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

Elissa T. Purnell, PhD; Harpal Singh, PhD

INTRODUCTION

Methemoglobin (MetHb) formation in erythrocytes can be induced by a variety of chemical compounds. 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 (Fe²⁺) to the ferric (Fe³⁺) 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. Much in the literature concerns the presence of MetHb reductase (NADH-diaphorase) in human erythrocytes and rat erythrocytes. 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. 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, pramaquine and primaquine, as well as other environmental chemicals. 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. Aniline and its analogs have been reported to cause an increase in MetHb levels in rats and dogs after inhalation therapy. Morphologic alterations have also been observed in rat erythrocytes treated with the N-hydroxy derivative of dapsone. 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. A number of published studies focused on MetHb formation due to aromatic amines and substituted derivatives of aniline. 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. The compound that elicited 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...
with exogenous concentrations of phe- 

nyldihydroxyamine (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 con- 

trolled to provide 12 hours of fluo- 

rescent 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-nitro- 

benzene, 1-Fluor-4-nitrobenzene, etc) 

purchased from Aldrich Chemical 

Company (Milwaukee, Wis). Each an- 

alog 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 de- 

scending 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 refrig- 

erated Eppendorf centrifuge (Model 

5403) for five minutes at 10,000 revolu-

tions per minute (21,780 g). The cells 

were centrifuged for seven minutes after 

the third wash to better pellet the 

erthrocytes. The supernatant was aspi- 

rated into a vacuum flask after each 

centrifugation to remove the serum and 

buffy coat, leaving the packed erythro-

cytes behind. The hematocrit of the 

resulting packed erythrocytes was de- 

termined 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 treat-

ment group and mixed with 5 mL ice 

cold hemolysis buffer (0.277% 

KH2PO4, .289% Na2PO4, and 0.05% 

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, 

Savanece, Ga.) and removed at the 

specified time for subsequent MetHb 

readings. Equivalent aliquots were col-

lected 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 hemol-

ysis buffer were capped and kept on ice 

until samples for all the time-points had 

been collected.

Two rows of disposable methacryl-

ate 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 Chemi-

cal Company, St. Louis, Mo.) was 
pipetted into the second and fourth 
cuvettes. We placed 20 µL of a 20% 
kation ferricyanide solution 

(K3Fe(CN)6; Sigma Chemical Co.) into 

cuvettes 3 and 4.

We put 1 mL hemolysis buffer/ 

erthrocyte 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) percent- 

ages in male dog erythrocytes exoge-

nously exposed to aniline analogs dem- 

onstrated 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. Methemo-

globin (MetHb) levels assayed 30 min-

utes after initial incubation of dog 

erthrocytes 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 graphi-

cally 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
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 (T₀) 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-
Erythrocytes 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 twofold 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 effect of inhaled aniline on MetHb induction has been reported in purebred beagles. 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-
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 (T₀) 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
CHEMICALY INDUCED METHEMOGLOBINEMIA – Purnell and Singh

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 exposed to 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. 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. 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. The effect of phenylhydrazine on the survival of 51Cr tagged rat erythrocytes has been reported. 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 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.

ACKNOWLEDGMENTS

The authors thank Dr. John E. Otis for assistance in the synthesis of aniline analogs, Dr. Lesley Mailler and staff at Skidaway Animal Hospital for providing whole blood samples and taking care of Pogo, and Mrs. Melva Coles Bostick for her technical assistance in conducting experiments.

Funding for this project was provided by Title III and NHLBI #5K14HI003540. All experiments were conducted with the approval of and in adherence with the established guidelines of the Institutional Animal Care and Use Committee of Savannah State University and NIH Assurance #A4216-01.

REFERENCES