THE HEMOTOXICITY OF *PARA*-SUBSTITUTED ANILINE ANALOGS IN DOG AND RAT ERYTHROCYTES: A SPECIES COMPARISON

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Introduction: Hemolytic anemia, the early removal of erythrocytes from the circulation, has been recognized as a side effect of drugs and other environmental chemicals. Formation of methemoglobin (MetHb) following chemical exposure is the first hemotoxic response in the induction of hemolytic anemia. The purpose of this study was to compare the species differences in the chemical induction of MetHb in dog and rat erythrocytes exposed to *para*-substituted halogenated aniline analogs (phenylhydroxylamine, *p*-bromo-, *p*-fluoro-, and *p*-iodo-phenyhydroxylamine).

Methods: Whole blood was collected from a healthy, male Dalmatian dog that weighed 51 lbs and male Sprague-Dawley rats that weighed 100–125 g. Cells were washed (×3) with phosphate-buffered saline supplemented with glucose (pH 7.4). Methemoglobin (MetHb) induction was determined by treating aliquots with pre-specified micromoles of the test agents. Aliquots (75 µL) were removed from each treatment group at specific time points and mixed with cold hemolysis buffer for MetHb determination. Methemoglobin (MetHb) was determined spectrophotometrically at 635 nm.

Results: Methemoglobin (MetHb) levels in dog erythrocytes treated with the four analogs increased continuously over 180 minutes and showed no signs of declining. Methemoglobin (MetHb) levels in rat erythrocytes, however, immediately increased and continued to rise and fall before gradually approaching control levels.

Conclusions: Our data demonstrated the species differences in the MetHb-inducing ability of the analogs tested in both dog and rat erythrocytes. The differences in the patterns associated with MetHb induction in the animal models used may be attributed to variations in the MetHb reductase enzyme in both species. (*Ethn Dis*. 2005;15 [suppl 5]:S5-81–S5-87)

Key Words: Dog Erythrocytes, Hemotoxicity, Methemoglobin, *Para*-Substituted Aniline Analogs, Rat Erythrocytes

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INTRODUCTION

Methemoglobin (MetHb) formation in erythrocytes can be induced by a variety of chemical compounds.[1] One of the first signs of a hemotoxic response after chemical exposure is an increase in MetHb levels. Methemoglobin (MetHb) is formed when the iron in hemoglobin is oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. Normal hemoglobin binds reversibly to oxygen, while the met-form of the molecule does not. The enzymatic mechanism by which MetHb can be reduced to normal hemoglobin is an intrinsic property of erythrocytes. Jensen showed that the oxidation of hemoglobin in the erythrocytes of fish and pigs was dependent upon temperature, and that the rate at which the process occurred varied among species.[2]

Much in the literature concerns the presence of MetHb reductase (NADH-diaphorase) in human erythrocytes and rat erythrocytes.[3–7] Kuma et al were able to separate three fractions of diaphorase from the erythrocytes of normal individuals and members of a family diagnosed with congenital methemoglobinemia.[4] Of the three fractions designated A–C, only B and C were associated with the cells from persons suffering from methemoglobinemia. The authors therefore concluded that diaphorase A was the only one of the three isolates that functioned in the reduction of MetHb in human erythrocytes.

Exposure to MetHb-inducing agents can lead to hemolytic anemia (the early removal of erythrocytes from the circulatory system at a rate that surpasses the bone marrow's ability to compensate). This disease state is a side effect of certain drugs, such as dapsone, pamaquine and primaquine, as well as other environmental chemicals.[1] Early removal of circulating erythrocytes can result in an increase in the amount of indirect bilirubin formed when hemoglobin is broken down.

The chemical aniline is used to manufacture dyes and antioxidants and has also been used to produce pharmaceutical products.[1] Aniline and its analogs have been reported to cause an increase in MetHb levels in rats and dogs after inhalation therapy.[8,9] Morphologic alterations have also been observed in rat erythrocytes treated with the *N*-hydroxy derivative of dapsone.[10] Specifically, the shape of the erythrocytes became echinocytic in a concentration-dependent manner associated with hemotoxicity. Damage to the circulating erythrocytes as a result of aniline hydrochloride exposure has also been associated with lesions of the spleen observed in rats.[11]

A number of published studies focused on MetHb formation due to aromatic amines and substituted derivatives of aniline.[12–14] These studies all looked at MetHb induction in vivo, while French et al ranked a number of direct-acting chemicals as well as several compounds that needed to be bioactivated, according to their ability to induce MetHb in Dorset sheep erythrocyte suspensions.[15] The compound that illicited the most potent induction of MetHb was a *para*-substituted analog, *p*-dinitrobenzene.

The objective of this study was to determine species differences in the induction of MetHb formation in rat and dog erythrocytes treated directly

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with exogenous concentrations of phenyhydroxylamine (PHA), *p*-fluoro-PHA, *p*-bromo-PHA, and *p*-iodo-PHA.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 100–125 g were obtained from Harlan Laboratories (Indianapolis, Ind). The rats were given food and water and acclimated for seven days before use in experimental protocols. The light cycle in the animal facility was timer controlled to provide 12 hours of fluorescent light and 12 hours of darkness each day. A male Dalmatian housed and cared for at Skidaway Animal Hospital (Savannah, Ga) served as a dog blood donor.

Chemicals

The *para*-substituted halogenated aniline analogs used in this project were synthesized at the Medical University of South Carolina (Charleston, SC) from respective starter compounds (eg, 1-Iodo-4-nitrobenzene, 1-Bromo-nitrobenzene, 1-Fluor-4-nitrobenzene, etc) purchased from Aldrich Chemical Company (Milwaukee, Wis). Each analog was synthesized by reduction with zinc dust and ammonium chloride. All buffer components were purchased from Sigma Chemical Company (St. Louis, Mo).

Blood Collection

Blood was collected from the descending aorta of anesthetized rats with a heparinized 22-gauge needle and 10-cc syringe. Dog blood was collected from a 51-lb Dalmatian immediately before use. The samples were washed three times with 50 mL phosphate-buffered saline supplemented with glucose (PBSG; pH 7.4) and placed in a refrigerated Eppendorf centrifuge (Model 5403) for five minutes at 10,000 revolutions per minute (21,780 *g*). The cells were centrifuged for seven minutes after the third wash to better pellet the erythrocytes. The supernatant was aspirated into a vacuum flask after each centrifugation to remove the serum and buffy coat, leaving the packed erythrocytes behind. The hematocrit of the resulting packed erythrocytes was determined and then adjusted to 40% by heparinized capillary tubes.

Methemoglobin Assay

Packed erythrocytes (2 mL) were pipetted into plastic scintillation vials. The control group was dosed with vehicle only (10 µL acetone), while the experimental groups received 100 µmol/L, 200 µmol/L, or 300 µmol/L of an aniline analog. For initial MetHb readings, 75-µL aliquots were immediately removed from each treatment group and mixed with 5 mL ice cold hemolysis buffer (0.277% KH_2PO_4, .289% Na_2PO_4, and.05% Triton-X 100). The vials were then placed in a constantly shaking 37°C (98.6°F) water bath (Precision Shallow Form Shaking Bath, Fisher Scientific, Suwanee, Ga.) and removed at the specified time for subsequent MetHb readings. Equivalent aliquots were collected at 5, 10, 15, 30, 60, 120, and 180 minutes and then mixed with hemolysis buffer before taking the MetHb reading. Duplicate samples for each time-point were collected. The centrifuge tubes containing the hemolysis buffer were capped and kept on ice until samples for all the time-points had been collected.

Two rows of disposable methacrylate cuvettes (Fisher Scientific; 1.5 mL) were set up in styrofoam holders for each sampling time-point. The first cuvette in each row remained empty, while 20 µL of a 10% potassium cyanide solution (KCN; Sigma Chemical Company, St. Louis, Mo.) was pipetted into the second and fourth cuvettes. We placed 20 µL of a 20% potassium ferricyanide solution ($K_3Fe(CN)_6$; Sigma Chemical Co.) into cuvettes 3 and 4.

