5-2014

Characterizing Neutrophil Behavior in Zebrafish (Danio rerio) in Response to Arsenic and Glucose

Jacob Longfellow
University of Maine - Main

CHARACTERIZING NEUTROPHIL BEHAVIOR IN ZEBRAFISH (*DANIO RERIO*) IN RESPONSE TO ARSENIC AND GLUCOSE

by

Jacob R. Longfellow

A Thesis Submitted in Partial Fulfillment

of the Requirements for a Degree with Honors
(Microbiology)

The Honors College

University of Maine

May 2014

Advisory Committee:
Carol H. Kim, Professor of Microbiology, Advisor
Michelle F. Goody, Postdoctoral Researcher, Molecular and Biomedical Sciences, Advisor
John T. Singer, Professor of Microbiology
Dr. Melissa Ladenheim, Adjunct Assistant Professor, Honors
Robert T. Wheeler, Associate Professor of Microbiology

Abstract

The innate immune system recognizes self from non-self and is involved in pathogen clearance. Neutrophils are innate immune cells that quickly migrate to areas of infection or wounding. Neutrophils phagocytize pathogens and produce a respiratory burst that kills infectious agents. However, inappropriate presence and function of neutrophils contributes to many chronic inflammatory diseases. Environmental toxicants and other ingested compounds are known to impact innate immunity and neutrophil behavior. Two compounds of importance to the Maine population are arsenic and glucose, due to the presence of arsenic in Maine well water and the high rate of obesity and diabetes in Maine. Since many Mainers and people worldwide are exposed to arsenic from the environment or have elevated glucose levels, it is important to understand how these compounds impact our health. To test the effects of these compounds on neutrophil behavior, we used a transgenic zebrafish line that allows for observation of neutrophil behavior. Zebrafish embryos were immersed in arsenic- or glucose-containing media and an immune response was stimulated through tail fin amputation or *Pseudomonas aeruginosa* injection. The total number of neutrophils per embryo was counted and the percentage of neutrophils that migrated to the site of infection or wound in control, arsenic- or glucose-treated zebrafish was calculated. We find that 1% glucose has no effect on the total number of neutrophils or the migration of neutrophils to a wound. However, treatment with 130 ppb arsenic affects neutrophil migration to a wound, with significantly fewer neutrophils being present at wound sites in arsenic treated zebrafish compared to controls.

Acknowledgments

I wish to thank my advisors, Dr. Carol Kim and Dr. Michelle Goody, for making this experience possible. I would also like to thank my committee members, Dr. Robert Wheeler, Dr. Melissa Ladenheim, and Dr. John Singer, for all of their help and support. Lastly, I would like to thank the Kim lab members and Dr. Clarissa Henry.

Table of Contents

List of Figures

Introduction

This study was done to assess the effects of arsenic and glucose on neutrophils. This study aimed to better characterize how these compounds affect our innate immune response to infection or wounding, so that we may be better informed as to what effects these compounds have when ingested into our bodies. This study is important due to the high concentrations of arsenic that have been discovered in Maine well water and the increasing rate of obesity and type 2 diabetes in Maine and the United States. With this study, we hope to increase our understanding of how these compounds affect our immune system and potentially offer support for increased regulations surrounding these compounds.

Innate Immune System:

The innate immune system is responsible for recognizing self from non-self and is involved in the clearance of pathogen infections. The innate immune system clears infections through a variety of methods including the use of: bacterial-killing substances, mucous membranes, and pro-inflammatory cells. The optimal response of the innate immune system is to release pro-inflammatory cells, such as neutrophils and macrophages, to the site of wounding and/or infection and once the site is clear of non-self organisms and materials, the correct response would be reverse migration or apoptosis of those pro-inflammatory cells (Reviewed in Guo et al., 2010). Both macrophages and neutrophils are migratory innate immune cells that destroy invading pathogens and produce a respiratory burst containing reactive oxygen species (ROS), but this study only examined neutrophils. The respiratory burst is indicative of the general

innate immune health of an organism, because the production of ROS is important in killing phagocytized pathogens. One important cell that produces ROS and thus contributes to the overall respiratory burst is the neutrophil.

