AFRL-RH-WP-TR-2014-0154

INTERACTION OF JET FUEL HYDROCARBON COMPONENTS WITH RED BLOOD CELLS AND HEMOGLOBIN

Jason J. Jacobsen
Anthony B. Polito III
AFIT/CI

Richard R. Chapleau, Ph.D.
Elizabeth I. Maurer
Jeanette S. Frey
Henry M. Jackson Foundation
for the Advancement of Military Medicine

Trevor J. Bihl
USAF AETC AFIT/ENS

Camilla A. Mauzy, Ph.D.
Bioeffects Division
Molecular Bioeffects Branch
2729 R Street, Bldg 837
Wright-Patterson AFB, OH 45433-5707

June 2014
Interim Report

Air Force Research Laboratory
711th Human Performance Wing
Human Effectiveness Directorate
Bioeffects Division
Molecular Bioeffects Branch
WPAFB, OH 45433-5707

NOTICE AND SIGNATURE PAGE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation; or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

Qualified requestors may obtain copies of this report from the Defense Technical Information Center (DTIC) (http://www.dtic.mil).

The experiments reported were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

(AFRL-RH-WP-TR - 2014 - 0154) has been reviewed and is approved for publication in accordance with assigned distribution statement.

MATTIE.DAVID.R.1230101880

Digitally signed by MATTIE.DAVID.R.1230101880
DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USAF, cn=MATTIE.DAVID.R.1230101880
Date: 2015.06.18 13:46:22 -04'00'

DAVID R. MATTIE, Work Unit Manager
Molecular Bioeffects Branch

POLHAMUS.GARRETT.D.1175839484

Digitally signed by POLHAMUS.GARRETT.D.1175839484
DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USAF, cn=POLHAMUS.GARRETT.D.1175839484
Date: 2015.06.24 21:12:02 -05'00'

GARRETT D. POLHAMUS, DR-IV, DAF
Chief, Bioeffects Division
Human Effectiveness Directorate
711th Human Performance Wing
Air Force Research Laboratory

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute the Government’s approval or disapproval of its ideas or findings.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
24-06-2014	Interim Technical Report	August 2013 - June 2014

4. TITLE AND SUBTITLE

Interaction of Jet Fuel Hydrocarbon Components With Red Blood Cells and Hemoglobin.

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jason J. Jacobsen, Anthony B. Polito III, Richard R. Chapleau, Elizabeth I. Maurer, Jeanette S. Frey, Trevor J. Bihl, *Camilla A. Mauzy

5d. PROJECT NUMBER
7757/H0D1

5e. TASK NUMBER

5f. WORK UNIT NUMBER
7757HD05/H0D1

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Air Force Materiel Command, Air Force Research Laboratory, 711th Human Performance Wing, Human Effectiveness Directorate, Bioeffects Division, Molecular Bioeffects Branch (RHDJ). Wright-Patterson AFB OH 45433-5707

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Materiel Command, Air Force Research Laboratory, 711th Human Performance Wing Human Effectiveness Directorate, Bioeffects Division, Molecular Bioeffects Branch (RHDJ), Wright-Patterson AFB OH 45433-5707

10. SPONSOR/MONITOR'S ACRONYM(S)
711 HPW/RHDJ

11.SPONSORING/MONITGENCY REPORT AGENCY REPORT Number
AFRL-RH-WP-TR-2014-0154

12. DISTRIBUTION AVAILABILITY STATEMENT

Distribution A: Approved for public release; distribution unlimited. (PA Case no 88ABW-2015-3106, 18 June 2015)

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This study examined the impact of five jet fuel hydrocarbon components on red blood cells (RBCs). We examined the biochemical changes to RBCs by measuring mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and red cell distribution width (RDW). Exposed cells were imaged using scanning electron microscopy (SEM) to verify morphological changes. The induction of oxidative stress was examined using glutathione (GSH) depletion as a marker of reactive oxygen species. Finally, microscale thermophoresis (MST) was used to determine the binding interactions between human hemoglobin and the test set of hydrocarbon compounds. For some chemicals, MCV (toluene, decane), RDW (toluene, octane, ethylbenzene), and MCH (ethylbenzene) values were sensitive to exposure incubation temperatures (room temperature versus 37 ^{o}C). SEM imaging indicated formation of ~1% crenated red blood cells in all lower dose exposure sets. Dose dependent oxidative stress was seen for all chemical exposures with the exception of high concentrations of tetradecane and toluene. MST revealed binding affinities between purified human hemoglobin monomer and the hydrocarbons decane (KD = 2.4 μM), tetradecane (KD = 8.8 μM), and octane (KD = 5.6 μM), with toluene demonstrating the tightest binding to hemoglobin at KD = 1.9 μM. Collectively, the apparent increase in the surface area of the cell membrane, GSH depletion, and interaction between the hydrocarbon and hemoglobin molecule may contribute to potential toxicity of these chemicals causing adverse effects on hemodynamics and circulatory function.

15. SUBJECT TERMS

JP-8; jet fuel; oxidative stress; toluene; decane; octane; tetradecane; ethylbenzene; MCV; MCH; exposure; RBC; thermophoresis; hemoglobin; leukocyte, scanning electron microscopy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
U				36	Camilla A. Mauzy
a. REPORT	b. ABSTRACT	c. THIS PAGE	SAR		19b. TELEPONE NUMBER (*Include area code)*
U	U	U			937-904-9535

THIS PAGE INTENTIONALLY LEFT BLANK.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

PREFACE

This research was accomplished at the Molecular Bioeffects Branch, Bioeffects Division, Human Effectiveness Directorate of the 711th Human Performance Wing (711 HPW/RHDJ) of the Air Force Research Laboratory (AFRL), Wright-Patterson AFB, OH, Dr. John J. Schlager, Branch Chief. This interim technical report was written for the Mixtures Toxicology Work Unit 7757HD05 in the Aerospace Toxicology program at AFRL.

Part of the reported experiments and data were completed in partial fulfillment for the Master Thesis requirements within Department of Immunohematology, Health Sciences from the School of Medicine and Health Sciences at George Washington University, Washington, DC, and were completed by author Jason Jacobson. His M.S.H.S thesis was submitted and defended successfully under the title "Effects of Jet Fuel Adducts on Red Blood Cells."

The use of pre-existing human blood samples in this study was reviewed by the AFRL Internal Review Board (IRB No. FWR20140022N), which determined that this study did not fall under the purview of human use regulations as per 32 CFR 219.109 (f).

ACKNOWLEDGEMENTS

This work was supported by the 711th Human Performance Wing's Human Effectiveness Directorate (RHDJ), Wright-Patterson AFB, OH. The authors would like to thank Maj. Paul Eden, Nicole Schaeublin, Christin Grabinski, Dr. Jeff Gearhart, and Dr. Peter Robinson at RHDJ for their help and advice with this study. Thanks go to Chris Ruark for his excellent content review and comments. We would also like to thank LtCol. Norman Fox (Laboratory Flight Commander), Mrs. Nersa Loh (Supervisor, Transfusion Services), and Mr. Dan Fischer (Supervisor, Hematology) at the 88th Medical group, Wright-Patterson AFB, for their invaluable assistance with acquiring pre-existing blood samples as well as hematology data.

SUMMARY

Jet propulsion fuel-8 (JP-8) is the primary military fuel used by the Department of Defense (DoD), which consumes more than 5 billion gallons each year. Jet propulsion fuel constitutes the largest chemical mixture exposure in the military, occupationally exposing an estimated 2 million people annually.[1] Limited evidence indicates some health consequences of human exposure to JP-8. To examine the impact of jet fuel exposures on erythrocytes, this study examined the impact of five jet fuel hydrocarbon components on red blood cells (RBCs). We examined the biochemical and morphological changes to RBCs by measuring mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and red cell distribution width (RDW). Exposed cells were also imaged using scanning electron microscopy (SEM) to verify morphological changes and to identify types of erythrocyte deformability. The induction of oxidative stress in chemically-exposed whole blood was examined using glutathione (GSH) depletion as a marker of reactive oxygen species. Finally, microscale thermophoresis (MST) was used to determine the binding interactions between human hemoglobin and the test set of hydrocarbon compounds. The results indicated that whole blood exposed to toluene increased the MCV. For some chemicals, MCV (toluene, decane), RDW (toluene, octane, ethylbenzene), and MCH (ethylbenzene) values were sensitive to exposure incubation temperatures (room temperature versus 37 ^{o}C). SEM imaging indicated formation of ~1% crenated red blood cells in all lower dose exposure sets. In the case of toluene, these morphological changes were seen at dose levels identified in human studies for non-lethal exposures. GSH levels significantly decreased for all chemical exposures with the exception of high concentrations of tetradecane and toluene. Finally, thermophoretic analysis revealed binding affinities between purified human hemoglobin monomer and the hydrocarbons decane, toluene, tetradecane, and octane, with toluene demonstrating the tightest binding to hemoglobin with a K_D = 1.9 μM, with decane K_D = 2.4, tetradecane K_D = 8.8, and octane K_D = 5.8. Ethylbenzene did not demonstrate any appreciable binding. Collectively, the apparent increase in the surface area of the cell membrane, GSH depletion, and interaction between the hydrocarbon and hemoglobin molecule may contribute to potential toxicity in these chemicals via adverse effects on hemodynamics and circulatory function.

Key Words: JP-8; jet fuel; oxidative stress; toluene; decane; octane; tetradecane; ethylbenzene; MCV; MCH; exposure; RBC; thermophoresis; hemoglobin; leukocyte, scanning electron microscopy

Interim report August 2013 to June 2014

1. INTRODUCTION

Jet propulsion fuel-8 (JP-8) is the primary military fuel used by the United States Air Force, Department of Defense (DoD) and North Atlantic Treaty Organization (NATO) allied countries.[1,32] It is a kerosene-based fuel with a complex composition containing more than 200 aliphatic and aromatic hydrocarbon compounds. The DoD uses more than 5 billion gallons each year with jet propulsion fuels constituting the largest single chemical exposure in the military, occupationally exposing an estimated 2 million people annually.[1] Occupational exposure to JP-8 occurs primarily during aircraft refueling and defueling, transportation and storage of fuel, general maintenance of aircraft and military vehicles, and cleaning and degreasing of parts.[43] Due to the widespread use of JP-8, there is a growing concern regarding the health consequences for fuel handlers, mechanics, and flight-line personnel who are regularly exposed. Past exposure studies have indicated an increased incidence of fatigue, emotional dysfunction, dermal irritation, headaches, postural sway imbalances, decreased attention spans, elevated white blood cell counts, as well as adverse effects on liver function and the respiratory system.[1,4]

One potential mechanism for JP8 toxicity involves increasing cellular oxidative stress. Increases in reactive oxygen species (ROS) create an oxidative environment which promotes apoptosis, in part, by depleting cellular antioxidants like glutathione (GSH) and inducing direct damage to DNA, proteins, and lipids.[5,6] Alterations in GSH levels can promote oxidative stress, potentially leading to apoptotic cell death.[7,8] JP-8 mixture exposures have been shown to alter immune function as well as trigger cellular apoptosis.[3,8] Such toxicity is in agreement with other petroleum hydrocarbon toxicity studies, which have been shown to elicit mechanisms dependent on oxidative stress, in addition to increasing lipid peroxidation and reducing enzymatic antioxidant defense mechanisms.[10,11] One such study using benzene, a component of JP-8, indicated that bioactivation of the chemical led to the formation of ROS decreasing antioxidant activity and consequently increasing oxidative stress.[1,10]

In addition to potential toxicity to exposed personnel, blood donations from an exposed individual may have transfusion efficacy, storage life, and tolerance altered by oxidative stress induced by hydrocarbon exposure. Such changes in the RBC rheological properties may contribute to poor outcomes for transfusion recipients.[12,13] Interestingly, in addition to ROS accumulation and concomitant oxidative stress, benzene exposures have demonstrated strong associations with altered hemoglobin and red cell indices. Hematological changes were examined by complete blood counts (CBCs) and the data reported a decrease in hemoglobin content and an increased mean corpuscular volume (MCV), a measure of RBC size.[1,14] These data indicate that hydrocarbon exposures may indeed alter RBC morphology thus affect efficacy of oxygen transport.

While hydrocarbon-induced oxidative stress may alter the RBC morphology, certain chemicals may also penetrate the RBC membrane and bind to the hemoglobin protein itself. Hemoglobin is

the major protein in red blood cells, constituting about 90% of the cell's dry weight.[15] Previous studies have demonstrated an interaction between several hydrocarbons and various proteins, as well as cellular membranes.[15] It is possible that covalent bonding of hydrocarbon chemicals to hemoglobin could initiate the hematological effects observed in cellular toxicity. The current study aims to provide a perspective of the potential effects of jet fuel components on red blood cells and hemoglobin.

2. MATERIALS AND METHODS

2.1 Chemicals

Tetradecane (MW: 198.39), Ethylbenzene (MW: 106.17), Octane (114.23), Toluene (MW: 92.14), and Decane (MW: 142.29) were obtained from Sigma (St. Louis, MO). Purity was >98% for octane, 99% for all other test hydrocarbons.

2.2 Blood Samples

The project was reviewed by the Air Force Research Laboratory IRB and determined exempt from human experimentation requirements as per 32 CFR32 219.101. Fresh whole human blood was purchased from Bioreclamation (Hicksville, NY). Whole blood was collected in ethylenediaminetetraacetic acid (disodium EDTA) tubes for SEM studies or in sodium citrate for exposure studies. Matching serum from the same subject was collected for use in exposure studies. Expired packed red blood cell (PRBC) units were obtained from the 88th Medical group for use in hemoglobin purification.

2.3 Chemical Exposures

Samples were prepared in decreasing concentrations by using serum as a carrier to dilute the test chemical to appropriate molarity prior to the addition to 260 µl of whole blood (**Table 1**). The addition of equal volumes (in this case, 40 µl) to the whole blood (WB) sample was necessary to insure that the RBC levels are equal throughout the dilution series. Controls were prepared by using 300 µl of whole blood only and 260 µl of whole blood mixed with 40 µl of serum. All samples were incubated for one hour at 22 ^{o}C (room temperature) or 37 ^{o}C prior to testing. Final Molarity of the hydrocarbon in the whole blood solution are indicated in **Table 2**.

Table 1. Dilution series for whole blood exposures**.**

Dilution	**Compound (C)** **volume**	**Serum (S)** **volume**	**(C+S)Volume added to Whole Blood**	**Whole Blood (WB) Used**
1	40 µl	0 µl	40 µl	260 µl
2	40 µl	40 µl	40 µl	260 µl
3	12 µl	108 µl	40 µl	260 µl
4	20 µl of Dil 3	180 µl	40 µl	260 µl
5	20 µl of Dil 4	180 µl	40 µl	260 µl
Control WB+S	0 µl	0 µl	40 µl	260 µl
Control WB	0 µl	0 µl	0 µl	300 µl

Table 2. Final Molarity in solution for whole blood exposures**.**

Dilution	**Final Molarity** in blood solution (M)				
	Toluene	**Octane**	**Decane**	**Tetradecane**	**Ethylbenzene**
1	1.25	0.82	0.68	0.51	1.09
2	0.63	0.41	0.34	0.26	0.54
3	0.13	0.08	0.068	0.05	0.11
4	0.013	0.008	0.0068	0.005	0.01
5	0.0013	0.0008	0.0006	0.0005	0.001

2.4 Clinical Blood Measurements

Clinical analyses of exposed blood samples were conducted using an automated Coulter LH 780 Hematology Analyzer (Beckman Coulter, Brea, California). Hematological data were analyzed using SigmaPlot (v.12.5) software (San Jose, California). Statistical differences between samples or sample/control ($p<0.05$) were assessed by one-way repeated measures analysis of variance (ANOVA). When normality was not justified, additional post-hoc analysis was performed using the Bonferroni or Kruskal-Wallis tests to determine pairwise comparisons.

2.5 Scanning Electron Microscopy (SEM)

2.5.1 Exposure of Whole Blood. Chemicals were diluted into matching serum as shown in **Table 3**. A total of 80 µl of three dilutions (1, 5, and 6) were added to 520 µl PRBCs. The blood/chemicals were incubated at either 37 ^{o}C or at room temperature (22 ^{o}C) for 1 hr. After the incubation, the solution was spun down at ~1000 *xg* for 5 min, and the supernatant removed. The cell pellet was gently washed three times using 1 ml sterile phosphate-buffered saline (PBS). The final cell pellet was resuspended in 500 µl PBS in preparation for SEM imaging (**Table 4**).

Table 3. Dilution series for whole blood exposures for SEM imaging. Dilutions 1 "High", 6 "low", and controls were used for imaging.

Dilution	**Compound** (C) **volume**	**Serum (S) volume**	**(C+S) Volume** added to Whole Blood (WB)	**WB Used**
1 "High"	80 µl	80 µl	80 µl	520 µl
3	12 ul	108 µl	0 µl	
4	10 µl of Dil 3	90 µl	0 µl	
5	10 µl of Dil 4	90 µl	80 µl	520 µl
6 "Low"	10 µl of Dil 5	190 µl	80 ul	520 µl
Control - Whole blood plus serum	0 µl	80 µl	80 µl	520 µl
Control - Whole blood	0 µl	0 µl	0 µl	600 µl

Table 4. Samples analyzed using SEM imaging.

Sample	Dilution			
	1	5	Low	UNDIL
Toluene 37 °C	X	X	X	
Toluene 22 °C	X	X		
Octane	X	X	X	
Decane	X	X	X	
Tetradecane	X	X	X	
Ethylbenzene	X	X	X	
WB+S Control				X
WB Control				X

2.5.2 Fixing Red Blood Cells. The exposed cells were centrifuged 10 min at ~1000 *xg*. The supernatant was removed and the cell pellet fixed by adding 2% paraformaldehyde (PF)/2.5% gluteraldehyde solution *v/v* with an incubation of 2 hr at room temperature (RT). After incubation, the cells were centrifuged and the solution removed. The cells were then washed three times using an equivalent volume of PBS. The cell pellet was dehydrated by adding 300 µl of 50% ethanol (EtOH) with a10 min incubation at RT. The cell solution was then centrifuged, the supernatant removed, and 300ul of 70% EtOH was added to the pellet and incubated 10 min at RT. The cell pellet was again harvested and washed two additional times using 70% EtOH with 10 min RT incubations. The cell pellet was washed one time using 300ul of 80% EtOH with a10 min incubation at RT, then again using 3000 µl 90% EtOH incubated 10 min RT. Three final washes using 300 µl 100% EtOH with 10 min RT incubations were conducted. Finally, 300 µl of 100% EtOH was added to the cell pellet and left at 4 °C until SEM imaging was conducted.

2.5.3 Preparation of SEM Stubs. After cell fixing, 400 µl of each cell solution was pipetted onto a clean aluminum SEM stub. The stub was dried at RT, and kept at RT until it was imaged.

2.5.4 SEM Imaging. The cells were imaged using a JOEL JSM-6610LV. The imaging settings (magnification and working distance) were optimized for each image. Sizes are indicated on each image.

2.6 Glutathione (GSH) Assay

The detection and quantification of glutathione (GSH) in red blood cells was performed by GSH-Glo™ Glutathione Assay (Promega Corporation, Madison, Wisconsin). Blood samples were exposed to two different chemical concentrations, either 10 µl chemical + 90 µl whole blood or 1 µl chemical + 99 µl whole blood. For controls, 100 µl whole blood was used. All of the samples were incubated for one hour at 22 °C prior to testing. The GSH assay procedure for whole blood

was conducted per manufacturer instructions. Luminescent emission was read using a Synergy HT multi-mode spectrophotometer and appropriate filters (Bio-Tek, Winooski, Vermont). The background GSH-dependent luminescence was subtracted from each determination. Changes from the average net signal for total GSH concentration to the net signals for reactions with test compound reflect the effect of the compound on the GSH levels. For analysis of glutathione, data were presented as individual compounds or group mean ± SD. Statistical analyses were conducted by 2-tailed Student's t test.

2.7 Hemoglobin Purification

Human hemoglobin (Hb) was purified from PRBCs using a modification the hemoglobin purification method of Sun et al.[16]

2.7.1 RBC Isolation from Whole Blood. A whole blood acid-citrate-dextrose (ACD) unit was centrifuged (Beckman Coulter, Brea, California) at 3700 *xg* for 30 minutes at 4 °C. The plasma was removed from the bottle and discarded. The remaining red blood cell pellet was re-suspended in a 0.9% isotonic saline solution and centrifuged at 3700 x*g* for 30 minutes at 4 °C. The wash process was performed three times until the supernatant was clear. After the final wash, the supernatant was removed and the red blood cell pellet resuspended with roughly three volume equivalents of cold 3.75 mM phosphate buffer (PB, pH = 7.2). The suspension was stored at -20 ^{o}C overnight. The suspension was thawed on ice and centrifuged at 3700 *xg* at 4 °C for 30 min. The supernatant was filtered through cheesecloth and centrifuged two additional times at 3700 x*g* at 4 °C for 30 min. The remaining solution was concentrated using a 4 mL 10 KD centrifugal filter (Millipore, Billerica, Massachusetts), and the retentate filtered through a 0.45 μm syringe filter (Corning, Corning, New York).

2.7.2 Anion Exchange Chromatography. Hemoglobin protein was purified from the filtered solution by separation using a Mono Q 4.6/100 PE (GE Healthcare, Pittsburg, Pennsylvania) anion exchange column run on an ÄKTA Explorer FPLC system (GE Healthcare, Pittsburgh, PA). The protein sample was injected onto the column using a low salt binding buffer (20 mM Tris-HCl pH 8.2). Elution from the column was achieved by using a linear gradient from 100% low salt buffer to 75% high salt buffer (20 mM Tris-HCl pH 8.2, 0.2 M NaCl) in five column volumes, followed by a step gradient to 100% high salt buffer.

2.7.3 Hemoglobin Characterization. To prepare the protein for microscale thermophoresis analysis, a HiPrep Buffer Exchange using a HiPrep 26/10 Desalting column from GE Healthcare was used to exchange buffers from the MonoQ elution buffer to 1X PBS. The various hemoglobin fractions were quantitated by using a Bradford assay. Approximately 7.8 mg of hemoglobin sample was concentrated into a total volume of 5 mL of Fischer PBS pH 7.5 buffer using an Amicon Centrifugal Filter Unit (Millipore, Billerica,

Massachusetts), with a final yield of 5 mL of a 1.5 mg/mL hemoglobin solution. SDS-polyacrylamide and native gel electrophoresis experiments of the purified human hemoglobin were performed under standard conditions. Analysis of the purified hemoglobin sample using SDS and native polyacrylamide gel electrophoresis (PAGE) indicated that the protein was >98% pure, and in monomer form composed of alpha and beta units (data not shown).

2.8 Microscale Thermophoresis (MST)

Purified hemoglobin was labeled with DyLight -650 (Pierce Thermo Scientific, Waltham, Massachusetts) and tested using a Monolith NT.115 (NanoTemper Technologies, Munich, Germany) to monitor thermophoretic changes during binding. The concentration of labeled protein was kept constant at 8.75 nM with 16 point 1:1 serial dilutions of organic compounds from 400 µM. MST measurements were performed in triplicate in hydrophilic treated capillaries (NanoTemper Technologies) using 50% LED and 80% IR-laser power. Laser on and off times were set at 30 sec and 5 sec, respectively.

2.9 MST data analysis

Normalized thermophoresis profiles were converted to bound fractions according to **Equation 1** and plotted as a function of compound concentration using Prism 5 (GraphPad Software, La Jolla, California).

Equation 1.

$$f_u = \frac{(F_x - F_{\quad})}{(F_{\quad x} - F_i)}$$

Where F_{max} = normalized thermophoresis for the unbound state, F_{min} = normalized thermophoresis for the bound state, F_{obs} = normalized thermophoresis for a given binding reaction.

3. RESULTS

3.1. Clinical Data from Exposed Whole Blood

Clinical data from the exposures were tested for statistical significant differences using two different methods: One-way ANOVA using Wilcoxon/Kruskal-Wallis with temperature as a variable factor (**Table 4**) and two-way ANOVA with exposure and temperature as factors (**Table 5**).

3.1.1 Mean Corpuscular Volume (MCV). The mean corpuscular volume (MCV) is a measurement of the average volume of the red blood cells present in the sample, and is calculated by dividing the hematocrit value by the total of RBCs in the sample (**Eq. 2**).

Equation 2. $MCV\ (\text{fL}) = \dfrac{hematocrit\ (\%\ as\ decimal)\ X\ 10}{RBC\ count\ (millions/\mu L)}$

This value can be used to estimate the variations in erythrocyte deformability. In this study, the MCV calculations were conducted by aperture-impedance for determination of MCV. The normal range for human adult MCV values are 80-94 fL, and all control MCV data fell well within the normal MCV range.[17]

Samples were tested for statistical significant differences within each temperature (22 ^{o}C and 37 ^{o}C) dosage sets, as well as comparison between the sets 22 ^{o}C versus 37 ^{o}C. Using the one-way ANOVA method with temperature as the factor, only toluene and octane indicated significant differences in the MCV data sets, and in both cases when compared between the two temperatures. Toluene-exposed cell sets incubated at sets 22 ^{o}C versus 37 ^{o}C demonstrated significant differences from control with a $p = 0.069$ (**Table 5, Fig 1A**). Octane behaved similarly to toluene, with single temperature analysis demonstrating no differences, whereas 22 ^{o}C vs 37 ^{o}C comparisons were different with a $p = 0.0010$ (**Table 5, Fig 1A**). Most of the median MCV values from dosed cells incubated at 22 ^{o}C or 37 ^{o}C during the one hr exposure did not differ enough to exclude random sampling variability **(Table 5, Fig 1A).**

Two-way ANOVA analysis with exposure time and temperature as factors and the MCV data as responses demonstrated that only tetradecane did not demonstrate a significant difference, with p values well above 0.05 (**Table 6**). MCV values with Toluene and decane exposures demonstrated a strong interaction with exposure levels and temperatures, with $p<0.0001$. The p value could not be calculated for ethylbenzene due to test error, as noted.

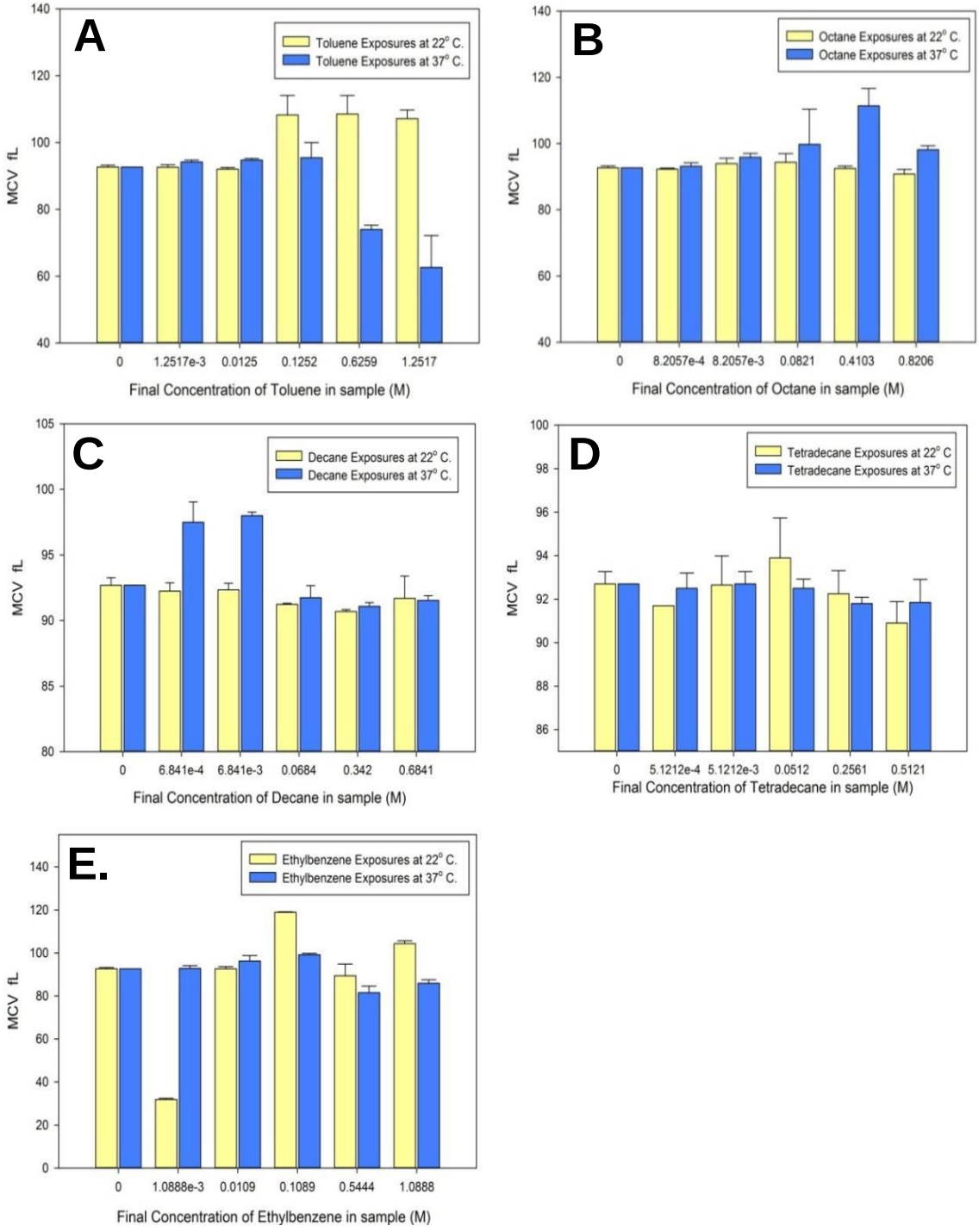

Figure 1. Mean corpuscular hemoglobin (MCV) for whole blood exposures. **A)** Toluene; **B)** Octane; **C)** Decane; **D)** Tetradecane**; E)** Ethylbenzene. See Tables 5 and 6 for discussions on significant changes.

3.1.2 Mean Corpuscular Hemoglobin (MCH). The mean corpuscular hemoglobin (MCH) value is an average of the hemoglobin mass per RBC, and is calculated by dividing the hemoglobin mass by the number of RBCs in the sample. Hemoglobin mass is determined by spectrophotometric absorption, while cell count are determined by centrifugation and pellet analyses against standards. The normal MCH range in humans is 27-31 pg/cell.[18] Exposed samples were tested for statistical significant differences in 22 ^{o}C and 37 ^{o}C dosage sets, as well as comparison between the sets 22 ^{o}C versus 37 ^{o}C.

Samples were tested for statistical significant differences using a one-way ANOVA and the 22 ^{o}C and 37 ^{o}C dosage sets, as well as comparison between the sets 22 ^{o}C versus 37 ^{o}C. None of the chemical exposures indicated significant differences in the MCH data sets (**Table 5, Fig 2E**). This was expected as the hemoglobin levels should remain steady in both temperature data sets.

Interestingly two-way ANOVA analysis with exposure and temperature as factors and the MCH data as responses demonstrated that several chemicals indicated significant changes (**Table 6**). Significant values were not seen with toluene, octane, and tetradecane. However, Decane demonstrated significance with exposure levels (p = 0.0180) and strongly significant differences with interaction between the two factors (p = 0.0016). Therefore for MCH values with decane exposures, temperature alone does not seem to alter MCH values as much as chemical concentration, although temperature can also modulate the MCH response to chemical exposures. As the viscosity of decane does not vary widely between room temperature and 37 ^{o}C (295 to 310 K), it is likely these changes are not due to alterations in viscosity.

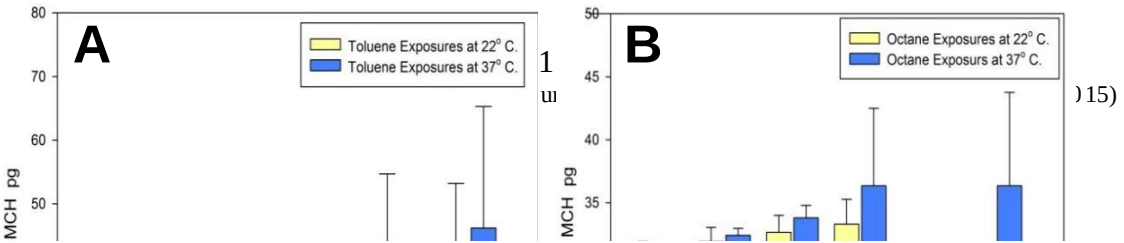

Figure 2. Mean corpuscular hemoglobin (MCH) for whole blood exposures. **A)** Toluene; **B)** Octane; **C)** Decane; **D)** Tetradecane**; E)** Ethylbenzene. See Tables 5 and 6 for discussions on significant changes.

3.1.3 ed Cell Distribution Width (RDW). Red cell distribution width (RDW) is a measurement of the variation, also called anisocytosis, in RBC cell size. While RDW is used

for determination of anemias, this measurement is also useful for determining changes in RBC morphology. RDW measurements are calculated using impedance and flow cytometry data (**Eq. 3**). The normal ranges are 11% to 14.5% RDW.[18]

Equation 3. $$RDW = \frac{one\ SD\ of\ RBC\ volume}{Mean\ cell\ volume} \times 100\%$$

Sample RDW data were analyzed using a one-way ANOVA with temperature as a factor using doses within each temperature (22 ^{o}C and 37 ^{o}C) sets, as well as comparison between the sets 22 ^{o}C versus 37 ^{o}C. Toluene and ethylbenzene did not indicate any temperature-sensitive alterations in RDW values (**Table 5; Figs 3A, 3E**). Octane, decane, and tetradecane demonstrated no statistically significant differences within the temperature groups, but strong differences between the temperature sets (**Table 5; Figs3B, 3C, 3D**)

Two-way ANOVA analysis with exposure and temperature as factors and the RDW data as responses demonstrated that several chemicals caused significant changes (**Table 6**) including toluene, octane, and decane. All three chemical exposure data indicated that temperature, chemical concentration, as well as interaction between temperature/exposure, were significantly altered RDW levels when compared to controls. Decane exposure data indicated that temperature/exposure were dependent, while significant (p = 0.03) was less than that seen with toluene and octane (with p = 0.002 and 0.0001, respectively). The effects of tetradecane on RDW values, using the two-way ANOVA, indicated that temperature strongly altered RDW values (p <0.0001) while chemical dose did not (p = 0.35 nor did there seem to be any additive effect with temperature/chemical dose (p =0.92). Ethylbenzene exposure data indicated that chemical dose (p <0.0001) strongly altered RDW values, which were not altered by temperature (p = 0.11) or interaction between the two factors (p = 0.26).

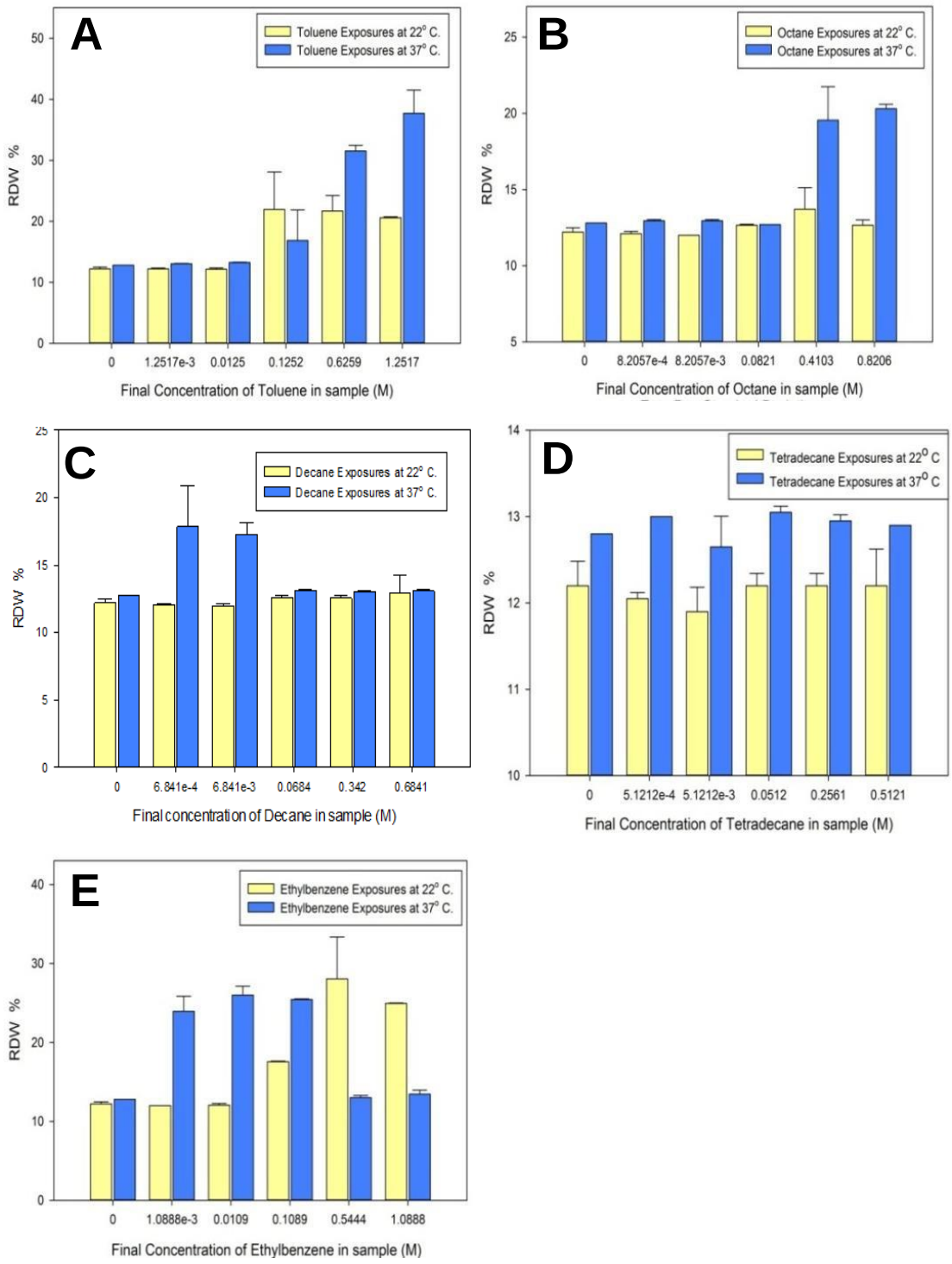

Figure 3. Red Cell Distribution Width (RDW) for whole blood exposures to: **A)** Toluene; **B)** Octane; **C)** Decane; **D)** Tetradecane**; E)** Ethylbenzene. See Tables 5 and 6 for discussions on significant changes. Error bars represent one standard deviation.