We put 1 mL hemolysis buffer/erythrocyte mixture into cuvettes 1 through 4. Spectrophotometric analysis was obtained at 640 nm with a Beckman Coulter Spectrophotometer (Model DU 640B).

The levels of induced MetHb assayed in this project are reported as averaged values plus or minus the standard error of the means (SEM) for four sets of experiments conducted in duplicate for each analog.

STATISTICS

Statistical analysis of the data was performed with DeltaGraph 4. The values obtained for MetHb were entered into a spreadsheet within the program in order to determine the SEM. Average values were used to graph data.

RESULTS

Methemoglobin (MetHb) percentages in male dog erythrocytes exogenously exposed to aniline analogs demonstrated a slow but steady increase throughout the time course of the experiments (Fig 1A–C). Samples taken five minutes after analog exposure revealed an almost linear increase in the percentage of MetHb. Methemoglobin (MetHb) levels assayed 30 minutes after initial incubation of dog erythrocytes with PHA, *p*-fluoro-, and *p*-iodo-PHA were all ≈13%, while cells treated with *p*-bromo-PHA yielded an average MetHb level of 8% ± 0.14%. After one hour of incubation with 100, 200, and 300 µmol/L of the various analogs, chemically induced MetHb levels for each compound were graphically distinguishable from each other (Fig 1A–C). The highest MetHb levels at this particular time point were observed in erythrocytes treated with 300 µmol/L of PHA (21.1% ± 1.2%) and *p*-fluoro-PHA (19.4% ± 5.5%). Dog erythrocytes treated with

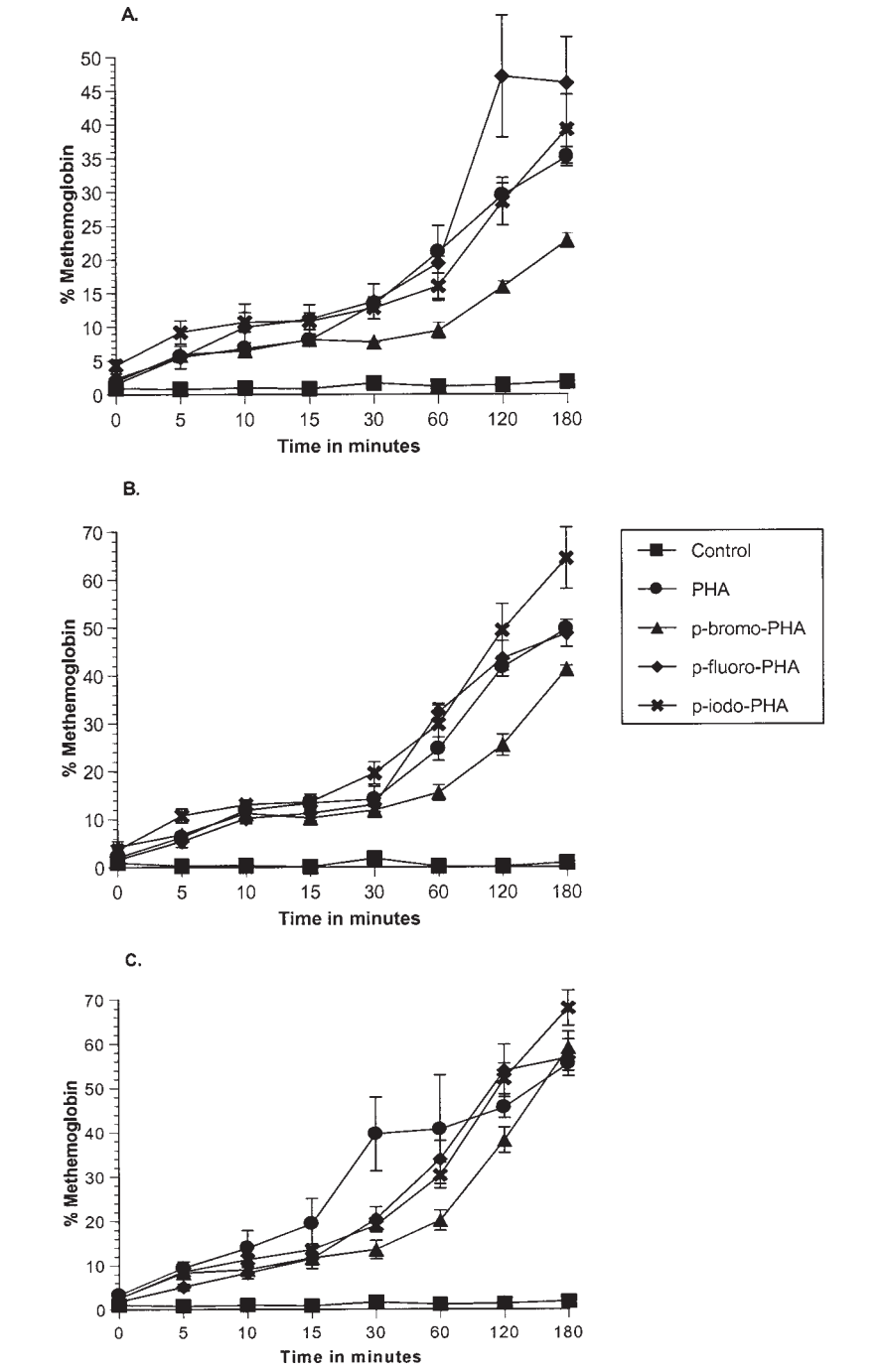

Fig 1. Comparison of four aniline analogs on the formation of methemoglobin in male dog erythrocytes. (A) Chemically induced MetHb levels in red blood cells treated with 100 μM of each analog. (B) Chemically induced MetHb levels in red blood cells treated with 200 μM of each analog. (C) Chemically induced MetHb levels in red blood cells treated with 300 μM of each analog

100 μmol/L of *p*-fluoro-PHA for 180 minutes (Fig 1A) demonstrated the highest MetHb levels of all the analogs tested (46.2% ± 5.1%), while the lowest induced MetHb levels were observed in cells incubated with *p*-bromo-PHA (22.8% ± 1%). Erythrocytes exposed to 200 and 300 μmol/L of *p*-iodo-PHA for 180 minutes revealed the highest MetHb levels (64.45% ± 6.4% and 68.03% ± 4%, respectively) as compared to the control and other analogs at these two chemical concentrations.

Methemoglobin (MetHb) levels did not rise above 50% in dog erythrocytes treated with 100 μmol/L of the four analogs. When incubated with 200 μmol/L of each analog, MetHb percentages greater than 50% were observed in dog erythrocytes exposed to *p*-iodo-PHA. The results show that an increase in analog concentrations to 300 μmol/L brought about an elevation in the MetHb levels associated with dog erythrocytes treated with *p*-bromo-, *p*-fluoro-, and *p*-iodo-PHA.

In contrast, male rat erythrocytes exposed to the same concentrations of aniline analogs demonstrated an initial (T_0) increase in MetHb levels as compared to the control percentages (Fig 2A–C). Within five minutes, assayed MetHb levels in cells treated with the four aniline analogs at all concentrations tested were elevated above the controls. Chemically induced MetHb percentages in rat erythrocytes displayed patterns of rising and falling as the experimental time progressed. Erythrocytes incubated in the presence of varying concentrations of aniline analogs revealed a consistent pattern with respect to the analogs capable of inducing the greatest and lowest MetHb levels. In all experimental analog concentrations, *p*-bromo-PHA yielded the highest MetHb percentages at 180 minutes post-treatment (50.7% ± 6.5%, 52.2% ± 10.1%, and 71.8% ± 1%, respective of increasing analog concentrations from 100 to 300 μmol/L). Rat erythrocytes treated with *p*-fluoro-PHA showed the lowest MetHb levels, ranging from 7.3% ± 2.3% to 13.3% ± 2.7%. In rat erythrocytes, *p*-bromo-PHA consistently increased MetHb levels above 50% throughout the duration of the experiments. Methemoglobin (MetHb) percentages in rat ery-