Neutrophils:

Neutrophils are innate immune cells that quickly respond and migrate to areas of infection or wounding. Neutrophils phagocytize pathogens by engulfing them into phagosomes where the pathogens are subsequently digested through oxygen-dependent and oxygen-independent pathways (Willey et al., 2010). One important method of oxygen-dependent phagocytosis is the production of a respiratory burst that kills infectious agents. However, recent research has shown that mice, both healthy and diabetic, that were depleted of their neutrophils healed wounds faster than wild type mice. This result suggests that although neutrophils facilitate pathogen destruction, they inhibit the healing process in damaged tissue (Dovi et al., 2003). The long-term and/or inappropriate presence of neutrophils in sites of wounding or infection is used medically to diagnose infection or inflammatory disease. This diagnostic technique is useful, because neutrophils do not naturally reside in healthy tissue, but remain in the bloodstream to circulate to damaged tissues and sites of infection (Kindt et al., 2007). Therefore, it can be understood that neutrophils are pro-inflammatory cells that can be damaging if they do not appropriately clear from sites of infection or wounding.

Similarly to pathogens and wounds, environmental factors such as the compounds that we ingest can influence cell behavior and potentially affect neutrophil function. Two compounds of concern to the Maine population are arsenic and glucose. Arsenic is found

in high concentrations in well water throughout the state. Arsenic is a poison that is known to affect the immune system and many other bodily systems, but the mechanism of action in not fully understood. Glucose is known to affect both the number and behavior of neutrophils in diabetics, and because of the rise in type 2 diabetes and obesity in the United States, it is important to understand how high blood glucose levels are impacting our health and immunity.

Arsenic:

Arsenic is a natural metalloid that is found in the earth's crust. The EPA has recommended that arsenic in drinking water not exceed 10 parts per billion (ppb), which was a recent reduction from the former limit of 50 ppb. Arsenic is of importance to Mainers due to the presence of high levels in their well water. In some areas of Maine, the concentration of arsenic exceeds 10 ppb, and one individual well was found to contain over 3 parts per million arsenic (Nielsen et al., 2010). Arsenic is a poison that when ingested at doses higher than 10 ppb could cause health issues ranging from muscle weakness and skin lesions to lung cancer and infertility. Children who ingest arsenic throughout their development are especially at risk during adulthood for multiple health issues. Long-term accumulation of arsenic in children has been shown to affect behavior, intellectual function, memory, and the nervous system (Kapaj et al., 2006). Arsenic, at unsafe levels, has been known to affect neutrophils, by causing neutropenia, the death of neutrophils. Evidence of neutropenia is observed in Acute Promyelocytic Leukemia (APL) patients who are given arsenic as a last-ditch treatment to control neutrophil levels. The studies show that apoptosis is induced in APL cells at high arsenic doses (65.0-260 ppb) (Guo-Qiang et al., 1997). Due to the presence of arsenic in our food and water and

its known health effects, it should be studied in greater depth in order to understand how it is affecting humans and specifically in the case of this study, the innate immune system and neutrophils.

Glucose:

Glucose is an ingredient in many foods that humans ingest, which makes it not very surprising that there are diseases, such as type 2 diabetes, that can brought upon by too much glucose intake. There are two types of diabetes: Type 1 and Type 2. Type 1 is usually diagnosed in children and is characterized by the lack of insulin production. Insulin is an important hormone that is necessary for the uptake of glucose from blood into cells. Type 2 diabetes is common among adults and is characterized by a variety of complications. One complication is insulin resistance, which is the result of overworked pancreatic beta cells, cells that produce insulin, that leads to their shutdown and the resultant high blood glucose levels. Another complication is called glucose toxicity and is characterized by beta cell dysfunction from high levels of blood glucose. The percent of Americans living with diabetes is 8.3% with 139 million new cases diagnosed in 2010. In addition, there are a predicted 79 million people who are pre-diabetic (American Diabetes Association, 2011).

