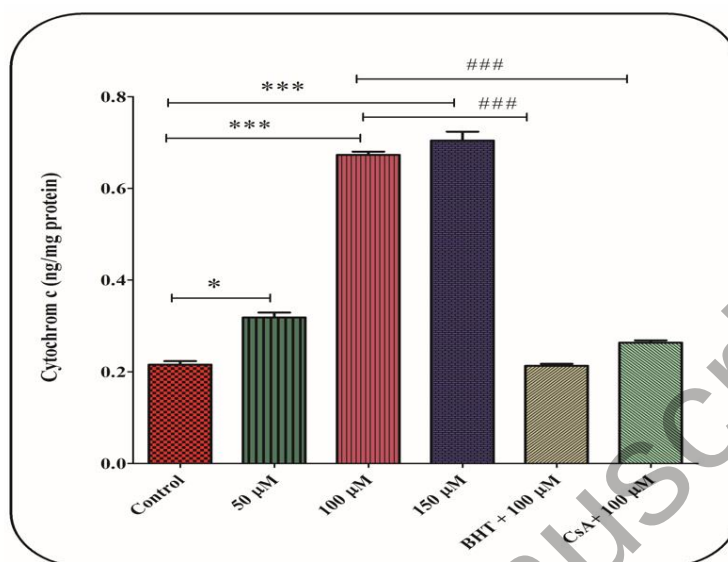


Figure 6: Effect of PPD (50, 100 and 150 μ M) on lysosomal membrane damage for 6, 12 and 18 hr. All results were showed as the mean $\pm$ S.D (n=3). *** $p < 0.001$ and **** $p < 0.0001$: significantly different from the vehicle-only group (PPD concentration = “0”).

Figure 6:

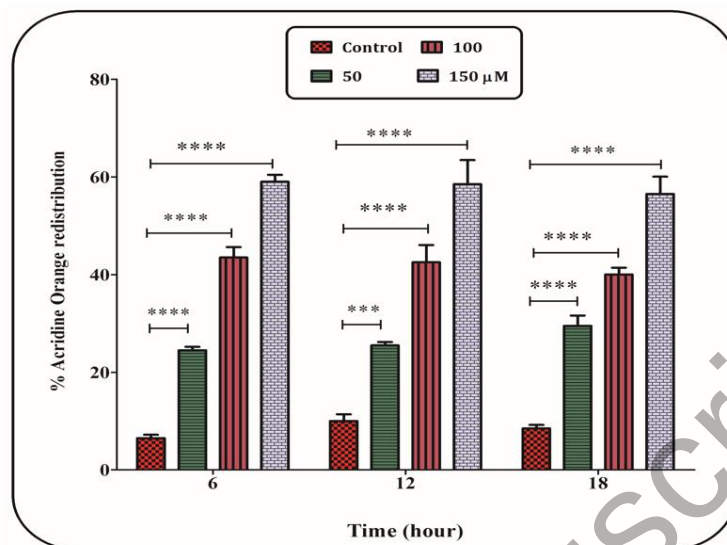


Figure 7: Effect of PPD (0-200 μ M) on LDH release for 24 hr. All results were showed as the mean $\pm$ S.D (n=3). * $p < 0.05$ and *** $p < 0.001$: significantly different from the vehicle-only group (PPD concentration = “0”).

Figure 7:

