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MECHANISTIC STUDY ON ANILINE-INDUCED ERYTHROCYTE TOXICITY*

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Strategies for the use of bio-indicators in the prediction of environmental damage should include mechanistic research. This study involves the relationship between the chemical structure and hemotoxic markers of aniline and its halogenated analogs. Aniline-induced methemoglobinemia, loss of circulating blood cells, blood stability, glutathione depletion and membrane cytoskeletal changes were assessed following exposure to phenylhydroxylamine (PHA), *para*-fluoro-, *para*-bromo-, and *para*-iodo in male Sprague-Dawley rats. Methemoglobin was determined spectrophotometrically at 635 nm. Erythrocyte depletion was investigated by loss of radioactivity in chromium-labeled red blood cells *in vivo*. Membrane proteins were analyzed by SDS-PAGE using red blood ghost cells treated with various aniline analogs. Results showed dose- and time-dependent changes in the induction of methemoglobin of up to 78 % with *para*-bromo PHA and 75 % with *para*-iodo PHA compared to 3 % to 5 % in control. Treated animals lost up to three times more blood from circulation compared to control within 14 days after treatment. Erythrocytes were more stable in buffer solution than in *para*-iodo-treated cells. Depletion of reduced glutathione in PHA and *para*-iodo-PHA treated red cells was also observed. Analysis of red cell skeletal membrane treated with *para*-iodo-PHA showed that protein band 2.1 became broader and band 2.2 diminished completely in some treatments. Dose- and time-dependent changes suggested the use of hemotoxic endpoints as potential biomarkers for assessing chemical and drug safety.

KEY TERMS: *cytoskeletal proteins, hemolytic anemia, hemotoxicity, methemoglobin, para-bromo-PHA, para-fluoro-PHA, para-iodo-PHA, phenylhydroxylamine*

Toxic metabolites responsible for the hemolytic activity of a variety of environmental chemicals, including compounds of industrial interest (aniline), agricultural chemicals (Propane), and drugs (dapson, phenacetin, primaquine) have been identified (1-8). The following major events lead to hemotoxicity: (1) the parent compounds in most cases are converted to their toxic metabolites (*N*-hydroxyarylamines) in the liver; (2) the *N*-hydroxyarylamines entering the RBCs react with oxyhemoglobin, with consequent reduction of RBC oxygen to active oxygen species (oxygen radicals/peroxide), that convert the sulfur

of hemoglobin sulfhydryl groups to free radicals (9-12); and (3) the hemoglobin sulfhydryl-free radicals attack and form adducts with the membrane skeletal proteins (spectrin, ankyrin, band 3 protein, and band 4.2 protein) (13). This modification of the skeletal protein meshwork changes the erythrocyte cell surface properties leading to premature expression of the cryptic senescent antigenic site and premature activation of the autoimmune-mediated mechanism by which "old" red cells are sequestered and phagocytized in the spleen.

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Methemoglobinemia and hemolytic anemia are two major toxic side effects known to accompany treatment with a variety of arylamine chemicals and drugs. Studies in rats with aniline and dapsone have led to the identification of *N*-hydroxy metabolites as the hemotoxic mediators for arylamine compounds (1, 14-15). There is little information, however, regarding the comparative hemotoxicity of halogenated arylamine derivatives, particularly industrial and agricultural chemicals, such as aniline which may also be metabolized to *N*-hydroxy hemotoxic agents.

Bioindicators are endpoints in events leading from exposure to toxic damage. Such markers are potentially useful for risk assessments by linking exposures to given amounts of a chemical with the induction of specific health effects. Strategies for the use of bioindicators in the prediction of environmental damage should include mechanistic research to determine the earliest steps leading to toxicity. For example, DNA and hemoglobin adducts formed by a chemical may be related to chemical and physical properties of a toxicant. Once the mechanism of disease is known, strategies can be developed using biomarkers for the risk assessment management. In general, DNA and hemoglobin adducts formed by exposure to a chemical are likely to lead to a toxic injury based on structural properties. Therefore, it is desirable to understand the relationship between exposures and the toxicity with respect to structure and physical properties of a chemical (16).

The crucial role of the membrane skeletal proteins and in particular the actin-spectrin meshwork in maintaining the structure of the red cell is now well established (17-21). Oxidative cross-linking between spectrin units via disulfide bridges form a high molecular weight protein (HMWP) spectrin complex (MW > 106) on prolonged incubation of normal red cells in the absence of glucose. The formation of the spectrin HMWP complex is thought to diminish the lateral mobility of transmembrane proteins and to "fix" the membrane skeletal protein into a rigid structure (22, 23). Similar rigidity can result from cross linkage involving spectrin, ankyrin, and band 3 proteins in a HMWP complex mediated by a calcium-activated transglutaminase, which links the side chain groups of lysine and glutamine (24-26).

Aniline (MW 93.12) is an oily liquid, colorless but darkens upon exposure to light and air. This chemical is used as a solvent in manufacturing dyes, medicinal products, resins, perfumes and shoe polish. Methemoglobin and hemolytic anemia effect of aniline are mediated by active/reactive metabolite(s)

formed during the hepatic clearance of the parent compounds (15). These chemicals do not cause hemoglobin oxidation when incubated with erythrocyte suspensions at physiologically relevant concentrations *in vitro* (27-28). Toxicity to erythrocytes results from active metabolites formed *in vivo* during hepatic clearance (29). However, very little is known regarding the effect of halogenated phenylhydroxylamines on erythrocytes in animals.