Table 5. One-way ANOVA statistical analyses of clinical data from test exposures. Data analyses were conducted using a one-way Wilcoxon ANOVA with temperature as the factor and MCV/MCH/RDW as responses. Significance ≤ 0.05 at presented in blue.

Chemical Exposure	Clinical Data	Exposure Set Analyzed	*p*-value	Comment
Toluene	**MCV**	22 °C	0.115	*comparison data indicate significant differences between incubation temperatures in higher dose groups.
		37 °C	0.1614	
		22 °C + 37 °C	**0.069***	
	MCH	22 °C	0.3373	Differences in the median values not great enough to exclude random sampling variability.
		37 °C	0.125	
		22 °C + 37 °C	0.4597	
	RDW	22 °C	0.148	No statistically significant differences between groups.
		37 °C	0.0855	
		22 °C + 37 °C	0.1093	
Octane	**MCV**	22 °C	0.232	*Analyses indicate differences in values dependent upon exposure incubation temperature.
		37 °C	0.227	
		22 °C + 37 °C	**0.0081***	
	MCH	22 °C	0.150	No statistically significant differences between groups.
		37 °C	0.255	
		22 °C + 37 °C	0.2810	
	RDW	22 °C	0.111	#Analyses indicate differences in values dependent upon exposure incubation temperature.
		37 °C	0.102	
		22 °C + 37 °C	**0.0010#**	
Decane	**MCV**	22 °C	0.333	No statistically significant differences between groups.
		37 °C	0.134	
		22 °C + 37 °C	0.2290	
	MCH	22 °C	0.098	No statistically significant differences between groups.
		37 °C	0.156	
		22 °C + 37 °C	0.901	
	RDW	22 °C	0.356	*comparison data indicate significant differences between incubation temperatures in higher dose groups.
		37 °C	0.134	
		22 °C + 37 °C	**0.0003***	
Tetradecane	**MCV**	22 °C	0.256	No statistically significant differences between groups.
		37 °C	0.574	
		22 °C + 37 °C	0.722	
	MCH	22 °C	0.724	No statistically significant differences between groups.
		37 °C	0.151	
		22 °C + 37 °C	0.346	
	RDW	22 °C	0.787	*comparison data indicate significant differences between incubation temperatures in higher dose groups
		37 °C	0.142	
		22 °C + 37 °C	**0.0001***	
Ethylbenzene	**MCV#**	22 °C	0.333	No statistically significant differences between groups.
		37 °C	0.086	
		22 °C + 37 °C	0.051	
	MCH	22 °C	0.165	No statistically significant differences between groups.
		37 °C	0.082	
		22 °C + 37 °C	0.0962	
	RDW	22 °C	0.088	No statistically significant differences between groups.
		37 °C	0.127	
		22 °C + 37 °C	0.096	

#Values for 1.0888 at 22 °C were not used for calculations on MCV for Ethylbenzene

Table 6. Two-way ANOVA statistical analyses of clinical data from test exposures. Data analyses were conducted using a two-way ANOVA with exposure and temperature as factors and MCV/MCH/RDW as responses. Significance ≤ 0.05 at presented in blue.

Chemical Exposure	Clinical Data	Factor	*p*-value
Toluene	**MCV**	**Temperature (22 °C vs 37 °C)**	**< 0.0001**
		Exposure	**0.0036**
		Interaction	**< 0.0001**
	MCH	Temperature (22 °C vs 37 °C)	0.9221
		Exposure	0.1832
		Interaction	0.4380
	RDW	**Temperature (22 °C vs 37 °C)**	**0.0055**
		Exposure	**< 0.0001**
		Interaction	**0.0020**
Octane	**MCV**	**Temperature (22 °C vs 37 °C)**	**0.0043**
		Exposure	**0.0476**
		Interaction	**0.0452**
	MCH	Temperature (22 °C vs 37 °C)	0.2057
		Exposure	0.5458
		Interaction	0.4720
	RDW	**Temperature (22 °C vs 37 °C)**	**<0.0001**
		Exposure	**<0.0001**
		Interaction	**0.0001**
Decane	**MCV**	**Temperature (22 °C vs 37 °C)**	**0.0002**
		Exposure	**0.0005**
		Interaction	**<0.0001**
	MCH	Temperature (22 °C vs 37 °C)	0.6214
		Exposure	**0.0180**
		Interaction	**0.0016**
	RDW	**Temperature (22 °C vs 37 °C)**	**0.0005**
		Exposure	**0.0055**
		Interaction	**0.0302**
Tetradecane	**MCV**	Temperature (22 °C vs 37 °C)	0.9838
		Exposure	0.1903
		Interaction	0.5538
	MCH	Temperature (22 °C vs 37 °C)	0.2115
		Exposure	0.6642
		Interaction	0.5916
	RDW	**Temperature (22 °C vs 37 °C)**	**<0.0001**
		Exposure	0.3461
		Interaction	0.9209
Ethylbenzene	**MCV**	**Temperature (22 °C vs 37 °C)***	**0.0602**
		Exposure*	**0.0005**
		Interaction*	*****
	MCH	**Temperature (22 °C vs 37 °C)**	**0.0558**
		Exposure	**0.0002**
		Interaction	**0.0002**
	RDW	Temperature (22 °C vs 37 °C)	0.1055
		Exposure	**<0.0001**
		Interaction	0.0264

**Values for 1.0888 at 22 °C were not used in MCV for Ethylbenzene. Therefore values for 1.0888 M at 37° C were necessarily left off. Additionally sufficient degrees of freedom did not exist to consider the interaction.*

3.2 Scanning Electron Microscopy (SEM) of Exposed Whole Blood

A small sample from the whole blood obtained from Bioreclamation was subjected to standard clinical analyses to determine clinical indices prior to exposures and imagining. Data indicated that the blood was well within normal parameters (**Fig. 4, Fig 5**).[19]

Test	Value
White Blood count (**WBC)**	8.6 $x10^3/\mu l$
Neutrophils %	73.1 %
Lymphocyte %	22.4 %
Monocyte %	3.5 %
Eosinophils %	0.7 %
Basophil %	0.3 %
Nucleated RBC %	0.0 %
RBC	5.50 $x10^6/\mu l$
Hemoglobin	16.1 g/dL
Hematocrit	48.6 %
MCV	88.4 fL
MCH	29.2 pg
MCHC	33.1 g/dL
RDW	13.4 %
Platelet	172 $x10^3/\mu l$
Mean Platelet Volume	9.7 fL

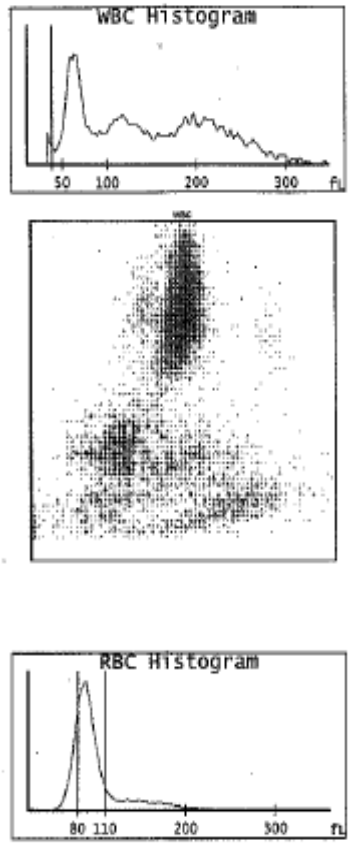

Figure 4. Clinical indices of whole blood sample used for SEM experiments.

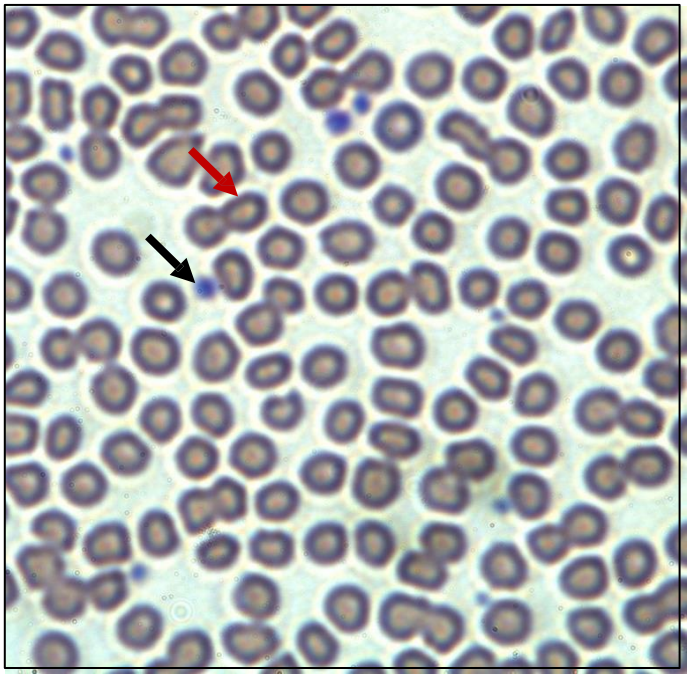

Figure 5. Blood smear of sample used for exposure studies**.** The majority of cells are RBCs (red arrow), with platelets indicated (black arrow).

Control and exposed red blood cells were examined by SEM imaging for the presence of crenation. Toxin damage can be indicated by the presence of crenated RBCs in the blood, with chemically-exposed RBCs exhibiting increases in the number and severity of speculated cells. As the chemical levels increase in the blood, RBC morphology alters from the normal shaped erythrocytes to the spiky ovoid echinocytes (**Fig. 6)**.

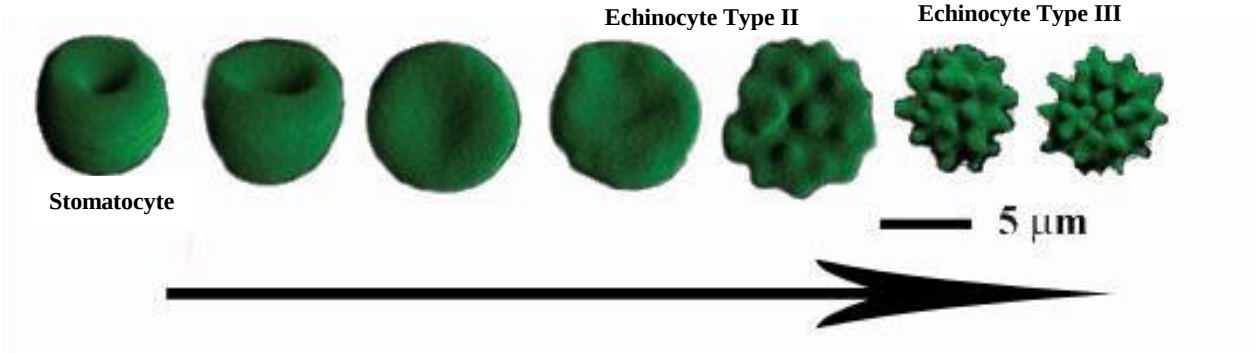

Figure 6. Changes in red blood cell morphology. Arrow indicates level of increasing chemical intercalation into the lipid bilayer. Confocal images adapted from Khairy et al. 2008.[20]

It has been suggested that the morphological changes induced by chemical exposures are a result of chemical insertion into the outer monolayer of the membrane bilayer. Differential membrane expansion is visually indicated by the increased formation of spicules.[21,22] Echinocytes can also result from poor technique during drying of the cells on the stub. The control samples show limited to no formation of echinocytes, an indication that the crenated cell formation is due to the chemical exposures and not the SEM cell preparation.

3.2.1 Toluene-Exposed Red Blood Cells. Blood samples were exposed to 0.625 M (high dose), 1.25 mM, and 0.626 μM toluene at 37 ^{o}C and 22 ^{o}C. As was found for nearly all chemicals tested, SEM preparation of the high dose samples resulted in formation of a jell-like mass. SEM analysis of high dose samples indicated significant cell denaturation as well as agglomeration **(Fig**. **7C**).

SEM at lower toluene concentrations (1.25 mM, and 0.626 μM) indicated the existence of RBC morphology changes at ~1% of the red blood cell mass (**Fig. 7A-7F**). The approximate quantitation of crenated cells did not demonstrate significant differences between incubation at 22 ^{o}C versus 37 ^{o}C. However, a more rigorous SEM interrogation of exposed cells may identify temperature sensitive changes in echinocyte formation. Of the echinocytes found, most are type III as indicated by spicule spacing (**Fig. 7D, 7E**). In addition to echinocytes, stomatocytes and knizocytes were seen at the lower toluene concentrations (**Fig. 7E, 7F**).