Glucose is a simple monosaccharide that is found in plants, and is absorbed directly into the bloodstream during digestion in humans. High levels of blood glucose have been known to affect the innate immune response by hindering a variety of neutrophil responses including: chemotaxis, phagocytosis, and bactericidal capacity (Reviewed in Guo et al., 2010). The impairment of neutrophil chemotaxis and

phagocytosis affects the ability of neutrophils to leave the extremities of humans with diabetes and can thus lead to chronic inflammation and/or infection, poor wound healing, and even amputation. The effect of high blood glucose on neutrophils potentially explains why wounds are notoriously difficult to heal in people afflicted with diabetes, and therefore, with just under 10% of the U.S. population diagnosed with diabetes, research on neutrophil behavior and migration may be key to understanding this devastating malady (American Diabetes Association, 2011); (Reviewed in Guo et al., 2010).

In addition to suppression of neutrophil activity, high levels of glucose have been shown to activate neutrophils. The activation of neutrophils can result in nonspecific damage to surrounding tissue and the suppression of neutrophils can lead to increased susceptibility to infections (Kummer et al., 2007). Furthermore, the neutrophils from diabetics who exhibit good control over their glucose levels produce significantly less ROS than people without good glycemic control. These results suggest that high blood glucose levels could cause nonspecific tissue damage from neutrophil-associated ROS (Karima et al., 2005); (Hand et al., 2007). Additionally, glucose can even act as a protective agent against the apoptosis of neutrophils. Apoptosis of neutrophils is a naturally occurring event that is important, because the chronic residence of neutrophils inhibits the resolution of the inflammatory response. One study showed that glucose maintains levels of intracellular ATP, which protect against spontaneous and anti-Fas antibody-induced neutrophil apoptosis (Healy et al., 2002). Most of the studies that have looked at neutrophils in response to glucose have been done in human neutrophils isolated from whole blood, and very few studies have looked at the effects of glucose on neutrophils in a living organism.

Zebrafish:

Zebrafish are an excellent animal model in which to analyze the effects of arsenic and glucose on neutrophil behavior because zebrafish only have innate immunity during the first few weeks of their life (Taylor et al., 2012). Zebrafish have been gaining support for their usefulness over the last few decades due to their optical transparency during their embryonic and larval stages. In addition, zebrafish are genomically and physiologically similar to mammals (Vascotto et al., 1997). They also have high fecundity and produce young *ex utero*, which allows for simplified injection and immersion in aqueous compounds, such as arsenic and glucose containing media. In order to visualize zebrafish neutrophils *in vivo,* we utilized a transgenic line of zebrafish where the myeloperoxidase (MPX) promoter drives green fluorescent protein (GFP) expression in neutrophils (Renshaw et al., 2006). The optical transparency of zebrafish also allows for visualization and imaging of infection with fluorescent pathogens, which would not be possible in other nontransparent animal models.

Previous studies done with zebrafish and these two ingested compounds have shown interesting results. Previous work from the Kim Laboratory showed that low concentrations of arsenic (2 and 10 ppb) caused a 50-fold increase in viral load and a 17-fold increase in bacterial load in zebrafish larvae. The cause of the increased pathogen load is likely due to the decreased respiratory burst response in zebrafish exposed to 2 and 10 ppb arsenic (Nayak et al., 2007). Zebrafish have also been incubated in glucose to mimic hyperglycemia and genes involved in glucose metabolism have been shown to respond similarly to high glucose levels in zebrafish and humans (Elo et al., 2007). Zebrafish embryos incubated in 1% glucose were found to have increased hematopoietic

stem cell (HSC) and red blood cell populations (Harris et al, 2013). As HSCs are precursors to innate immune cells including macrophages and neutrophils, it is possible that glucose could impact innate immunity and neutrophils by regulating the number of neutrophils during development. Zebrafish embryos are an appropriate model system for experimenting with arsenic and glucose, and should help us answer our two major research questions: Do either arsenic or glucose cause a change in the total number of neutrophils in the zebrafish, and do either arsenic or glucose cause a change in the migration of neutrophils to infections or wounds in the zebrafish?