In this study, we present mechanistic data on the relationship between the chemical structure and blood cell biomarkers as toxic side-effects using aniline and its halogenated analogs. A number of toxic metabolites that induce methemoglobin are also responsible for hemolytic activity. This study examines the methemoglobinemic and hemolytic capacities of structurally related halogenated arylhydroxylamines with decreasing electronegativity such as *N*-hydroxy derivatives of aniline phenylhydroxylamine: *para*-fluoro-, *para*-bromo-, and *para*-iodo-phenylhydroxylamines and others with special reference to red cell morphological changes caused by cytoskeletal protein alterations. The stability of certain selected hydroxylamine/nitroso pairs in the buffer and in the presence of red cells, and the extent to which the hydroxylamines bind covalently to the hemoglobin were also determined in addition to assessing the depletion of reduced glutathione in treated red blood cells.

MATERIALS AND METHODS

Synthesis of arylhydroxylamines

Various halogenated hydroxylamines (phenylhydroxylamine, *para*-fluoro-PHA, *para*-bromo-PHA, *para*-iodo-PHA and *para*-chloro-PHA) were synthesized from the corresponding nitroanilines by zinc dust/NH₄Cl reduction or by catalytic reduction with hydrogen (15, 18). Both techniques have been used routinely for the preparation of arylhydroxylamines in the literature. The following synthesized arylhydroxylamines were crystallized, purified and stored at -80 °C: phenylhydroxylamine, *para*-fluoro-, *para*-bromo-, and *para*-iodo-phenylhydroxylamines. Structures of these analogs were confirmed by MS, NMR and HPLC (30).

Animals

Male Sprague Dawley rats weighing 100 g to 125 g were used for this study. The usage of rats falls into two

categories: (1) RBC donors, (2) hemotoxicity assays. For RBC donation, the animals were placed under ether anesthesia and blood was obtained by cardiac puncture. For the hemotoxicity studies, the animals received ^{51}Cr -tagged red blood cells intravenously via tail vein under light ether anesthesia. Serial blood samples were taken subsequently from the orbital sinus. At the end of all experiments, the animals were placed under ether anesthesia and sacrificed by decapitation. There were 4 to 6 animals per dose per treatment. The animals were housed and treated avoiding unnecessary discomfort, distress, pain and injury. The protocol for animal used in this study was in compliance with our Institutional Animal Care and Use Committee.

Statistical analysis of the data was performed with DeltaGraph 4. The values obtained were entered into a spreadsheet within the program in order to determine the standard error of mean if applicable. Average values were used to graph data.

Blood Collection

Whole blood was collected from male Sprague-Dawley rats via aortic puncture. The blood was washed at 37 °C with PBSG (pH 7.4) then centrifuged at 10,000 rpm for 5 min in a desktop Eppendorf Centrifuge 5403. The resulting RBCs were washed twice more with the final wash lasting for 7 min to better pellet the cells. The final pellet was diluted with phosphate buffered saline solution with glucose (PBSG) to yield a 40 % hematocrit.

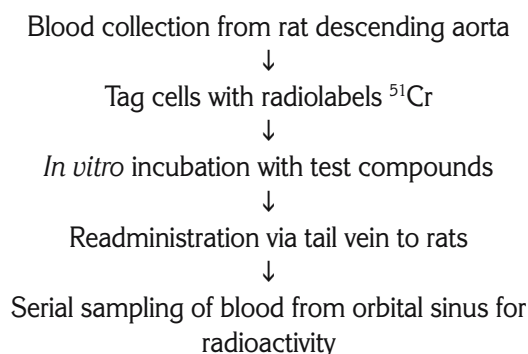
Methemoglobin Assay

The halogenated phenylhydroxylamines ranging from 30 $\mu\text{mol L}^{-1}$ to 300 $\mu\text{mol L}^{-1}$ were prepared in acetone. Ten μL of the test analog was added to the PBSG. Two mL aliquots in scintillation vials for incubations were placed in a 37 °C shaker bath, and methemoglobin levels were measured at various time points ranging from 0 min to 240 min. At specific time points, 75 μL of the 40 % hematocrit blood was pipetted into 5 mL of the hemolysis buffer and vortexed. One mL of the hemolyzed blood sample was pipetted into each of the four cuvettes to determine absorbance that was measured at 635 nm on an UltraSpec 2000 UV/VIS Spectrophotometer. Incubations in the presence and absence of synthesized aniline analogs were performed in triplicate.

Measurement of the hemolytic response

Rat erythrocytes were collected and washed three times with PBSG. The cells were then labeled with

radioactive sodium chromate (0.1 mCi/1.0 mL of packed cells) and incubated for 15 min at 37 °C in a shaker bath, before being washed once again with PBSG. The hematocrit was adjusted to 40 % prior to treatment of cells with specific doses of chemical compounds. The cells were incubated for 2 h at 37 °C before injecting into rats (0.5 mL per animal). The initial blood sample of 75 μL (T_0) was taken from the orbital sinus within 30 min after the infusion of tagged erythrocytes in rats. Control incubations received acetone as a vehicle. The treated erythrocytes were administered via tail vein to isologous rats as previously described elsewhere (14, 31). Serial blood samples (75 μL) from each rat were then collected into heparinized capillary tubes at designated intervals for 14 days. Samples were counted in a well-type γ -counter, with the counts per minute above background expressed as a percentage of the T_0 sample. Data were expressed in terms of percent decrease in values relative to the ^{51}Cr values of the red cells in that animal at time zero (T_0) in the control. A schematic presentation of this procedure is shown below.