One subtle difference between the 22 and 37 ^{o}C incubation cell sets was the formation of granulated RBC cells at 22 ^{o}C (**Fig. 7E**, **blue arrow**). The granulated cells were identified at larger numbers than those seen at the same toluene exposure incubated at 37 ^{o}C (**Fig 7F**, other image data not shown). This membrane disruption may be indicative of moderate chemical exposures (see **Fig. 6**) or may be 'ghosts'. RBC ghosts are hemolyzed cells which no longer contain hemoglobin and are mostly empty structures held in place by a spectrin cytoskeleton.

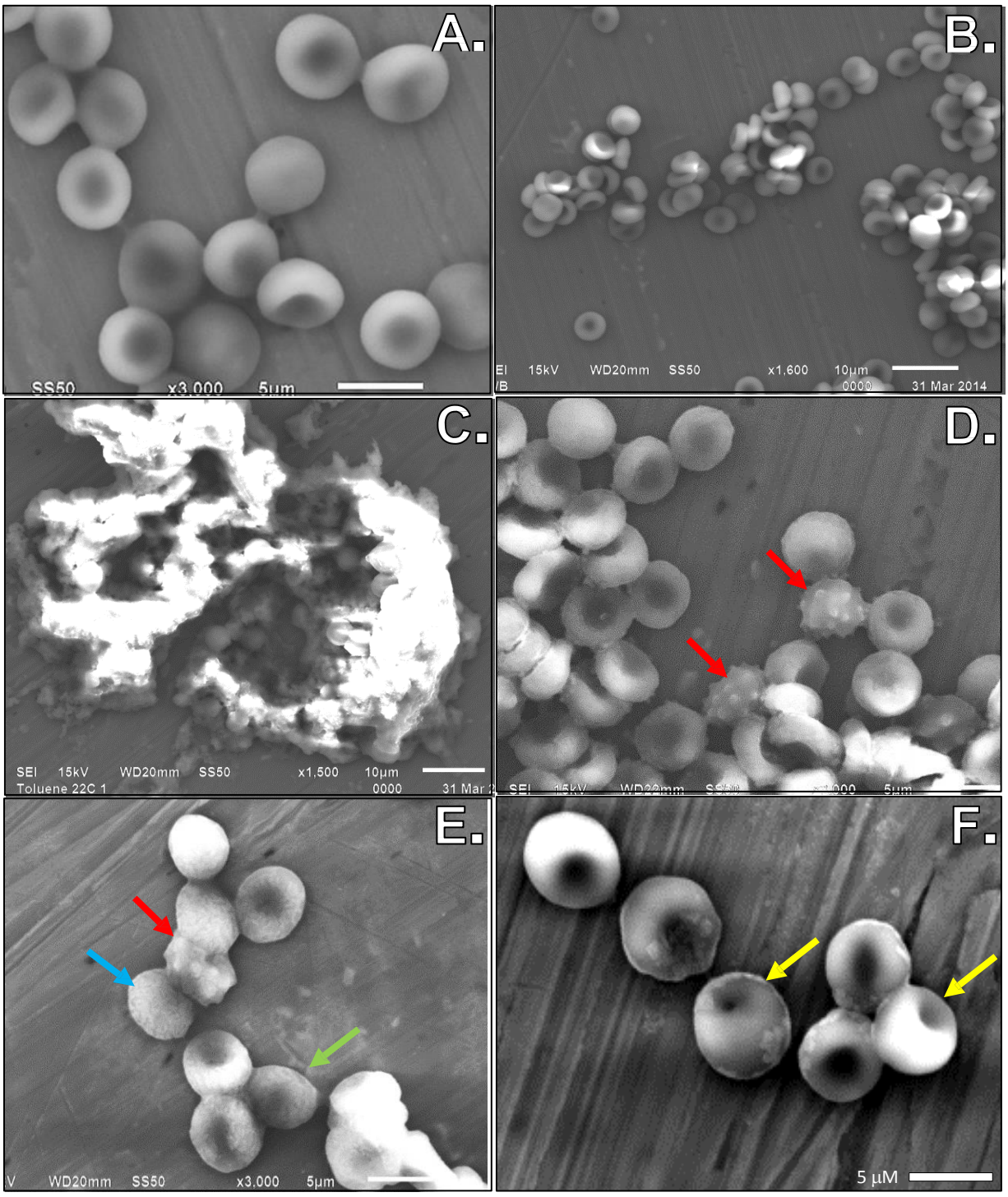

Figure 7. SEM images of Control and toluene-exposed RBCs. **A)** Control – whole blood plus serum carrier; **B)** Whole blood; **C)** 0.625 M toluene at 37 ^{o}C; **D)** 0.626 µM toluene at 37 ^{o}C; **E)** 1.25 mM toluene at 22 ^{o}C; **F)** 1.25 mM toluene at 37 ^{o}C.
Red = Echinocyte; **Green** = possible Knizocyte; **Yellow** = Stomatocyte; **Blue** = unknown membrane disruption.

3.2.2 Octane-Exposed Red Blood Cells. Blood samples were exposed to 0.4103 M (high dose) and 0.821 mM octane and incubated at 37 ^{o}C for one hr. As was found for nearly all chemicals tested, SEM preparation of the high dose samples resulted in formation of a jell-like mass (data not shown). Examination of the 0.821 mM octane-exposed whole blood revealed crenated cell morphologies at < 1% RBC cell mass. Crenated RBC cell shapes included echinocytes, stomatocytes, and well defined knizocytes (**Fig. 8B**).

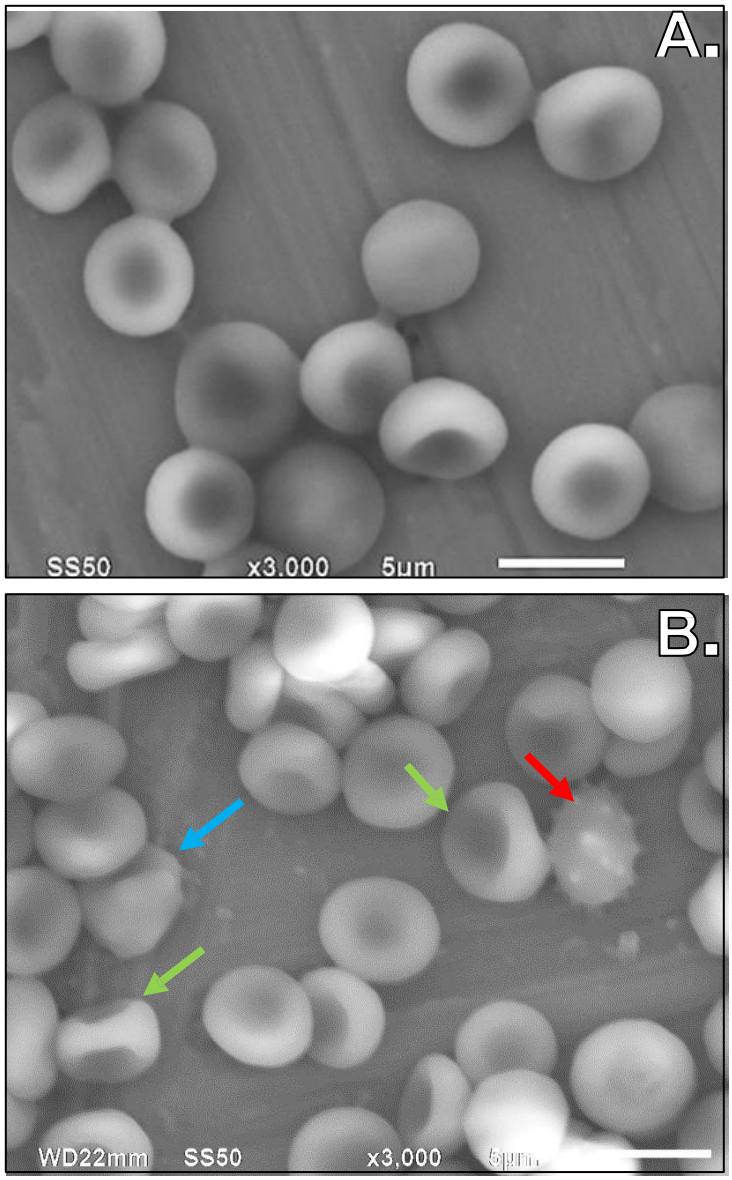

Figure 8. SEM images of Control and octane-exposed RBCs. **A)** Control – whole blood plus serum carrier; **B)** 0.821 mM octane at 37 ^{o}C.
Red = Type III Echinocyte; **Blue** = Type II Echinocyte; **Green** = Knizocyte.

3.2.3 Decane-Exposed Red Blood Cells. Blood samples were exposed to 0.342 M (high dose) and 0.684 mM decane and incubated at 37 ^{o}C for one hr. Unlike the other chemicals tested, SEM preparation of the 0.342 M decane-exposed (high dose) samples resulted in ~95% formation of nearly spherical stomatocytes, with <1% echinocytes/knizocytes (**Fig 9B**). For the 0.684 mM and 0.342 µM decane exposures, less than 1% crenated RBCs were seen. Of those, the majority were Type II echinocytes (**Fig 9C**, **Fig 9D**).

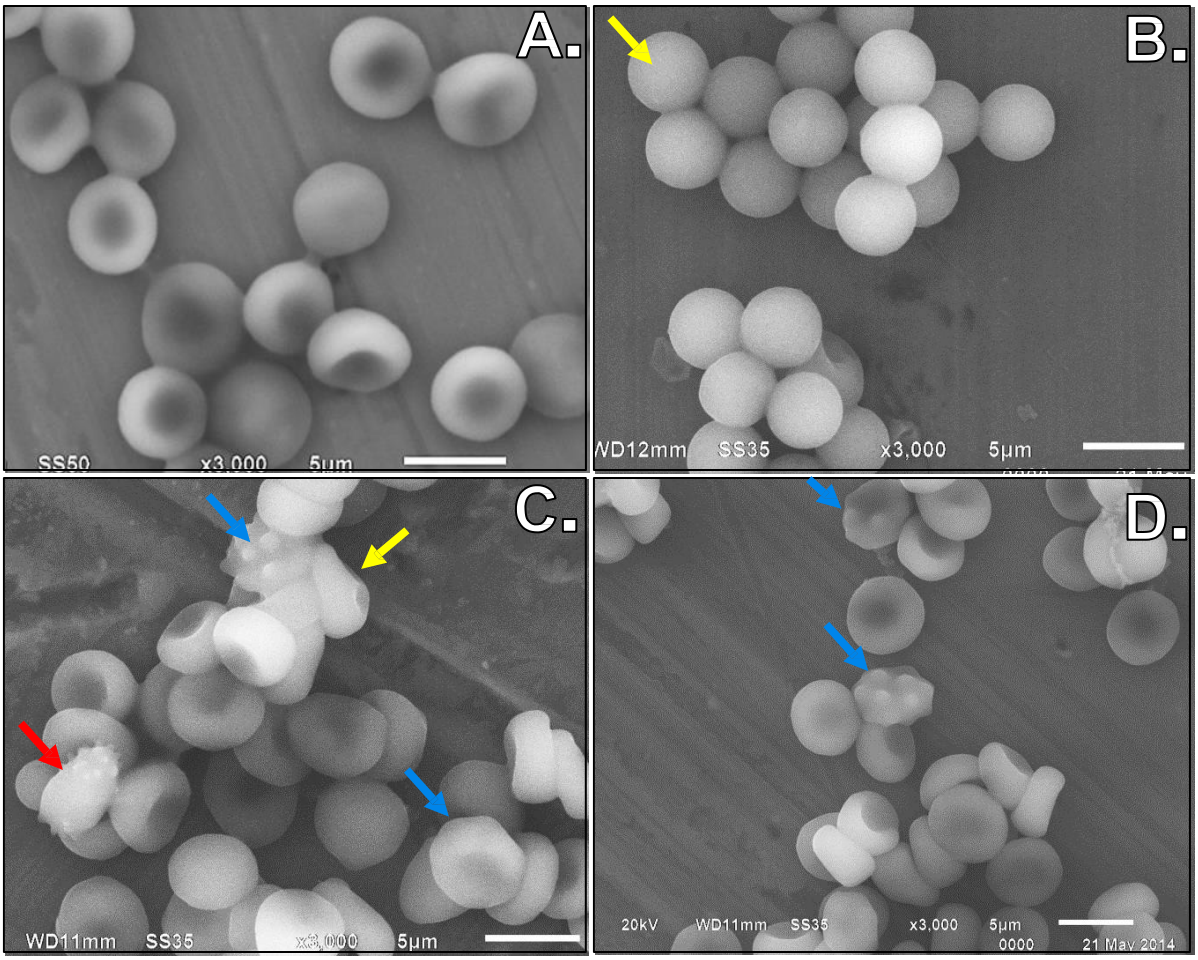

Figure 9. SEM images of Control and decane-exposed RBCs. **A)** Control – whole blood plus serum carrier; **B)** 0.342 M decane at 37 ^{o}C; **C)** 0.684 mM decane at 37 ^{o}C; **D)** 0.342 µM decane at 37 ^{o}C.
Blue = Type II Echinocyte; **Red** = Type III Echinocyte; **Yellow** = Stomatocyte; **Green** = Knizocyte.

