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Para-tertiary butyl catechol induces eryptosis *in vitro via* oxidative stress and hemoglobin leakage in human erythrocytes

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ABSTRACT

Exposure of human population to industrial chemicals is believed as a significant contributing factor to the outgrowth of occupational diseases especially in developing countries due to improper safety measures and sanitary conditions. Para-tertiary butylcatechol (PTBC) widely employed in petrochemical, thermofax and phototypesetting industries, induces melanocytotoxicity and contact dermatitis leading to occupational leukoderma/vitiligo. Few vitiligo patients were reported for oxidative stress-induced hemolytic anemia and thrombocytopenia, however its impact on blood components is still not clear. Erythrocytes are the major cell population in circulation and play a prominent role in various diseases. In this work, the effect of PTBC on human erythrocytes is evaluated *in vitro*. PTBC induces oxidative stress-mediated eryptosis (erythrocyte death) causing detrimental changes such as depleted antioxidant levels, altered surface morphology, hemoglobin denaturation and heinz body formation. These findings validate that PTBC could induce toxic effects on human erythrocytes. Exposure of humans to toxic chemicals constitutes an important issue in various industries; one such issue is the exposure of PTBC at work place resulting in a spectrum of dermal complications. Therefore, it is imperative to appraise the long-term toxicities in order to further delineate the mechanisms of resultant disorders associated with PTBC and to establish the therapeutic interventions.

1. Introduction

The production and utilization of catechol and its derivatives in industries for various purposes is tremendously growing. Para-tertiary butyl catechol (PTBC), one such catechol derivative, has been largely used as an antioxidant in rubber, plastic, paint, petroleum, thermo fax and phototypesetting industries and as polymerization inhibitor in the manufacture of butadiene, styrene, vinyl acetate and other reactive monomer streams and also as stabilizer in the production of polyurethane foam (Hillen et al., 2001). Majority of PTBC exposure to humans is through both inhalation and dermal absorption. Hence, PTBC is known to amass occupational risks such as skin rashes, dermal inflammation, contact dermatitis, leucoderma followed by vitiligo upon its recurrent contact with skin (Minamoto et al., 2002).

Formerly, Gellin disclosed depigmenting efficacy of PTBC in black guinea pigs and subsequent studies displayed that PTBC could modify various oxidative stress parameters in melanocyte and keratinocyte cell lines (Mansur et al., 1978). Since PTBC mimics tyrosine structure, it competes with tyrosinase, decreases melanin pigment eumelanin formation, and rather increases pheomelanogenesis. Further, PTBC is shown to deplete glutathione system and sulphur content of melanocytes by producing reactive quinones causing cell death (Kawashima et al., 1984). Although the usage of PTBC is worldwide and large, the prevailing literature of PTBC has focused only on melano-cytotoxicity followed by depigmentation on human and animal skin. It is reported that PTBC concentration of up to 0.5% can induce occupational vitiligo and contact sensitivity when patch tested on human skin (Horio et al., 1977). Furthermore, few studies have reported dermal absorption and retention of PTBC in plasma (Black and Mathews, 2000), therefore, we can speculate the deposition of PTBC on skin/lungs and its absorption into blood stream. Unfortunately, the evidences concerning its toxicity on blood and other health issues after persistent exposure to PTBC is highly inadequate. On account of this, our study for the first time, evaluated the effect of PTBC on human erythrocytes *in vitro,* as erythrocytes are major cell population of blood and play a critical role in various diseases such as anemia and respiratory disorders.

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The stability of RBC/erythrocyte membrane is very important to transport oxygen through capillaries. The structure of the erythrocyte membrane allows it to undergo large deformations while maintaining its structural integrity. However, erythrocytes have been a virtuous model for oxidative stress due to the existence of oxy-hemoglobin, a powerful promoter of oxidative stress, hence are highly vulnerable. Erythrocyte exposure to xenobiotic or auto-oxidation of oxy-hemoglobin generates various free radicals such as superoxides, peroxides and hydroxyl radicals, which have been postulated to impair erythrocyte rheology, despite of having an efficient endogenous antioxidant system (Pandey and Rizvi, 2011). Oxidative stress has been reported to play an important role in the progression of diabetes, cancer, cardiovascular diseases, arthritis, ulcers, pneumonia, vitiligo and many other neurological diseases (Mohanty et al., 2014; Harris, 2016). The free radicals formed during oxidative stress effectively impair the fundamental cellular functions of erythrocytes eliciting intracellular calcium and phosphatidyl serine (PS) exposure, which eventually leads to eryptosis (cell death). Eryptosis is characterized by erythrocyte shrinkage, cell membrane blebbing, and cell membrane scrambling with PS translocation to the erythrocyte surface triggering the phagocytosis of the cells (Lang and Lang, 2015). Accelerated eryptosis may contribute to anemia and is enhanced in numerous clinical conditions like malaria, sickle cell anemia, diabetes, sepsis, hepatic and renal insufficiency, Wilson's disease, hemolytic uremic syndrome, hyperphosphatemia, G6PD-deficiency and chronic kidney disease (CKD) (Officioso et al., 2016).

In addition, increased oxidative stress affects several parameters of RBC function and integrity such as changes in membrane fragility, increase of lipid peroxidation, oxidation of protein sulfhydryl groups, activation of proteolysis, modifications of erythrocyte energy metabolism and extensive oxidation and denaturation of hemoglobin (Hb) results in the formation of methemoglobin (MtHb), hemichromes and subsequent Heinz body formation (Sugawara et al., 2013). In this study, we have evaluated whether PTBC was able to trigger eryptosis in human erythrocytes. Data from our results indicate that PTBC induces hemolysis, eryptosis and Heinz body formation in erythrocytes. In view of this, further an elaborative work is required to determine its hemotoxicity in industrial workers.