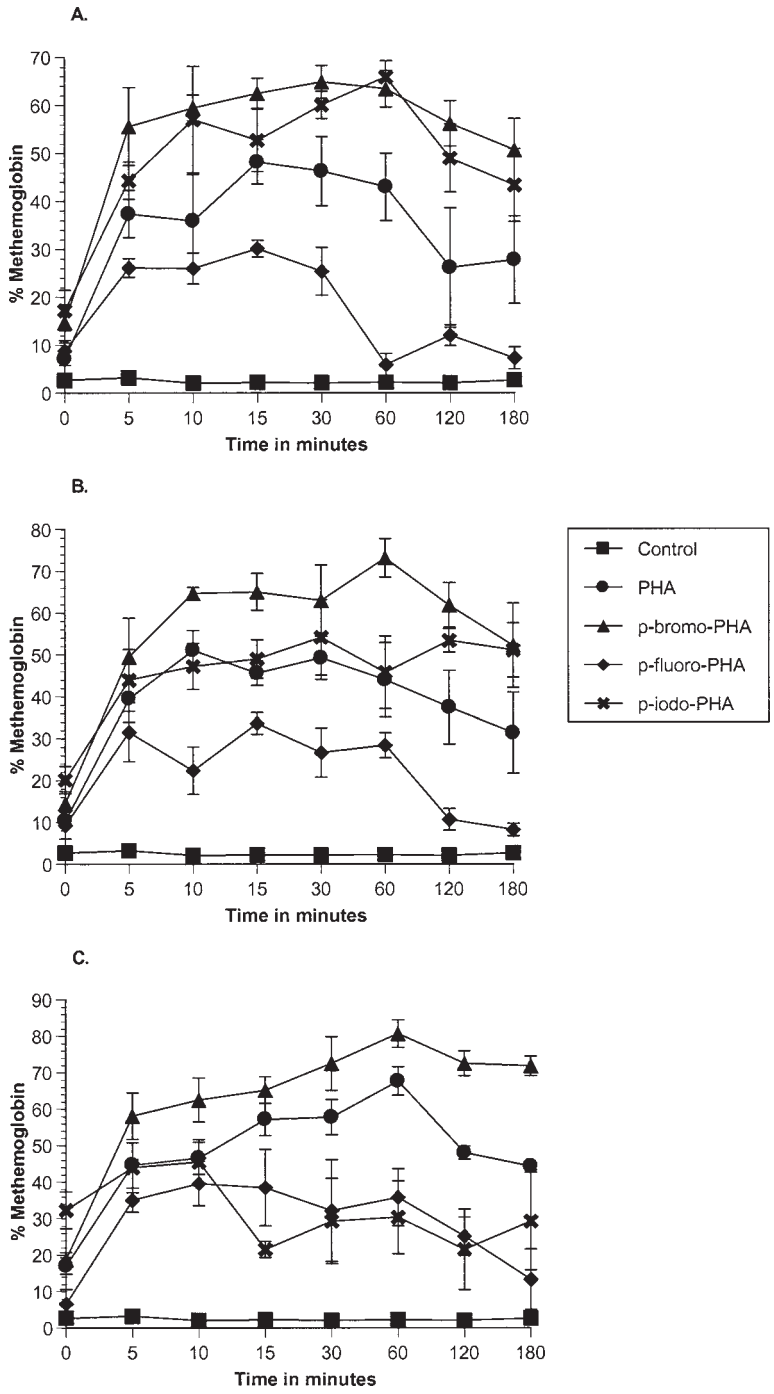

Fig 2. Comparison of four aniline analogs on the formation of methemoglobin in male rat erythrocytes. (A) Chemically induced MetHb levels in red blood cells treated with 100 μM of each analog. (B) Chemically induced MetHb levels in red blood cells treated with 200 μM of each analog. (C) Chemically induced MetHb levels in red blood cells treated with 300 μM of each analog

throcytes remained <50% with all concentrations of *p*-fluoro-PHA.