Stability Study

To compare the effects of the arylhydroxylamine series with respect to stability, red cell suspensions were incubated at 37 °C for up to 2 h in saline buffer. For estimation of hydroxylamine/nitroso pairs, aliquots of the red cell or buffer incubates were treated with ferricyanide to oxidize all remaining hydroxylamine to its nitroso-derivative, which was then extracted with ethyl acetate and measured by HPLC-EC. At various intervals, aliquots were withdrawn and assayed for the substituted phenylhydroxylamine/nitrosobenzene. Briefly, a standard curve was generated for each arylhydroxylamine by injection of various concentration of its nitroso derivative onto the HPLC column. Detection was done by both a UV monitor and by an electrochemical detector. The *para-chloro-*

PHA was used as an internal standard. Decay rates for hydroxylamine/nitrosobenzene pairs were calculated by plotting peak height ratios of the test agent *para*-iodo-PHA against the internal standard.

Glutathione (GSH) Depletion in Erythrocytes Suspensions

Diethyl maleate (DEM) was used to deplete GSH in red cell suspensions (8, 11). Briefly, DEM ($750 \mu\text{mol L}^{-1}$) dissolved in acetone was added to packed red cells. After a 15-min incubation at 37°C , the red cells were analyzed for GSH content in cells treated with PHA and *para*-bromo-PHA by HPLC-EC. Under these conditions, GSH was reduced to about 5 % of initial levels. The cells were resuspended to a 40 % suspension in PBSG and used on the same day that they were collected.

Electrophoretic Analysis

The procedure involved treating rat packed red cells with *para*-iodo-PHA or acetone (for control),

lysing the red cells by hypotonic ice-cold sodium phosphate buffer (5 mmol L^{-1} , pH 8) before running the treated cells and a standard marker through the SDS-PAGE mini-gels at 120 mA for 45 min after two hours of incubation. The proteins were stained with Coomassie Blue. The red cell membrane protein bands were identified according to the molecular weight with the aid of protein standard in Lane 1 (control) and markers in Lane 8.

RESULTS

Methemoglobin

The data revealed dose-dependent changes in the induction of methemoglobin following treatment with aniline-analogs tested (Figure 1 A-D). The MetHb induction peaked to 35 % at $50 \mu\text{mol L}^{-1}$, 56 % at $100 \mu\text{mol L}^{-1}$ and to 67 % at $150 \mu\text{mol L}^{-1}$ within 20 min, before declining after treatment with *para*-fluoro-PHA. At the end of 240 min, MetHb remained