Major Findings:

The major finding that will be discussed below is that 130 ppb arsenic treated 3 day old zebrafish had reduced neutrophil migration to a wound compared to controls. These findings suggest that arsenic at this concentration and for this duration is impairing the migration of neutrophils in zebrafish. The data also show that arsenic at this concentration is not causing neutropenia since the total neutrophil number is not decreased in the arsenic treatment group. Glucose (1%) was not found to significantly affect total neutrophil number or migration in response to wounding.

Materials and Methods

Zebrafish Care and Maintenance/Transgenic Lines:

Zebrafish embryos were collected at the one-cell stage of development from natural spawnings of adult AB or MPX transgenic zebrafish. They were kept at 28.5℃ on a 16 hour light and 8 hour dark cycle. Their egg water, the medium in which zebrafish embryos were grown, and any additions to it were changed every 24 hours. Any dead zebrafish were collected with a plastic pipette and disposed of daily. The zebrafish that were not used were euthanized by immersion in a lethal dose of tricaine followed by proper disposal. The zebrafish were dechorionated manually using forceps one day before wounding or infection. The zebrafish line expressing GFP in response to the neutrophil-specific myeloperoxidase promoter, which allows for visualization of neutrophils in alive and dead zebrafish (Renshaw et al., 2006) was a kind gift from the Renshaw Laboratory.

Arsenic Preparation and Treatment:

Sodium arsenate anhydride ($NaAsO_2$) stock solution was prepared at 10 mM. 0.5 mL of a 0.1 mM solution and 49.5 mL of egg water were added to a deep petri dish containing about 75 zebrafish. This medium now contains 1 μM of sodium arsenate anhydride, which is equivalent to 130 ppb arsenic. A control was kept that contained about 75 zebrafish in 50 mL of egg water without arsenic. The egg water with and without arsenic was changed every 24 hours. The arsenic stock was stored at 4℃ for up to a month.

Glucose Preparation and Treatment:

Anhydrous dextrose (glucose or D-glucose) was weighed out to 1.0 g and mixed into 100 mL of egg water to achieve a 1% glucose solution, which was used as the medium to grow about 75 zebrafish. The egg water and glucose were changed daily.

Bacterial Preparation and Injection:

Pseudomonas aeruginosa stock (PA14 (p6771):dTomato stock number 1544) was obtained from the -80℃ freezer and a small amount was added to a flask containing 50 mL of LB broth and 0.0375 g of ampicillin. The *P. aeruginosa* was cultured overnight at 30℃ with shaking. After the overnight period, 500 µL of bacterial solution was subcultured in 25 mL of LB broth and 0.01875 g of ampicillin for five hours. Then the culture was harvested at 5000 rpm for 10 minutes at 4℃. The supernatant fraction was removed and the pellet was resuspended with 5 mL of PBS. A second centrifugation at 5000 rpm for 10 minutes at 4℃ was completed. Next, the liquid was aspirated off and two more PBS pellet resuspensions were repeated. Finally, 3 mL of PBS was added and then vortexed for 5 seconds. The amount of bacteria was quantified by placing a 1:10 dilution of the subculture (60 µL of culture and 540 µL of PBS) into a Beckman DU640B spectrophotometer and reading at 600 nm, but before this concentration could be read a blank (600 µL of PBS) was measured at 600 nm. The target concentration was set at 9000 colony forming units (CFU) to adequately infect the zebrafish. Phenol Red solution was added to both the bacterial sample and the PBS sample at a dilution of 1:20 to ensure that each fish was properly injected in the hindbrain.

The target drop size of the injection was calibrated to 1.5 nL using a micrometer slide. The zebrafish were lightly anesthetized using a sublethal dose of tricaine (168 mg/L) before being lined up on a warmed agarose plate. Each fish was injected with either *P. aeruginosa* or PBS into their hindbrain through the otic vesicle (ear). The fish were then returned to their respective treatments in 50 mL of egg water and then placed in a 28℃ incubator.