3.2.4 Tetradecane-Exposed Red Blood Cells. Blood samples were exposed to 0.2561 M (high dose), 0.5121 mM, and 0.256 µM tetradecane and incubated at 37 ^{o}C for one hr. As was found for nearly all chemicals tested, SEM preparation of the high dose samples resulted in formation of a jell-like mass (data not shown). Examination of the 0.5121 mM tetradecane-exposed whole blood revealed crenated cell morphologies at < 1% RBC cell mass. RBC identified within the SEM images included type II and type III echinocytes and well defined knizocytes (**Fig. 10B**). More type II echinocytes and knizocytes were seen in these samples compared to the other chemical exposure SEMs, indicating less chemical insertion into the lipid bilayer of the RBC.

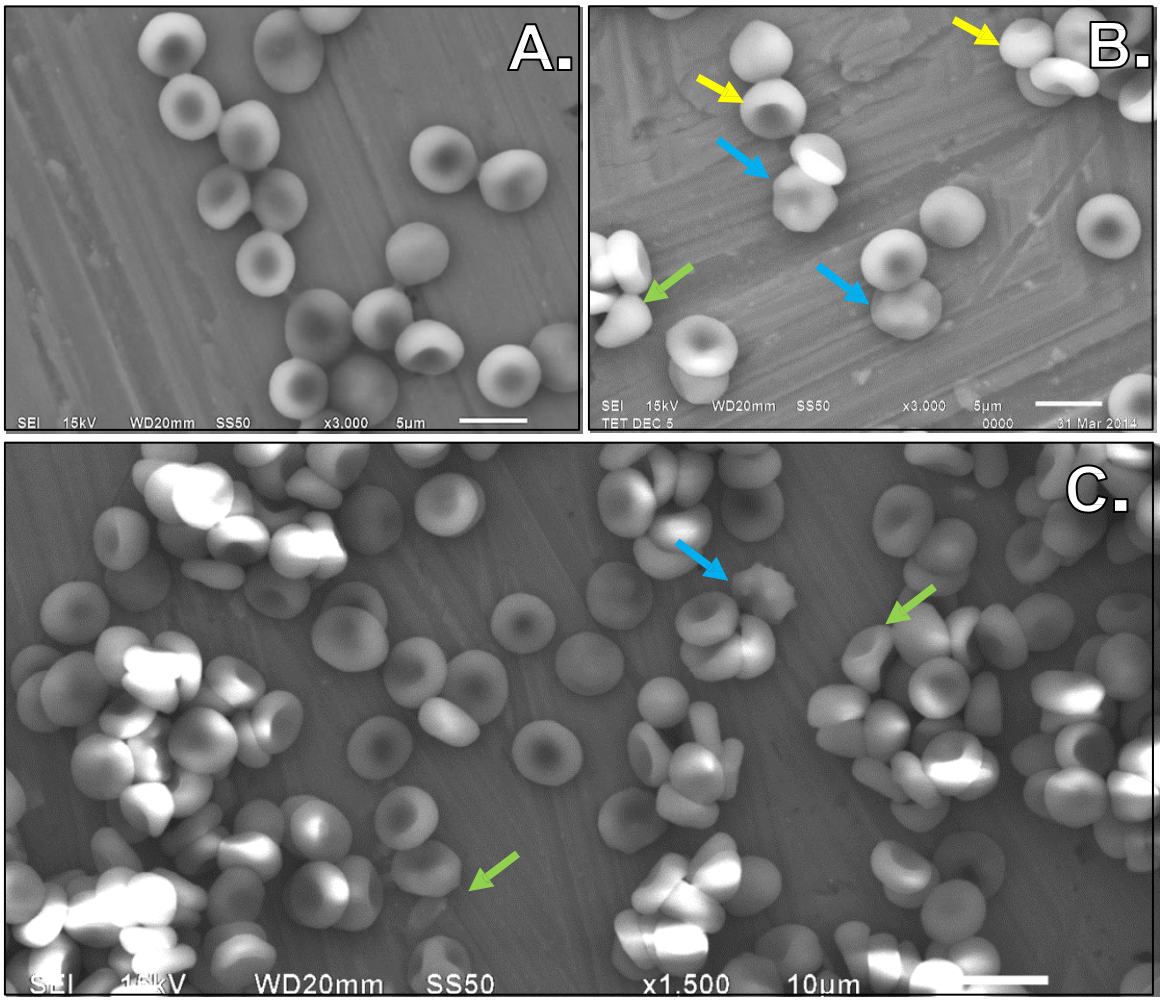

Figure 10. SEM images of Control and tetradecane-exposed RBCs. **A)** Control – whole blood plus serum carrier; **B)** 0.5121 mM tetradecane at 37 ^{o}C; **C)** 0.256 µM tetradecane at 37 ^{o}C. **Blue** = Type II Echinocyte; **Yellow** = Stomatocyte**; Green** = Knizocyte.

3.2.5 Ethylbenzene-Exposed Whole Blood. Blood samples were exposed to 0.544 M (high dose) and 0.1088 mM ethylbenzene and incubated at 37 ^{o}C for one hr SEM preparation of the high dose samples resulted in formation of a jell-like mass (data not shown). Examination of the 0.1088 mM ethylbenzene-exposed whole blood revealed crenated cell morphologies at < 1% RBC cell mass. RBC identified within the SEM images included type II and type III echinocytes and well defined knizocytes (**Fig. 11C, 11D**).

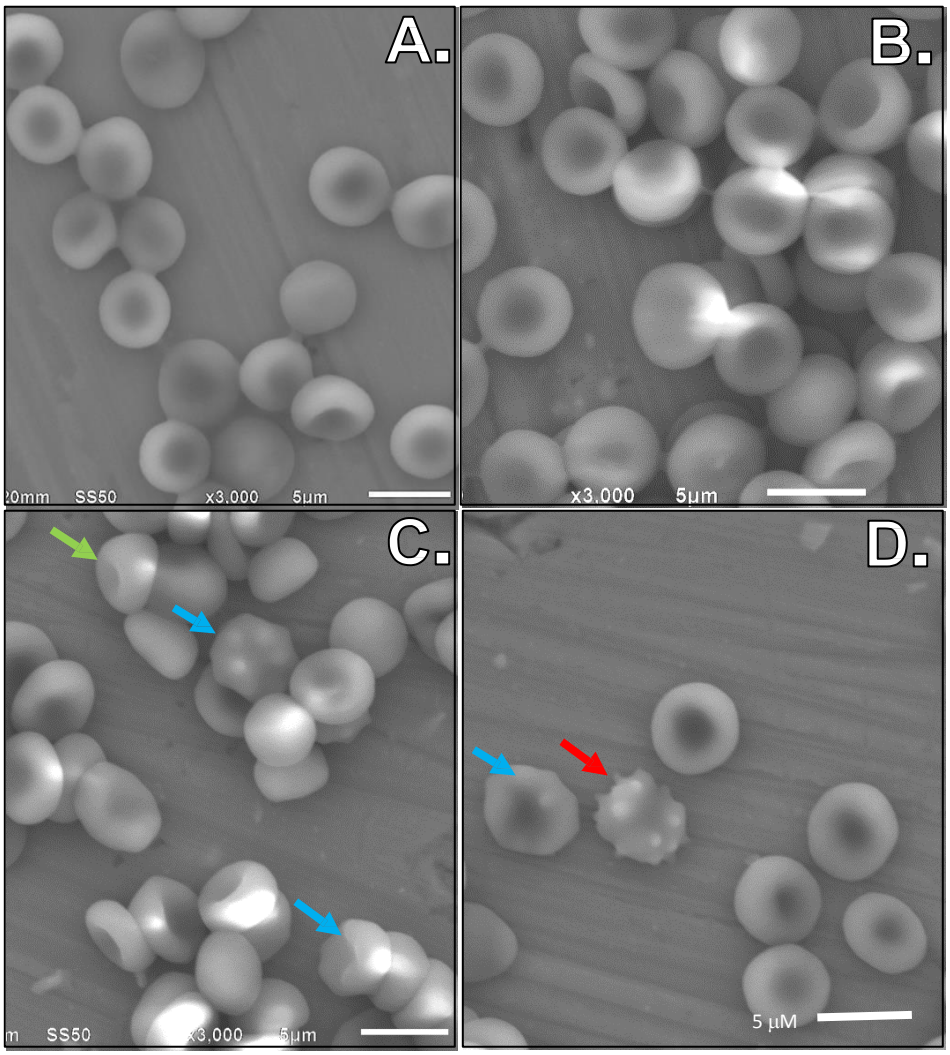

Figure 11. SEM images of Control and ethylbenzene-exposed RBCs. **A and B)** Control – whole blood plus serum carrier; **C and D)** 0.1088 mM ethylbenzene at 37 ^{o}C.
Red = Type III Echinocyte; **Blue** = Type II Echinocyte; **Green** = Knizocyte.

3.3 Glutathione Analysis

Oxidative stress due to the increased level of reactive oxygen species (ROS) in whole blood was measured by GSH depletion. When compared to non-treated control, significant decreases were observed in the GSH levels of all the chemical exposures with the exception of the high concentrations for tetradecane (.051212 M) and toluene (0.125172 M) (**Fig 12**). Of the five chemicals tested, ethylbenzene exposures depleted GSH the most, exhibiting GSH levels ~24% compared to control.

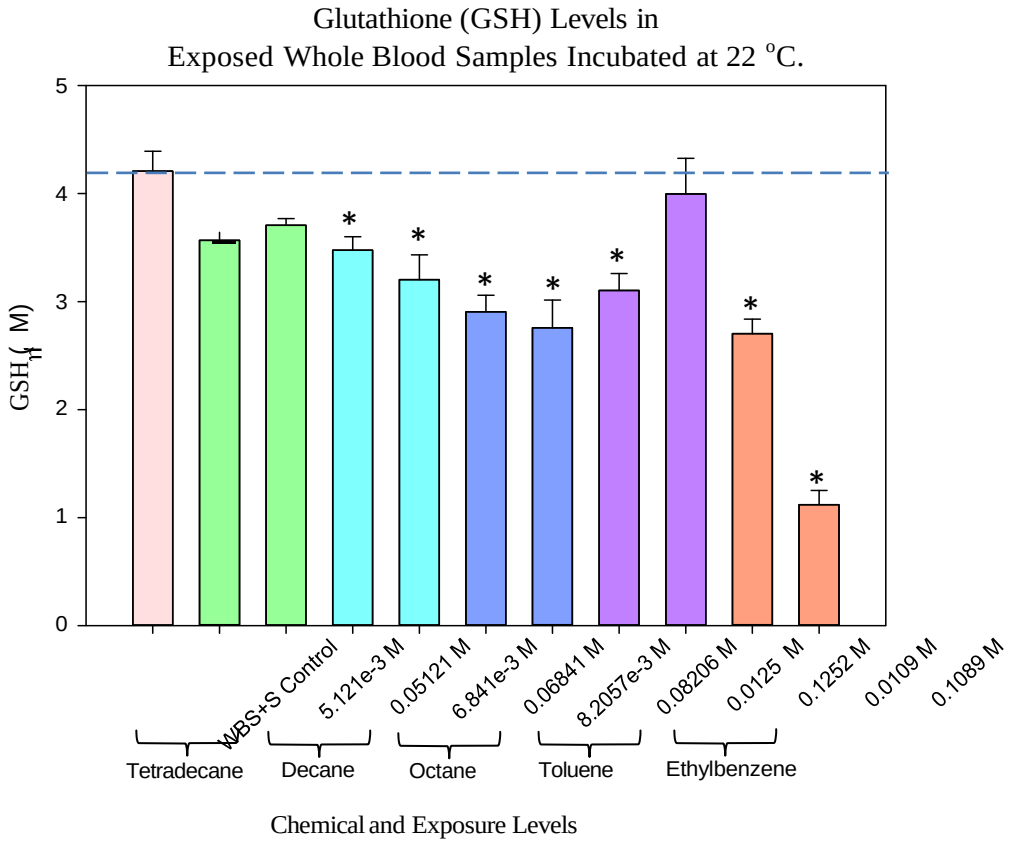

Figure 12. Glutathione levels in exposed whole blood samples incubated 1 hr at 22 °C. Samples statistically different ($p < 0.05$) from Control are indicated ($*$). Error bars indicate one standard deviation.