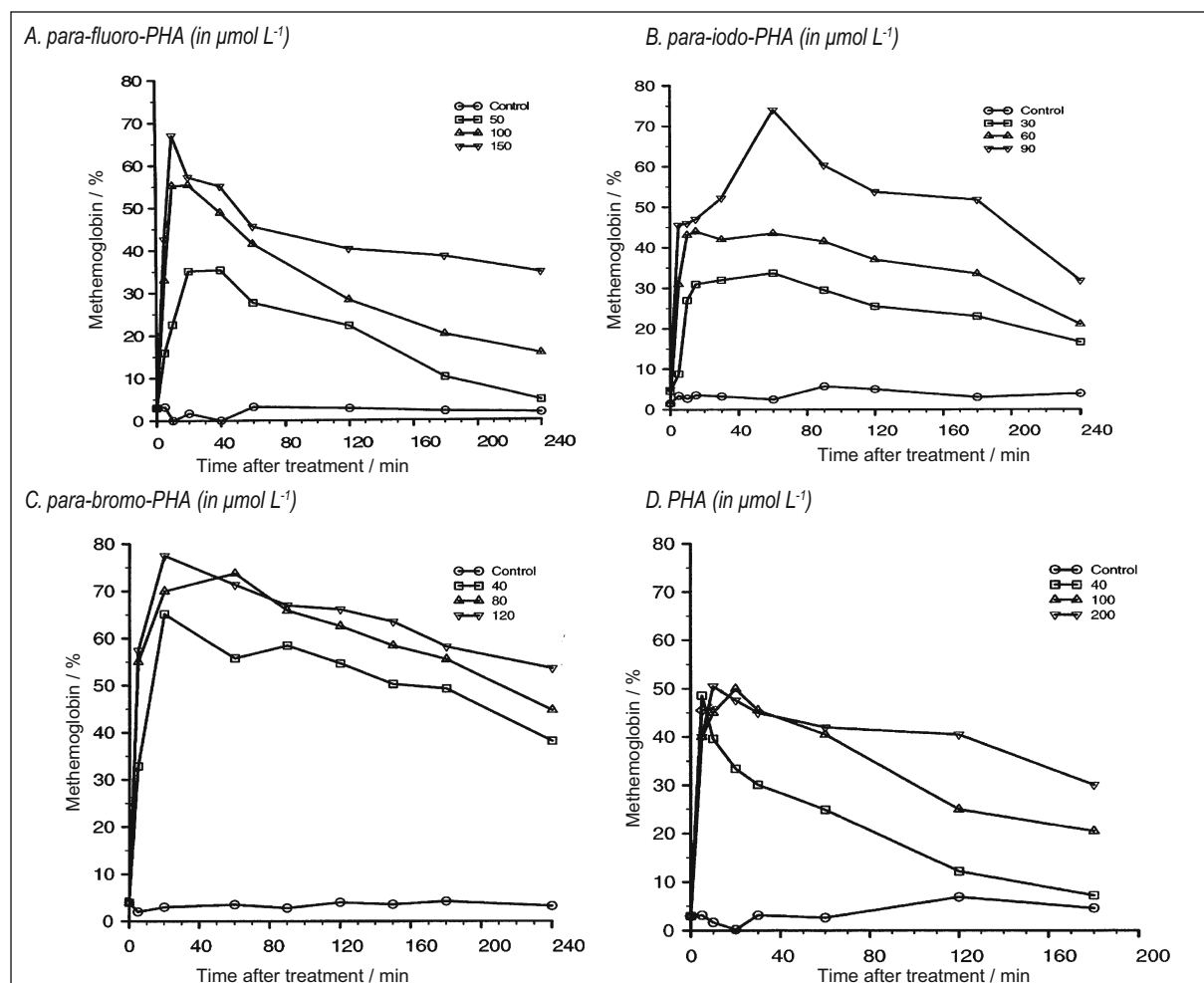


Figure 1 Time course of percent methemoglobin formation with (A) *para*-fluoro- (B) *para*-iodo- (C) *para*-bromo- and (D) phenylhydroxylamine.

elevated from 5 % to 42 % depending on the dose compared to 2 % in the control. Similarly, *para*-iodo-PHA induced MetHb that peaked at almost 75 % within 60 min at 90 $\mu\text{mol L}^{-1}$ before declining. The MetHb remained elevated to 40 % (almost 20-fold) compared to control at 240 min post-treatment with this agent. With *para*-bromo-PHA, the MetHb level peaked to 78 % at 120 $\mu\text{mol L}^{-1}$ within 20 min before declining. At the end of 240 minutes, the MetHb level stayed 40 % to 60 % depending on the dose compared to 3 % in control. The MetHb induction with PHA was brisk, but the magnitude of the response was relatively lower compared to other analogs tested. The data showed the highest MetHb peak with *para*-bromo-PHA followed by *para*-iodo- and *para*-fluoro-PHA. In another experiment, the methemoglobin induction capacity of four halogenated aniline analogs was compared at 100 $\mu\text{mol L}^{-1}$, 200 $\mu\text{mol L}^{-1}$, and 300 $\mu\text{mol L}^{-1}$. The comparison of these four analogs showed that *para*-bromo-PHA was the most potent in inducing MetHb, followed by *para*-iodo-PHA at 100 $\mu\text{mol L}^{-1}$ and 200 $\mu\text{mol L}^{-1}$ (Figure 2). With *para*-bromo-PHA, MetHb peaked to 62 % at 100 $\mu\text{mol L}^{-1}$, 70 % at 200 $\mu\text{mol L}^{-1}$ and 75 % at 300 $\mu\text{mol L}^{-1}$ within 60 min before declining.

Hemolytic Anemia

The halogenated aniline hydroxylamines produced dose-dependent reduction in the labeled erythrocytes, indicating the loss of blood cells from circulation (Figure 3 A-C). The most pronounced reduction was observed at doses from 175 $\mu\text{mol L}^{-1}$ to 250 $\mu\text{mol L}^{-1}$. The dose of 100 $\mu\text{mol L}^{-1}$ appeared to be the threshold level, and the dose of 325 $\mu\text{mol L}^{-1}$ did not show much further reduction in the red cell radioactivity in this study. Based on the loss of labeled erythrocytes from circulation, it appeared that *para*-iodo-PHA was 2.5 times more toxic (50 % red cell reduction in 2.5 days) than *para*-fluoro-PHA (50 % red cell reduction in 7 days) and 1.5 times more toxic (50 % red cell reduction in 4 days) than *para*-bromo-PHA after treatment with 175 $\mu\text{mol L}^{-1}$. All tested phenylhydroxylamines showed the potential of being active metabolites to mediate aniline-induced hemolytic anemia resulting from erythrocyte loss.