Figure 3A–C demonstrates the difference in chemically induced MetHb in dog and rat erythrocytes as a result of treatment with 100, 200, and 300 μmol/L of *p*-bromo-PHA. The initial MetHb percentages observed in rat erythrocytes following exposure to *p*-bromo-PHA were >12% for all concentrations tested. Initial MetHb levels in dog erythrocytes exposed to the same chemical were 2%–6%. Five minutes after exposure to *p*-bromo-PHA, rat erythrocytes showed a better than two-fold increase in mean MetHb levels, while MetHb percentages in dog erythrocytes displayed slight increases regardless of concentration.

DISCUSSION

As evidenced by these experiments, *para*-substituted aniline analogs caused an increase in the MetHb levels associated with Dalmatian dog and Sprague-Dawley rat erythrocytes in vitro. The MetHb levels induced in dog erythrocytes after initial exposure to each concentration of an aniline analog ranged from 1% to 5%. According to the data, *p*-iodo-PHA was the most potent MetHb inducer in dog erythrocytes treated with 200 and 300 μmol/L.

A previous study on the affect of inhaled aniline on MetHb induction has been reported in purebred beagles.[10] The author exposed both male and female beagles to aniline vapor for four hours. The data indicated MetHb levels rose to a maximum value of 5% during three hours (after inhalation). By the final hour of aniline inhalation therapy, MetHb levels showed signs of decreasing in three of the dogs but continued to rise in one dog. Pauluhn also administered oral doses of aniline (equivalent to inhaled concentrations) to beagles and noted resultant MetHb levels that were slightly greater than 26%. The reason for the difference in the MetHb levels induced by the two routes of exposure was unclear to the author. He postulated that the levels of MetHb associated with inhalation exposure could have been due to reduction in the amount of inhaled aniline remaining within the respiratory tract, or the fact that aniline is bioacti-