3.4 Microscale Thermophoresis Analysis

Microscale thermophoresis (MST) is an equilibrium-based, immobilization-free analytical method for monitoring binding phenomena. This method used visible light to excite a fluorescent molecule/protein, after which the rate at which the fluorescence within the illuminated region is observed as a thermal gradient was applied. This thermophoretic mobility is a function of the thermal and mass diffusion coefficients (**Eq. 4**).

Equation 4.

$$\frac{h}{} = \exp(- \ _T\Delta \ )$$

Where c_{hot} = molecule concentration in the hot area, c_{cold} = molecule concentration in the cold area, S_T = Soret coefficient (ratio of mass diffusion to thermal diffusion). Under constant buffer conditions, a biomolecular binding event induced changes in size, charge, solvation entropy, conformation, or a combination of these, and was directly observed as changes in thermophoretic mobility. For testing chemical binding, DyLight-650 labeled hemoglobin was tested to resolve specific from non-specific binding to organic compounds, and to determine affinities where appropriate. The hemoglobin binding interactions were examined using each of the chemical compounds and affinity (K_D) quantitated for decane, toluene, tetradecane, and octane (**Fig. 13**). Analyses using ethylbenzene did not demonstrate any appreciable binding to hemoglobin (data not shown).

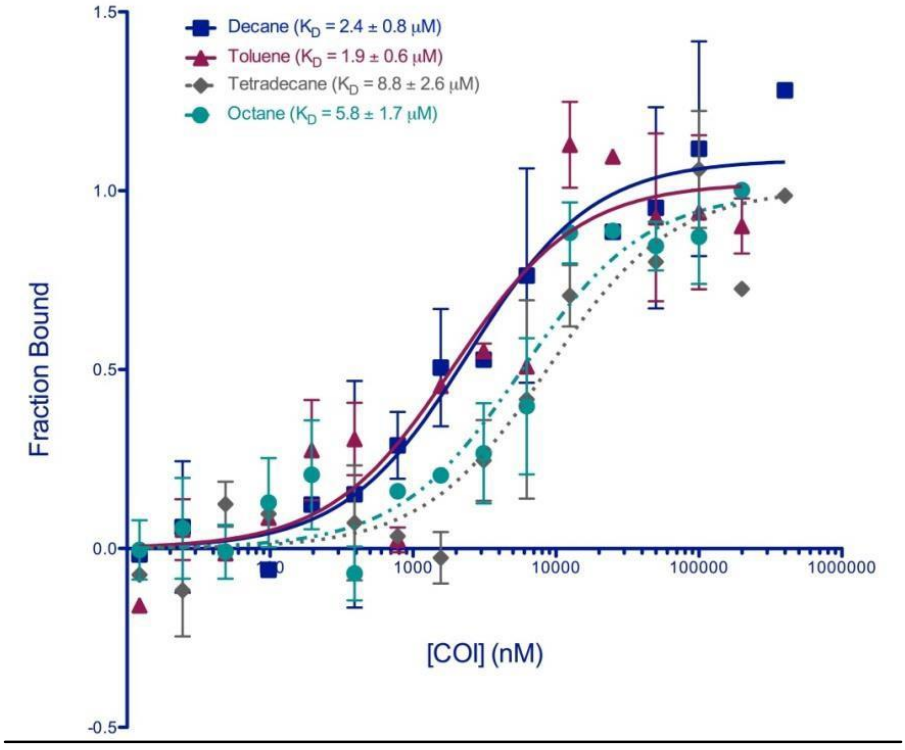

Figure 13. Chemical/hemoglobin binding at 25 °C. COI = concentration of ligand. Error bars represent one standard deviation from data generated from triplicate experiments.

4. DISCUSSION

Given the widespread use of JP-8 jet fuel in the military and the potential risk of exposure, it is important to understand potential mechanisms for toxicity at the cellular level. Though the occupational hazards of some hydrocarbon chemicals are well-documented, the mechanism of toxicity in biological membranes remains unsettled.[24] Evidence suggests that biological membranes with bilayers have a higher affinity for hydrophobic compounds than more-hydrophilic cell walls.[25] Therefore if present within the blood stream, a hydrocarbon-based chemical may insert into the lipid bilayer of the RBC, possibly aided by hydrophobic surface proteins (as seen with toluene[26]) and induce membrane structure alterations. In addition, if the chemical passes entirely through the bilayer to enter into the RBC cytoplasm, it may bind to hydrophobic regions of the hemoglobin structure. Previous reports have indicated that increased toxicity corresponds with a decrease in the viscosity of the hydrocarbon used.[24]

Lam et al. provided important information on how organic solvents are transported by blood.[27] Rats were exposing to a set of organic chemicals and their blood examined to determine how much of these solvents were sequestered inside the RBC. In examining toluene exposure data, it was seen that *in vitro* results were comparable to *in vivo* outcomes – in both cases approximately 66% of the solvent found in the blood was found within the RBC and not in the plasma. Interestingly, comparable *in vitro* testing with toluene and human blood indicated that only 43% of the chemical was found within the RBC. Data from Lam et al. indicate that the level of RBCs uptake of chemicals from the blood is *species dependent*.[27] Of the chemicals tested, the more hydrophobic chemicals displayed the most variance between organisms. Further analyses from exposed rat whole blood indicated that the bulk (51-96%) of the chemical was found to be associated with proteins, most likely hemoglobin. These data were supported by Gargas et al., who also found species differences in blood:air partition coefficients.[28] Due to these suggested differences seen between animal and human blood[29] we used human blood samples in all analyses.

This study was a preliminary examination of the effects of jet fuel components on their ability to induce changes in RBC membrane structure. In addition, the binding affinity of these chemicals to purified human hemoglobin was examined. Of the many components of JP-8, we examined only five: octane, decane, tetradecane, toluene, and ethylbenzene. JP-8 jet fuel contains 0.46% octane, 2.7% decane, 2.33 % tetradecane, 0.21% toluene, and 0.19% ethylbenzene as determined by ion chromatography.[30] Exposure doses were applied using a range extending from blood levels seen in realistic exposure scenarios[31] to unrealistically high levels in the molar range. We recognize the fact that, at the high dose levels, two behaviors may occur. First, at high levels the hydrophobicity of the hydrocarbon chemical may drive the formation of a micelle within the whole blood, resulting in sequestration of the chemical away from RBCs. If micelles formed, the behavior of the RBCs and its membranes would reflect clinical values seen at lower doses. Alternatively, such a high level of chemical may denature the RBCs within the blood, rendering

clinical data useless. Thus the data presented - for the upper dose levels - must be interpreted with caution.

RBC Morphological Alterations

An examination of the effects of concentration and temperature on chemical alteration of RBC volumes indicated that MCV values for toluene exposures at 22 ^{o}C were significantly higher than controls. The MCV differences seen in incubation temperatures may be due to inherent Coulter Analyzer errors, as the MCV value may change 2-4 fL.[32] However, these data indicate that MCV values increase, not decrease, with increasing dosages. Most likely this apparent increase in the surface area of the membrane (as indicated by MCV) may result from the accumulation of compound in the membrane bilayer, which can lead to an alteration of the membrane structure and function.[33] Interestingly, increased toluene doses, incubated at 37 ^{o}C, demonstrated the opposite effect – MCV values decreased with increasing doses of chemical. While the temperature dependency may reflect solubility and thus effective exposure concentrations for each of the hydrocarbon compounds, the differences in the chemical partition coefficient alone cannot explain the MCV shift in the case of toluene. We hypothesize that the changes in MCV suggest the compound may be accumulating in the cell membrane or further inserting into the cytoplasm with potential attachment to the hemoglobin protein. Normal clinical analyses for RDW and MCH values in blood are not temperature sensitive, therefore the differences seen in incubation temperatures of the dose sets most likely reflect actual cellular changes. The RDW values suggest that RBC structural variation, and therefore chemical influx into the cell, may be greater within the body (37 ^{o}C) than with *in vitro* testing.

To further examine the potential changes seen in red blood cell membrane structure using a more sensitive technique, scanning electron microscopy (SEM) imaging was utilized.[34] As crenation may naturally occur upon aging of blood,[35] we were careful to use fresh blood in all analyses. In addition, it has been shown that removal of albumin can cause RBCs to crenate into smooth spheres.[36] Therefore it is important that whole blood rather than isolated RBCs are used in the examination of chemical-induced morphological changes in the RBC.

SEM images of exposed blood indicated that low levels of RBC morphological changes were occurring upon exposures in all samples tested. In all cases, with the exception of decane, exposures at high doses denatured the cells into an undefined cell mass. While we estimate crenated cell types at ~ 1%, our SEM testing was not extensive enough to provide quantitative differences between the low/mid dose exposure groups. Interestingly, at high doses of decane the RBCs did not seem to denature but formed nearly 100% spherical stomatocytes (**Fig. 9B**). Echinocytes and knizocytes *were* seen at the lower dose exposures to decane, so it is unclear why this less severe structural change was seen unless the high dose images are of RBC 'ghosts' rather than stomatocytes. This discrepancy could be clarified using electrophoresis to quantitate the level of RBC ghosts.[37]

These data also provide important technical insights to parameters required for analysis of chemical-induced changes in the blood. The inability of MCV values to identify low level RBC morphology variations suggest that Hematology Analyzers may not be sensitive enough, for research, to reveal structural deformations. Conventional clinical methodology utilizes electrical impedance to measure the mechanical deformation of RBC membranes, whereas SEM uses electron beam scanning to provide high resolution (> 1 nm) images of the cell surface. Our original intent in examining the effects of incubation temperatures on exposures was to identify changes seen in banked blood for transfusions as well as *in situ* human exposures. In the data seen, there is no indication that room temperature induces more damage than that seen at body temperature. The MCV increases seen in the higher toluene doses at 22 ^{o}C toluene (**Fig 1A, yellow bars**) may reflect some of the issues previously discussed, rather than an indication of increased toxicity for room temperature-stored blood. The variation seen between the two temperature data sets strongly suggests care must be taken when conducting such blood exposure studies and that 37 ^{o}C incubations should be used.

Hydrocarbon Chemical Binding to Hemoglobin

Our studies found significant binding interaction between the test set of hydrocarbon chemicals and purified hemoglobin. Specifically, our results revealed an affinity between purified human hemoglobin monomer to the hydrocarbons decane, toluene, tetradecane, and octane, with toluene demonstrating the tightest binding (K_D = 1.9 μM). Interestingly, ethylbenzene did not bind purified Hb monomer in any demonstrable manner, unlike suggested in Béliveau et al.[38] However, for technical reasons ethylbenzene binding to Hb should be repeated to verify this result.

Once inside the RBC, it could be proposed that chemical accumulation on the hemoglobin as well as membrane integrity of the erythrocyte, could initiate changes in the structure, decreasing oxygen carrying capacity. It is also possible that the direct chemical attachment to the hemoglobin could impede oxygen binding by steric hindrance of the heme binding site. Toluene is used to initiate crystal growth in crystallographic studies of hemoglobin and these data hint at potential binding regions on the hemoglobin protein, primarily at Trp14α within oxyhemoglobin.[39] Wiester et al. proposed that hemoglobin may play a role in the variability seen in between different organisms with respect to volatile organic chemical (VOC) carrying capacity of the blood.[40] Although his experiments indicated the variation seen was most likely independent of Hb type *within* each specie, it was suggested that hydrophobic sites on the surface of plasma/heme proteins could play a role. We suggest that RBC surface proteins, perhaps even the antigen proteins, may be a factor in the ability of the hydrophobic chemicals to insert into the lipid bilayer.

Oxidative Stress induced by Hydrocarbon Exposures

GSH provides protection against oxidative stress in cells, and is essential for maintaining hemoglobin in its native form in RBCs.[41] The oxidative attack of ROS in erythrocytes is

targeted to hemoglobin and the cell membrane. One salient finding of the present study was that jet fuel adducts induce oxidative stress in whole blood, most likely due to the major target of ROS - the red blood cells and associated hemoglobin proteins. While this study tested only whole blood, it is presumed that the majority of GSH response seen was found in the RBCs. A follow-on study using isolated RBCs would clarify this finding. We found significant decreases in the GSH concentrations, thus concluding that the antioxidant deficiency is most likely a direct result of hydrocarbon-induced oxidative damage to the exposed RBCs. We had theorized that decreased GSH levels, thus higher oxidative stress, should be found at higher dose exposures. However, we encountered a confounding observation in that the GSH levels for the lower concentrations of tetradecane and toluene were higher rather than lower. It is possible that the assumed biological effects were not seen as a result of differences in the solubility of the compound, as previously discussed. This relationship should be further examined using additional low level dilutions to correlate the uptake of the compound with its biological effects, as well as examining temperature sensitivities as conducted in the toluene exposure set.