Blood Stability

Figure 4 shows that *para*-iodo-PHA is much less stable in blood than in the buffer. For instance, *para*-iodo-PHA lost 50 % of its stability in blood within 7

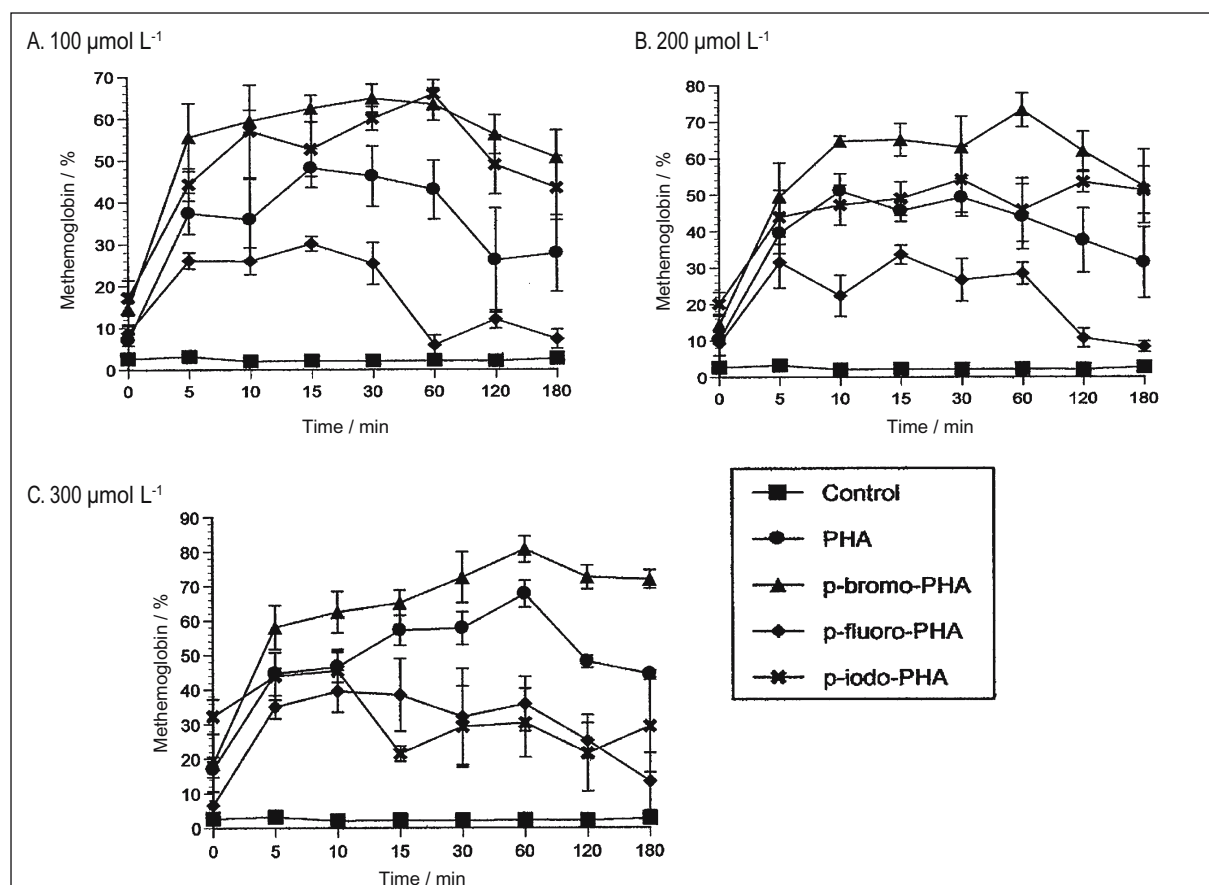


Figure 2 Comparison of four analogs on the formation of methemoglobin in male rat erythrocytes. (A) 100 $\mu\text{mol L}^{-1}$, (B) 200 $\mu\text{mol L}^{-1}$, and (C) 300 $\mu\text{mol L}^{-1}$.