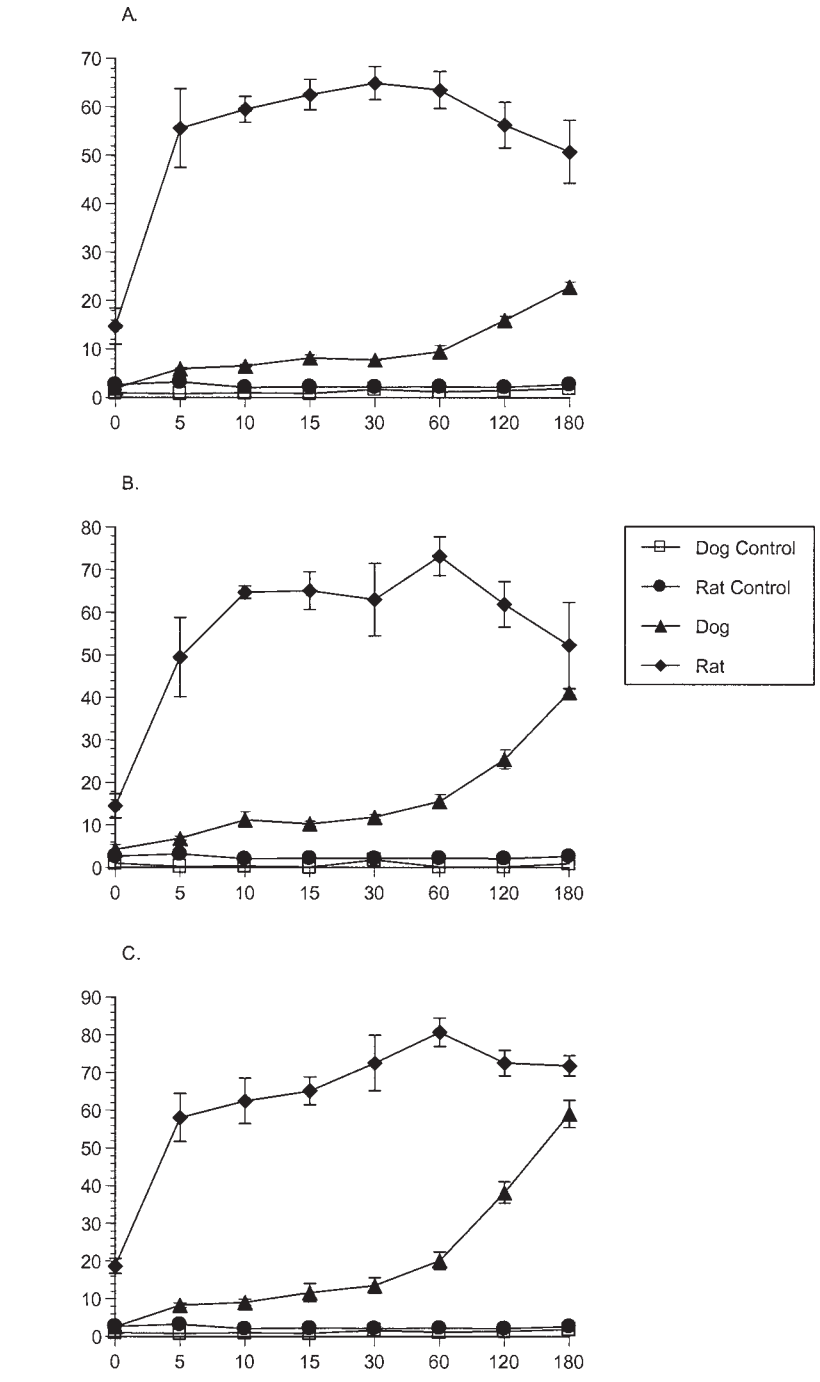

Fig 3. Comparison of chemically induced methemoglobin levels in dog and rat erythrocytes treated with p-bromo-PHA: (A) 100 μM, (B) 200 μM, and (C) 300 μM

vated in the gastrointestinal tract and liver.

The three-hour duration of the present project did not reveal any indication of an overall decrease in dog MetHb levels. An observation in dog erythrocytes treated with 100 μmol/L of *p*-fluoro-PHA is the occurrence of the MetHb peak after 120 minutes of exposure. A decrease in the mean MetHb levels of the erythrocyte samples followed 180 minutes of exposure to *p*-fluoro-PHA. In contrast, dog erythrocytes treated with this same analog at a concentration of 200 or 300 μmol/L displayed the highest levels of MetHb at the 180-minute sampling point. Another observation is the greater than two-fold increase in the MetHb levels associated with dog erythrocytes exposed to 300 μmol/L of PHA for 30 minutes. The sharp increase in MetHb levels at this particular time point was not noted in any of the other aniline analogs tested.

A concentration-dependent dose response to treatment was seen with 200 and 300 μmol/L of the analogs. For this reason, additional experiments were carried out for a total of 300 minutes to ascertain the point at which the percentage of chemically induced MetHb would begin to decline (unpublished data). The levels of MetHb continued to rise after five hours of chemical exposure, which suggests a deficiency in the MetHb reductase enzyme system in dogs.

Closer observations of Figure 2 indicate the highest MetHb levels were associated with rat erythrocytes after 60 minutes of exposure to *p*-iodo- and *p*-bromo-PHA. At a concentration of 100 μmol/L, *p*-iodo-PHA demonstrated the highest concentration of MetHb (65.89% ± 3.4%), while cells treated with the same concentration of *p*-bromo-PHA had a MetHb concentration of 63.43% ± 3.8%. After 60 minutes of exposure to 200 and 300 μmol/L of *p*-bromo-PHA, MetHb levels were at 73.18% ± 4.6% and 80.69% ± 3.8%, respectively. However, rat erythrocytes treated with 300 μmol/L of *p*-iodo-PHA displayed the lowest MetHb levels after 60 minutes of incubation (30.31% ± 10%). The peak in MetHb levels for this same aniline analog at the greatest concentration tested occurred after 10 minutes (45.46% ± 6.2%).