2. Materials and methods

2.1. Materials

Calcein-2AM, 2′,7′, Dichlorodihydroflourescin diacetate (CM-H_2DCFDA), Flourescin isothiocyanate (FITC)-labelled annexin V, Fluo-4AM, Calcium ionophore (A23187), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), Glyceraldehyde-3-Phosphate, Glutaraldehyde, Glutathione reductase (GR), Leupeptin hydrochloride, Benzamidine hydrochloride, CHAPS, dithiothreitol (DTT), Sodium orthovanadate (Na_3VO_4), 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), Anti-Hb antibody, calpain antibody and Para tertiary butyl catechol (PTBC) were procured from Sigma Chemicals, USA. Alexa Fluor 488 goat anti-rabbit secondary antibody, Phalloidin, Dihydroethidium (DHE), Amplex red, BODIPY 581/591 C11 and Monochlorobimane (MCB) were procured from thermo fisher scientific, USA. Lactate Dehydrogenase (LDH) kit was from Agappe diagnostics Limited, India. Reduced (GSH) and oxidized (GSSG) glutathione, reduced and oxidized nicotinamide adenine dinucleotide phosphate (NADPH and $NADP^+$ respectively), reduced nicotinamide adenine dinucleotide (NADH), N-ethylmaleimide [NEM], *o*-phthalaldehyde [OPT], 2,6-dichlorophenolindophenol, 2,4 dinitrophenylhydrazine, 5,5′-dithiobisnitrobenzoic acid (DTNB), glucose 6-phosphate, $NaNO_2$, Phenyl hydrazine, were from Sisco Research Laboratory (Mumbai, India). And all other fundamental chemicals were of analytical grade.

AAPH is a free radical generator, initiate oxidation reactions *via* both nucleophilic and free radical mechanisms (Zheng et al., 2016). Hydrogen peroxide acts as cytotoxic agent and depletes antioxidant levels in RBC (Qasim and Mahmood, 2015). Phenylhydrazine causes Heinz body formation and deformability of erythrocytes (Berger, 2007). Sodium nitrite-induces met-hemoglobin formation and membrane damage in human erythrocytes (Guo et al., 2013; Ansari et al., 2016). These small molecules have got prominence as a model oxidant to study oxidative susceptibility of erythrocytes. Therefore, we have selected these molecules as positive controls to study PTBC induced oxidative stress in erythrocytes.

2.2. Isolation of human erythrocytes/RBCs

Erythrocytes were isolated from the blood obtained from healthy human donors provided with informed consent, in accordance with the guidelines of Institutional Human Ethical Committee (IHEC-UOM No.112/Ph.D./2015-16) guidelines, University of Mysore, Mysuru. Freshly drawn blood was anti-coagulated with acid citrate dextrose and centrifuged to pellet erythrocytes (Sugawara et al., 2013).

2.3. Hemolysis assay

Erythrocytes (5% hematocrit) in Ringer solution [(in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2- ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$, pH 7.4], were incubated independently with indicated concentrations of PTBC (50–200 μM) and AAPH (5 mM) at 37 °C for different time intervals. The absorbance of supernatant was measured against blank at 415 nm for Hb leakage (Beckman coulter DU-730, USA). Untreated erythrocytes lysed in distilled water represent 100% hemolysis (Dai et al., 2006).

2.4. Methemoglobin levels and methemoglobin reductase activity

Erythrocytes (5% hematocrit) in ringer's solution were independently treated with different concentration of PTBC (50–200 μM), $NaNO_2$ (2 mM) and PHZ (100 μM) and incubated for different time intervals at 37 °C and cells were lysed with 2 mM Phosphate buffered saline (PBS) pH 7.4 containing 1% Triton X-100 (1:1, *v*/v) and the absorbance of hemolysate was read at 630 nm to determine MetHb formation. The MetHb reductase activity was determined from the rate of decrease in absorbance at 600 nm after incubation of treated and untreated hemolysate with NADH and 2,6-dichlorophenolindophenol as the electron acceptor (Ansari et al., 2015).

2.5. Flow cytometric analysis [FCM]

Erythrocytes were stimulated independently with PTBC (50–200 μM), H_2O_2 (2 mM), AAPH (5 mM) and A23187 (10 μM) for 1 h at 37 °C and incubated again for 20 min with fluorescent probe CMH_2DCFDA: to detect endogenously generated reactive oxygen species (Excitation: 488 nm, Emission: 530 nm), DHE: to detect superoxides (Peshavariya et al., 2007) (Excitation: 405 nm, Emission: 570 nm), BODIPY: to detect lipid peroxides (Excitation: 581 nm, Emission: 591 nm), (Fu et al., 2009), Fluo-4AM: to detect intracellular calcium (Excitation: 494 nm, Emission: 506 nm), (Glushakova et al., 2013), MCB: to detect endogenous glutathione levels, (Excitation: 370 nm, Emission: 478 nm), (Raththagala et al., 2006), Annexin V conjugated to FITC: to detect PS scrambling (Excitation: 496 nm, Emission: 516 nm), (Lang et al., 2015) and Calcein: to detect the extent of cell death (Excitation: 495 nm, Emission: 515 nm), (Bratosin et al., 2005). After incubation, excess dye was removed by washing followed by re-suspending the cells with 200 μL ringer solution. The cells were analyzed using FACSVerseTM flow cytometer (BD Biosciences, USA).