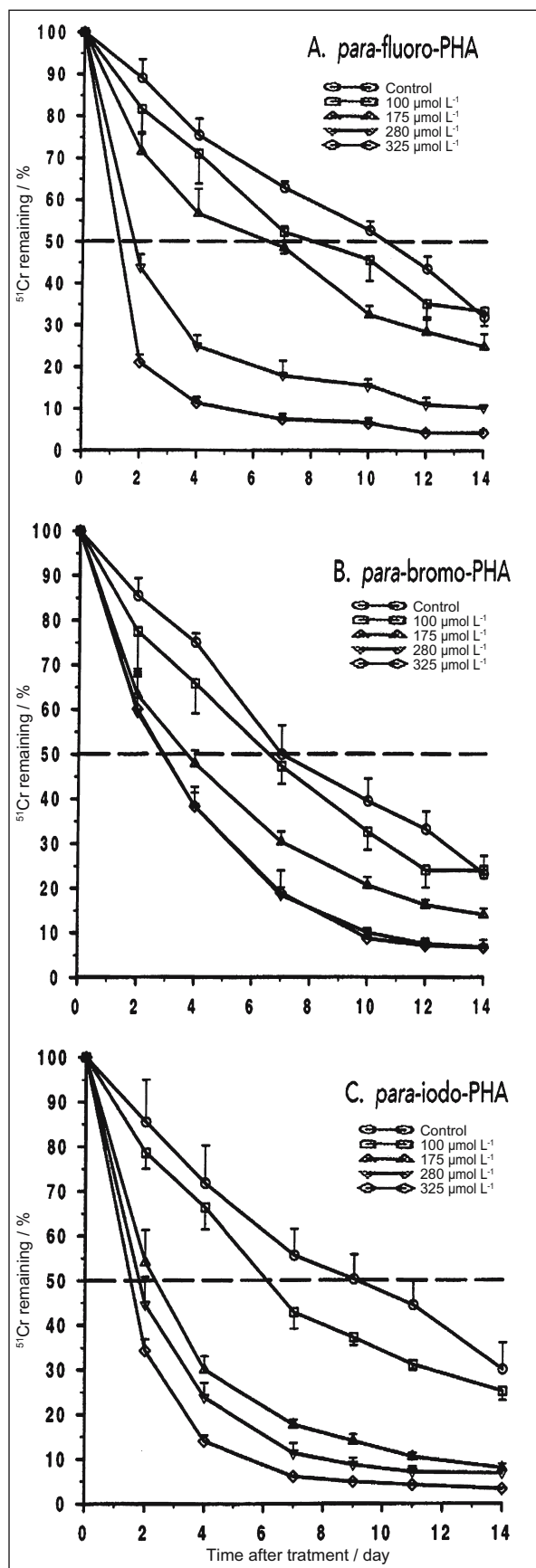


Figure 3. Percent ⁵¹Cr remaining in erythrocytes after treatment with (A) para fluoro- (B) para-bromo- and (C) para-iodo-PHA.

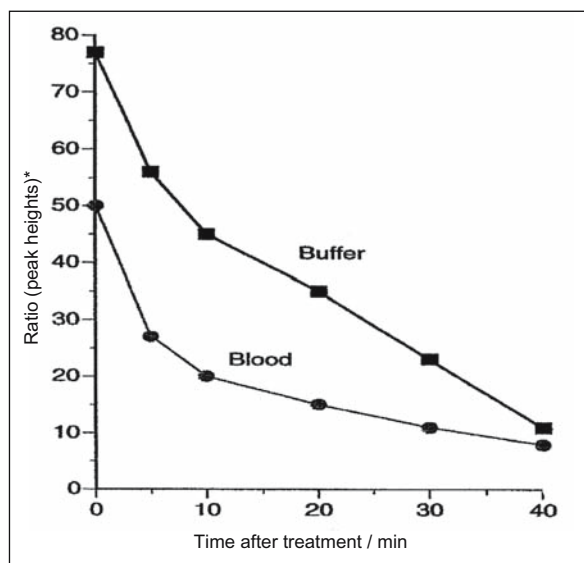


Figure 4 The stability of erythrocytes in the buffer and blood at 200 μmol L⁻¹ with para-iodo-PHA. (*test agent/internal standard, para-Chloro-PHA).

min compared to 18 min in the buffer relative to their respective peak heights.

Glutathione depletion

Extensive clinical and basic studies showed that administration of hemolytic compounds depleted erythrocyte-reduced glutathione. Our study with 110 μmol L⁻¹ of PHA or para-bromo-PHA confirmed GSH depletion in rat erythrocytes (Figure 5). For example, GSH was depleted to almost 70 % within 5 min with PHA and to 55 % within 15 min with para-bromo-PHA, but it remained at the original value or slightly higher in the control for the test period.

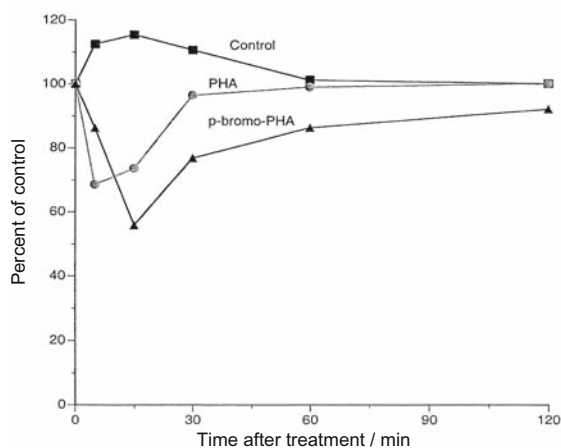


Figure 5 Glutathione (GSH) depletion in rat erythrocytes treated with 110 μmol L⁻¹ of PHA or para-bromo-PHA.

Electrophoretic changes

Figure 6 shows changes in red cell cytoskeletal membrane proteins after treatment with *para*-iodo-PHA. The Band 2.1 diminished in intensity or was lost completely at 200 $\mu\text{mol L}^{-1}$ (Lanes 4 and 5) or 300 $\mu\text{mol L}^{-1}$ (Lanes 6 and 7) compared to control (Lane 1). In contrast, Band 1 (spectrin) was broadened and diffused markedly in rats treated with *para*-iodo-PHA, especially at 200 $\mu\text{mol L}^{-1}$ or higher (Lanes 4-7) compared to control (Lane 1). Bands 4.1 and 4.2 also diminished in intensity at 200 $\mu\text{mol L}^{-1}$ higher or higher (Lanes 4-7) compared to control. In addition, the appearance of membrane-bound hemoglobin monomers (at the bottom) was observed at doses of 200 $\mu\text{mol L}^{-1}$ or higher (Lanes 4-7). Band 3 also showed splitting at the bottom following treatment with higher doses (Lanes 4-7). Alterations in the cytoskeletal protein patterns are positive precursors for changes in the cell membrane morphology. Because spectrin and ankyrin are the proteins that function in the cytoskeletal structure, red blood cells are likely to change morphologically and may become echinocytic in shape, triggering the immune response for their premature removal from circulation. The available preliminary electrophoretic data indicate that there are definite changes in the rat erythrocyte skeletal membrane proteins after exposure to *para*-iodo-PHA.