The initial (T_0) MetHb levels detected in rat erythrocytes were higher in cells treated with *p*-iodo-PHA at all concentrations used. However, after five minutes of incubation, the percentages of chemically induced MetHb were

highest in cells treated with *p*-bromo-PHA. The mean percentages of MetHb assayed in rat erythrocytes rose and fell throughout the 180-minute experimental period. At the end of the sampling time, two constants were demonstrated: *p*-bromo-PHA yielded the highest MetHb percentages, and *p*-fluoro-PHA showed MetHb levels that were near or below initial values. Though MetHb levels detected in cells treated with 100 µmol/L of *p*-iodo-PHA for 60 minutes (65.89% ± 3.4%) were slightly higher than the percentages assayed in cells exposed to *p*-bromo-PHA (63.43% ± 3.8%), *p*-bromo-PHA appeared to be the most potent MetHb-inducing agent in rat erythrocytes regardless of concentration.

The effect of halogenated aniline derivatives on erythrocytes is well documented.[13–15] In concurrence with the present study, Valentovic et al, showed that a halogenated aniline analog could bring about an increase in the percentage of MetHb associated with rat erythrocytes and that the induction of MetHb decreased in exposed erythrocytes within four hours.[14]

In an attempt to rank MetHb inducing agents, French et al were able to determine that *para*-nitrobenzene was the most effective inducer of MetHb in Dorset sheep erythrocytes, followed by an *ortho*-substituted version of the same compound.[15] The effect of phenylhydroxylamine on the survival of ^{51}Cr tagged rat erythrocytes has been reported.[11] The results of this group's work demonstrated that aniline does not possess hemolytic activity in vitro; however, its metabolite, PHA, was found to be hemotoxic to erythrocytes.

The halogens as a group require one electron in their outermost shell to make stable configurations. The sharing of electrons with nonmetals that are not as electronegative causes the halogens to act as oxidizing agents when their outer shell is completed. Fluorine is the most electronegative of all of the halogens (3.98 on the Pauling scale). As evidenced by the data, dog erythrocytes treated with 100 µmol/L of *p*-fluoro-PHA for 180 minutes displayed the highest MetHb levels as compared to dog erythrocytes exposed to vehicle alone, or the other aniline analogs used. Iodine has an electronegativity of 2.66. Dog erythrocytes exposed to 200 and 300 µmol/L of each halogenated analog revealed higher levels of MetHb associated with *p*-iodo-PHA 180 minutes after treatment.

In rat erythrocytes, exposure to *p*-bromo-PHA elicited higher MetHb percentages than the other *para*-substituted analogs at concentrations of 200 and 300 µmol/L. The same trend was observed in rat erythrocytes exposed to 100 µmol/L of aniline analogs up to 30 minutes. After 60 minutes of treatment, rat erythrocytes incubated with *p*-iodo-PHA displayed slightly higher MetHb percentages than rat erythrocytes exposed to *p*-bromo-PHA. The mean MetHb levels noted in dog erythrocytes treated with *p*-bromo-PHA were lower than the percentages recorded for the other halogenated aniline analogs. The electronegativity of the *para*-substituted aniline analogs used in this study as well as other factors may play a role in the ability of these compounds to induce a hemolytic response in the form of an increase in MetHb percentages.

Dog and rat erythrocytes behaved differently with regard to the chemical induction of MetHb by halogenated *para*-substituted aniline analogs. The differences in MetHb induction between erythrocytes from the two species incubated with the various aniline analogs may indicate species variations in the MetHb reductase enzyme. The results of this project support the conclusion that species differences exist in the chemical induction of MetHb. Further studies are necessary to determine the relationship of chemically induced MetHb percentages and the activity of the MetHb reductase enzyme over time within the same sample of erythrocytes.

IMPLICATIONS FOR IMPROVING HEALTH DISPARITIES

A number of drugs are known to induce hemolytic anemia. Understanding the exact nature of how these drugs alter erythrocytes, thus targeting them for premature removal, could be vital in the pharmaceutical modification of the compounds or the development of new drugs.

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