Future Directions

We postulate that the apparent increase in the surface area of the cell membrane, GSH depletion, and interaction between the hydrocarbon and hemoglobin molecule, contribute to negatively impact hemodynamics and circulatory function. As shown by this study, the data suggest that jet fuel adducts do induce oxidative effects in red blood cells, but the extent of toxicity and its subsequent effects on RBC functionality and morphology *in vivo* remain unclear. The data presented suggests that even at no observed adverse effect levels (NOAEL) of chemical concentrations in the blood, jet fuel components could initiate changes within the RBC (**Fig 14**) including oxidative stress and membrane structural alterations.

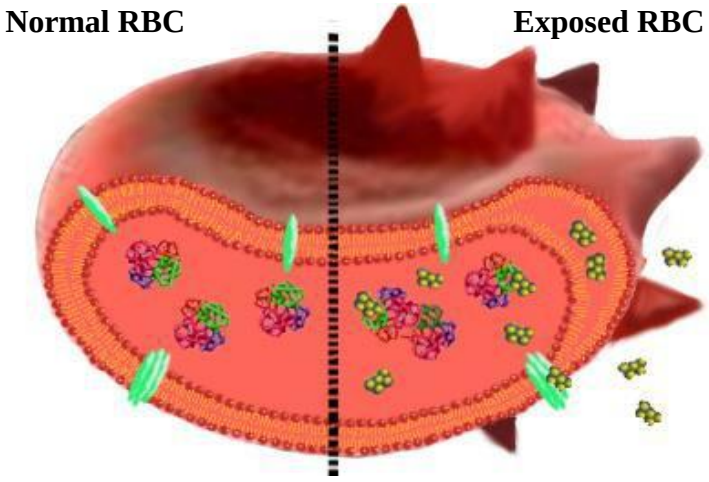

Figure 14. Chemical interactions with RBCs. Interactions identified with this study include embedment within the membrane bilayer as well as attachment to hemoglobin. Potential sites of attachment include membrane receptors, not examined in this study.

Since RBC morphology dictates both rheology and ultimately oxygen transport,[42,43] these changes could lead to anoxemic conditions within the organism. Even though morphological changes have been shown to be reversible,[44] the binding affinity of the chemical for hemoglobin[45] may play an important role in the longevity of the altered RBC structure. Once 'captured' inside the cytoplasm, the K_D of Hb binding may limit outflux of the chemical back into the plasma. If so, then the chemical affinity for hemoglobin may be the rate limiting step for RBC return to the dicocyte (normal) structure. Of the chemical series tested, octane > toluene > decane > tetradecane >>> ethylbenzene for effects on RBC dynamics. However, GSH data indicative of oxidative stress do not reflect this ranking, with ethylbenzene > octane > decane and with tetradecane and toluene exhibiting less, not more, oxidative stress with increased doses. Perhaps ethylbenzene, within the cell yet not bound to Hb, is freely available to react to increase ROS within the cell. Future studies should focus on clarifying these uncertainties.

5. REFERENCES

1. Ritchie G, Still K, Rossi J, Bekkedal M, Bobb A, Arfsten D. Biological and health effects of exposure to kerosene-based jet fuels and performance additives. *J Toxicol Environ Health B Crit Rev.* (2003) **6**:357-451.

2. National Research Council. Permissible Exposure Levels for Selected Military Fuel Vapors Washington, DC: The National Academies Press. (1996).

3. Erdem O, Sayal A, Eken A, Akay C, and Aydın A. Evaluation of genotoxic and oxidative effects in workers exposed to jet propulsion fuel. *Int Arch Occup Environ Health*. (2012) **85**:353-361.

4. Keil D, Dudley A, EuDaly J, Dempsey J, Butterworth L, Gilkeson G, Peden-Adams M. Immunological and hematological effects observed in B6C3F1 mice exposed to JP-8 jet fuel for 14 days. *J Toxicol Environ Health A.* (2004) **67**:1109-1129

5. Lang E, Qadri SM, Lang F. Killing me softly - suicidal erythrocyte death. *Int J Biochem Cell Biol*. (2012) **44**:1236-1243.

6. Ghashghaeinia M, Cluitmans JC, Akel A, Dreischer P, Toulany M, Köberle M, Skabytska Y, Saki M, Biedermann T, Duszenko M, Lang F, Wieder T, Bosman GJ. (2012). The impact of erythrocyte age on eryptosis. *Br J Haematol* **157**:606-614.

7. Ghaffari S. Oxidative Stress in the Regulation of Normal and Neoplastic Hematopoiesis. (2008) *Antioxid Redox Signal*. (2008) **10**:1923–1940.

8. Townsend, D.M., Tew, K.D. and Tapiero, H. The importance of glutathione in human disease. *Biomed. Pharm*. (2003) **57**:145–55.

9. Boulares A, Contreras F, Espinoza LA, and Smulson M. Roles of oxidative stress and glutathione depletion in JP-8 jet fuel-induced apoptosis in rat lung epithelial cells. *Toxicol Appl Pharmacol.* (2002) **180**:92-99.

10. Veraldi, A., Costantini, A., Bolejack, V., Miligi, L., Vineis, P., & van Loveren, H. Immunotoxic effects of chemicals: A matrix for occupational and environmental epidemiological studies. *Am J Ind Med*. (2006) **49**:1046-1055.

11. Oyebisi A, Akhigbe R, Anigbogu C. Oxidative status in rat kidney exposed to petroleum hydrocarbons. *J Nat Sci Biol Med*. (2013) **4**:149–154.

12. Chaudhary R, Rahul K. Oxidative injury as contributory factor for red cells storage lesion during twenty eight days of storage. *Blood Transfus*. (2012) **10**:59-62

13. Sparrow RL. Red blood cell storage and transfusion-related immunomodulation. *Blood Transfus.* (2010) **8**:s26–30.

14. Tsai SP, Fox EE, Ransdell JD, Wendt JK, Waddell LC, Donnelly RP. A hematology surveillance study of petrochemical workers exposed to benzene. *Regul Toxicol Pharmacol*. (2004) **40**:67-73.

15. Andrade CT. Purification and characterization of human hemoglobin: effect of the hemolysis conditions. *Int J Biol Macromol.* (2004) **34**:233-240.

16. Sikkema J, de Bont JA, Poolman B. Interactions of cyclic hydrocarbons with biological membranes. *J Biol Chem*. (1994) **269**:8022-8028

17. Sun G, Palmer A. (2008). Preparation of Ultrapure Bovine and Human Hemoglobin by Anion Exchange Chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci.* **867**:1-7.

18. Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd Edition. Walker HK, Hall WD, Hurst JW, editors. Boston: Butterworths; 1990.

19. National Library of Medicine, National Institutes of Health. RBC indices. MedlinePlus Medical Encyclopedia. (February 2012). Retrieved January 15, 2013 from www.nlm.nih.gov/medlineplus/ency/article/003648.htm

20. King M. Commond Blood Analysis Data. Medical Biochemistry Website. http://themedicalbiochemistrypage.org/bloodtests.php

21. Khairy K, Foo JJ, and Howard J. Shapes of Red Blood Cells: Comparison of 3D Confocal Images with the Bilayer-Couple Model. *Cell Mol Bioeng. Cell Mol Bioeng.* (2010) **1**:173–181.

22. Sheetz MP, Singer SJ. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc Natl Acad Sci U S A.* (1974) **71**:4457-4461.

23. Lim HWG, Wortis M, and Mukhopadhyay R. Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: evidence for the bilayer- couple hypothesis from membrane mechanics. *Proc Natl Acad Sci U S A.* (2002) **99**:16766-16769

24. Sikkema, J. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev* **59**:201-222.

25. Sikkema J, de Bont JA, Poolman B. Interactions of cyclic hydrocarbons with biological membranes. *J Biol Chem*. (1994) **269**:8022-8028.

26. Korpela M, Tähti H. The effect of *in vitro* and *in vivo* toluene exposure on rat erythrocyte and synaptosome membrane integral enzymes. *Pharmacol Toxicol*. (1988) **63**:30-32.

27. Lam C-W, Galen TG, Boyd JF, and Pierson A. Mechanism of Transport and Distribution of Organic Solvents in Blood. *Toxicol Appl Pharmacol.* (1990) **104**:117-129.

28. Gargas ML, Burgess RJ, Voisard DE, Cason GH, and Anderson ME. Partition coefficients of low-molecular weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharmacol*. (1989) **98**:87-99.

29. Wiester MJ, Winsett DW, Richards JH, Doerfler DL, and Costa DL. Partitioning of Benzene in Blood: Influence of Hemoglobin Type in Humans and Animals. *Environmental Health Perspectives* (2002) **110**:255-261.

30. Tremblay RT, Martin SA, Fisher JW. Novel characterization of the aerosol and gas-phase composition of aerosolized jet fuel. *Inhal Toxicol* (2010b) **22**:394-401.

31. Couper F and Logan BK. Drugs and Human Performance Fact Sheets. Final Report, National Highway Traffic Safety Administration, 400 Seventh St., SW. Washington, DC 20590. http://www.nhtsa.gov/people/injury/research/job185drugs/toluene.htm

32. Young M and Lawrence ACK. The influence of extraneous factors on Coulter S measurement of the mean corpuscular volume. *J. Clin. Path*. (1975) **28**:12-15.

33. Karabulut I, Balkanci Z, Pehlivanoglu B, Erdem A, Fadillioglu, E. Effect of toluene on erythrocyte membrane stability under in vivo and in vitro conditions with assessment of oxidant/antioxidant status. *Toxicol Ind Health.* (2009) **25**:545-550.

34. Youngchan Kim, Kyoohyun Kim and YongKeun Park (2012). Measurement Techniques for Red Blood Cell Deformability: Recent Advances, Blood Cell - An Overview of Studies in Hematology, Dr. Terry Moschandreou (Ed.), ISBN: 978-953-51-0753-8, InTech, DOI: 10.5772/50698.

35. Danon D1, Marikovsky Y. The aging of the red blood cell. A multifactor process. *Blood Cells*. (1988) **14**:7-18.

36. Furchgott RF, Ponder E. Electrophoretic Studies on Human Red Blood Cells. *J Gen Physiol*. (1941) **24**:447-57.

37. G. Schwoch, H. Passow Preparation and properties of human erythrocyte ghosts. Molecular and Cellular Biochemistry (1973) **2:**197-218

38. Béliveau M and Krishnan K. Estimation of rat blood:air partition coefficients of volatile organic chemicals using reconstituted mixtures of blood components. *J Toxicol Environ Health A.* (2000) **60**:377-89.

39. Park S, Yokoyama T, Shibayama N, Shiro Y, Tame J. (2006). 1.25 Å Resolution Crystal Structures of Human Haemoglobin in the Oxy, Deoxy and Carbonmonoxy Forms. *J Mol Biol*. **360**:690–701.

40. Wiester MJ, Winsett DW, Richards JH, Doerfler DL, and Costa DL. Partitioning of Benzene in Blood: Influence of Hemoglobin Type in Humans and Animals. Environmental Health Perspectives (2002) **110**:255-261.

41. Pandey K, Rizvi S. Biomarkers of oxidative stress in red blood cells. Biomedical Papers Of The Medical Faculty Of The University Palacký, Olomouc, Czechoslovakia [serial online]. (2011) **155**:131-136.

42. Turchetti V, Leoncini F, De Matteis C, Trabalzini L, Guerrini M, Forconi S. Evaluation of erythrocyte morphology as deformability index in patients suffering from vascular diseases, with or without diabetes mellitus: correlation with blood viscosity and intra-erythrocytic calcium. Clin Hemorheol Microcirc. (1998) **18**:141-149.

43. Doyle MP1, Galey WR, Walker BR. Reduced erythrocyte deformability alters pulmonary hemodynamics. J Appl Physiol (1985). **67**:2593-2599.

44. Alhanaty E and Sheetz MP. Control of the Erythrocyte Membrane Shape: Recovery from the Effect of Crenating Agents. *J Cell Biol.* (1981) **91**:884-888.

45. Chushak YG, Chapleau RR, Frey JS, Mauzy CA, Gearhart JM. Identifying Potential Protein Targets for Toluene using Molecular Similarity Search, in Silico Docking and in Vitro Validation. Toxicology Research, Advanced Online article published 2 Feb 2015 DOI: 10.1039/C5TX00009B

6. LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

ANOVA	analysis of variance
C	chemical
CBC	complete blood count
COI	concentration of ligand
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
KD	kilodalton
ACD	acid citrate dextrose (solution)
FPLC	fast protein liquid chromatography
GSH	glutathione
hr	hour
Hb	hemoglobin
IR	infrared
LED	light emitting diode
MCV	mean corpuscular volume
MCH	mean corpuscular hemoglobin
MCV	mean corpuscular volume
MST	microscale thermophoresis
NOAEL	no-observed-adverse-effect level
PAGE	polyacrylamide gel electrophoresis
RBC	red blood cell
RDW	red cell distribution width
RM ANOVA	repeated measures analysis of variance
ROS	reactive oxygen species
RT	room temperature
S	serum
sec	second
SD	standard deviation
SEM	scanning electron microscopy
VOC	volatile organic chemical
WB	whole blood