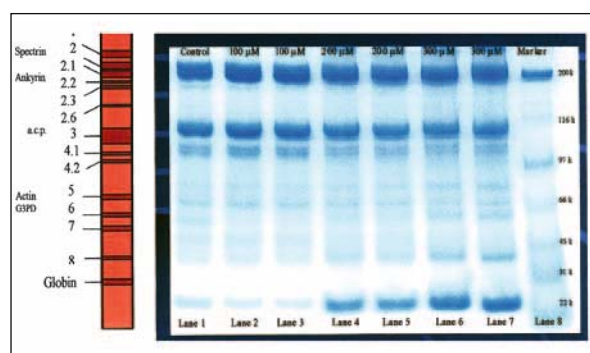


Figure 6 Electrophoretic band pattern changes in male rat erythrocytes following treatment with *para*-iodo-PHA.

DISCUSSION AND CONCLUSION

The erythrocytic effect of phenylhydroxylamines varied with dose, time and the electronegativity of halogenated analogs. The *para*-fluoro-PHA produced the quickest response at 150 $\mu\text{mol L}^{-1}$ by elevating MetHb to almost 70 % but the highest MetHb peak of 78 % occurred with *para*-bromo-PHA. MetHb steadily declined with time, but still remained significantly

elevated above control. The most pronounced effect of halogenated phenylhydroxylamines on the loss of erythrocytes from circulation occurred at doses from 175 $\mu\text{mol L}^{-1}$ to 250 $\mu\text{mol L}^{-1}$. The dose of 100 $\mu\text{mol L}^{-1}$ appeared to be the threshold level, and the dose of 325 $\mu\text{mol L}^{-1}$ did not show any further reduction in the circulating erythrocytes, as indicated by the loss of radioactivity in the labeled blood cells. Results indicated that the *para*-iodo-PHA or *para*-bromo-PHA was 2.5 times more toxic than *para*-fluoro-PHA in reducing circulating red cells. The phenylhydroxylamines tested in this study appeared to be active metabolites that may mediate aniline-induced erythrocyte destruction in circulation. The study showed the following hemotoxic response of the tested phenylhydroxylamines: *para*-iodo-PHA > *para*-bromo-PHA > *para*-fluoro-PHA > PHA. It was also noted that *para*-iodo-PHA and other phenylhydroxylamines tested were much less stable in blood than in the buffer. Reduced glutathione depletion in rat erythrocytes treated with 110 $\mu\text{mol L}^{-1}$ of PHA or *para*-bromo-PHA was also observed, and was markedly higher with *para*-bromo-PHA than with PHA. Glutathione prevents the oxidation and denaturation of hemoglobin and other critical RBC proteins by inhibiting the accumulation of peroxide compounds (4, 12).

Aniline induced dose-dependent methemoglobinemia in rats after intraperitoneal injection, but did not cause methemoglobin formation *in vitro*, indicating that methemoglobinemia after treatment with aniline is mediated by one or more toxic metabolites formed during the hepatic clearance (27). Phenylhydroxylamine, nitrosobenzene or other breakdown products of aniline metabolism have not been demonstrated in urine. However it has shown that nitrosobenzene is present in blood after aniline administration (32, 33). Production of nitrosobenzene and/or phenylhydroxylamine from aniline has also been observed in microsomal systems (27) and in isolated perfused rat liver (34). The time course of methemoglobinemia in rats given aniline halogenated hydroxylamines is much different from that observed in rats treated with other phenylhydroxylamines. In general, halogenated aniline induces a much longer-lasting methemoglobinemia for a given peak level than did PHA, as was observed in this study. Whereas the metabolites produced parallel increases in peak and total methemoglobinemia with increasing dose, higher doses of aniline analogs were associated with a disproportionate increase in the duration and level

of methemoglobinemia (35, 36). This was confirmed in our study.

Among the halogenated anilines, the *para*-substituted compounds have been found to be most active in cats (37, 38) and dogs. In dogs, the high yield of ferrihemoglobin by 4-chloroaniline is due to the slow elimination of the parent amine and its *N*-hydroxy metabolite. For example 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene accumulate to high concentrations, up to 2×10^{-4} mol L⁻¹, and probably produce most of the ferrihemoglobin (38). Ferrihemoglobin formation by halogen-anilines has also been observed in humans (39-40).

It is generally known that hemolytic arylamines require metabolism to *N*-hydroxylamines to produce toxic effects on red blood cells. The reactive *N*-hydroxy metabolites subsequently react with oxyhemoglobin to produce reactive oxygen species and sulfur-center free radicals of hemoglobin resulting in the formation of adducts with a variety of membrane-associated proteins altering cytoskeletal structure and function. These membrane alterations result in the premature removal of damaged cells from the circulation by the spleen. This study compared the hemotoxicity of a variety of laboratory synthesized arylhydroxylamine analogs of aniline whose selection was based on their differences in electronegativity. Aryl hydroxylamines selected for examination vary in electron donation/withdrawal in the *para* position and hence were expected to vary the stability of the generated hydronitoxide radical that might act as an intermediary in inducing hemotoxic damage. All phenylhydroxylamines in the present studies acted as probable mediators of aniline-induced hemolytic anemia in rats. The phenylhydroxylamine mediated hemoglobin oxidation is proposed to occur via a "coupled oxidation" with oxyhemoglobin in the erythrocyte, forming methemoglobin, nitrosobenzene and partially reduced oxygen species (e.g., peroxide and/or superoxide) (27). Nitrosobenzene thus formed may be bound to deoxyhemoglobin or may be reduced to phenylhydroxylamine by a NADPH dependent diaphorase, leading to the formation of a redox cycle producing many equivalents of methemoglobin for each equivalent of phenylhydroxylamine (41). The cycle is depleted eventually by side reactions involving phenylhydroxylamine or nitrosobenzene, or by the reduction of phenylhydroxylamine to aniline within erythrocytes (27, 41, 42). The findings presented here support that several aniline metabolites of known structure mediate the hemolytic effect of aniline-related

compounds. Although it has been proposed previously that *N*-hydroxydapsone might mediate the hemolytic effects of dapsone (28), *N*-hydroxyl metabolites have not otherwise been considered as mediators of the hemolytic effects of aniline, primaquine or other hemolytic drugs (5). Further experiments are required to determine whether aniline-induced hemolytic anemia as a result of premature sequestration of abnormal cells from circulation is an appropriate model for inducing similar responses by primaquine and other drugs which are aniline derivatives. Our data indicated that *N*-hydroxyl metabolites should be considered as possible mediators of chemical-induced methemoglobin and hemolytic anemia, and thus are potential biomarkers for assessing chemical injury to erythrocytes.

The role of cytoskeletal membrane proteins, especially actin-spectrin meshwork, in maintaining the erythrocytic structural integrity is well documented and may be under genetic control. Oxidative cross-linkage between spectrin units through disulfide bridges can be provoked by oxidants diminishing the cellular integrity resulting in abnormal morphology (43, 44). The precise role of this meshwork in the dynamics of membrane alterations is not yet understood. However, the importance of the meshwork in determination of the shape of the red cell has been clearly documented on hereditary erythrocyte disorders such as spherocytosis (HS), elliptocytosis (HE) and pyropoikilocytosis (23, 45). Relative deficiency in protein 4.1 has been associated with one form of HE, and a deficiency in spectrin with an autosomal recessive HS. Crosslinkage between spectrin units and spectrin, ankyrin and band 3 have been associated with permanent stabilization of shape of the cells. These morphological changes may trigger premature splenic sequestration of abnormal erythrocytes.

In conclusion, this study shows that hemotoxic agents which produce hemolytic anemia may induce methemoglobin and even cytoskeletal changes in the red cell membrane proteins. These changes may be used as an early indicator of potential toxicants to red cell and thus may aid in the development of safer drugs or industrial chemicals and pesticides.

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Sažetak

ISTRAŽIVANJE MEHANIZMA TOKSIČNOSTI ANILINA U ERITROCITIMA

Strategije primjene biopokazatelja za predviđanje štete u okolišu trebaju u obzir uzeti istraživanja mehanizama djelovanja. Ovo istraživanje propituje odnos između kemijske strukture i hemotoksičnih pokazatelja djelovanja anilina i njegovih halogeniranih analoga. Nakon izlaganja mužjaka štakora soja Sprague-Dawley *para*-fluoro-, *para*-bromo- i *para*-jodofenilhidroksilaminu, utvrđena je methemoglobinemija uzrokovana anilinom te pad broja krvnih stanica u krvotoku i stabilnosti krvi, gubitak glutaciona i promjene na membrani stanice. Methemoglobin je određivan spektrofotometrijski na 635 nm. Pad broja eritrocita mjeren je *in vivo* s pomoću eritrocita obilježenih radioaktivnim kromom. Membranske su bjelančevine analizirane s pomoću SDS-PAGE, rabeći eritrocite bez hemoglobina (engl. *ghost cells*) kojima su dodani različiti analozi anilina. Nalazi upućuju na promjene indukcije methemoglobina ovisno o dozi i vremenu djelovanja do 78 % s *para*-bromo-fenilhidroksilaminom te do 75 % s *para*-jodofenilhidroksilaminom u usporedbi s 3 % do 5 % u kontrolnih uzoraka. U razdoblju od 14 dana nakon tretiranja izložene životinje izgubile su tri puta više krvi iz krvotoka od kontrolnih. Eritrociti su bili stabilniji u puferskoj otopini negoli u stanicama kojima je dodan *para*-jodofenilhidroksilamin. Zamijećen je i pad glutaciona u eritrocitima kojima je dodan fenilhidroksilamin odnosno *para*-jodofenilhidroksilamin. Analizom membrane eritrocita kojima je dodan *para*-jodofenilhidroksilamin zamijećeno je da se u pojedinim obradama proširila proteinska vrpca 2.1, a potpuno smanjila proteinska vrpca 2.2. Zamijećene promjene uvjetovane dozom i vremenom upućuju na primjenu hemotoksičnih parametara kao mogućih biopokazatelja u procjeni sigurnosti lijeka odnosno kemikalije.

KLJUČNE RIJEČI: *bjelančevine citoskeleta, hemolitička anemija, hemotoksičnost, fenilhidroksilamin, methemoglobin, para-bromofenilhidroksilamin, para-fluorofenilhidroksilamin, para-jodofenilhidroksilamin*

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