Tail Fin Tip Amputation:

After 72 hours post fertilization (hpf), the zebrafish were placed in a sublethal dose of tricaine to render them immobile. They were then placed on a warmed agarose plate and had the tip of their caudal tail fin cut off with a sterile scalpel (Brad Parker Rib-Back carbon steel surgical blade no. 10) under a dissecting microscope (Olympus SZ61), while making sure not to damage the notochord (Figure 1). The fish were then returned to their respective treatments in 50 mL of egg water and then placed in a 28℃ incubator.

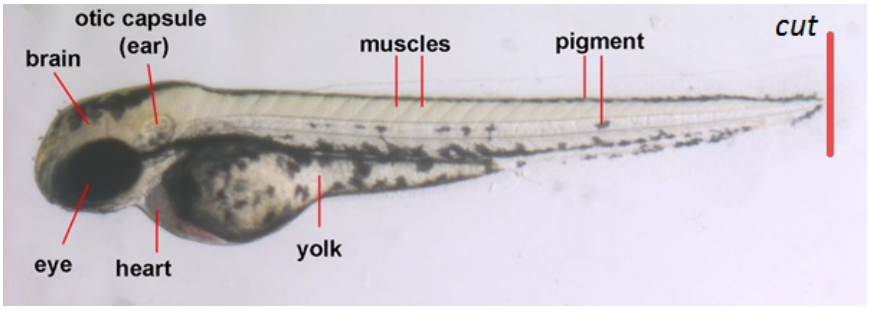

Figure 1: Caudal Tail Fin Amputation. The tip of the caudal tail fin was amputated just posterior to the notochord of a lightly anesthetized zebrafish. Image modified from www.nc3rs.org.uk.

Fixing:

After the wounding or injection of the zebrafish, they were fixed with 4% para-formaldehyde, which both kills them and crosslinks their proteins into a rigid structure making them more durable for long-term storage and imaging. After an overnight period of incubation (4℃) in fixative, the fish were rinsed out of fixative using PBS containing 0.1% Tween. This washing was done three times with PBS 0.1% Tween and then fish were left in PBS 0.1% Tween in a dark refrigerator at 4℃ for up to a month before being imaged.

Mounting:

In order to prepare the zebrafish for imaging, it was necessary to mount the fish on microscope slides. The zebrafish were pipetted out of their PBS 0.1% Tween solution and into a deep petri dish with about 25 mL of PBS. Under a dissecting microscope, the fish were manually de-yoked using sharp needles to pry the yolk sac from the rest of the embryo. Next four dollops, the size of a pin head, of vacuum grease were applied to the corners of one side of a microscope slide at about a centimeter from the edge of the slide. One zebrafish was then removed from the deep Petri dish and placed on the slide in-between the four dollops of vacuum grease and, using the sharp needles, the fish was decapitated in order to ensure clearer images and allow for better positioning of the fish. Lastly, a glass coverslip was placed upon the vacuum grease and pressure was gently applied to each corner. This was repeated twice more for two more fish on the same microscope slide (Figure 2).

Figure 2: Mounting Zebrafish on Microscope Slides. This cartoon shows that three zebrafish per microscope slide were mounted and imaged. The zebrafish had their yolk sacs and heads removed and discarded when imaging wounded fish (for infected fish the heads were saved and imaged). The dark gray circles represent small dollops of vacuum grease on which a glass cover slip was placed. The corners of the cover slip were firmly pressed to keep the zebrafish from moving during transportation to the microscope.

Imaging:

Images were acquired on a Zeiss Axio Imager.Z1 with an Apotome attachment running Axio Vision software. The 5X objective was used to image the body of the fish and the 10X objective was used to image the tail for wounding experiments and the head for infection experiments. Images were acquired by Dr. Michelle F. Goody.

Counting/Quantifications of neutrophils:

The images of the zebrafish embryos were then viewed using Axio vision software in order to count the number of neutrophils in each zebrafish (Figures 3 & 4). Neutrophils were counted by hand by two separate people at separate times. The number of neutrophils in the tail (wounding experiment), head (infection experiment), and trunk (both experiments) were recorded in an excel document.

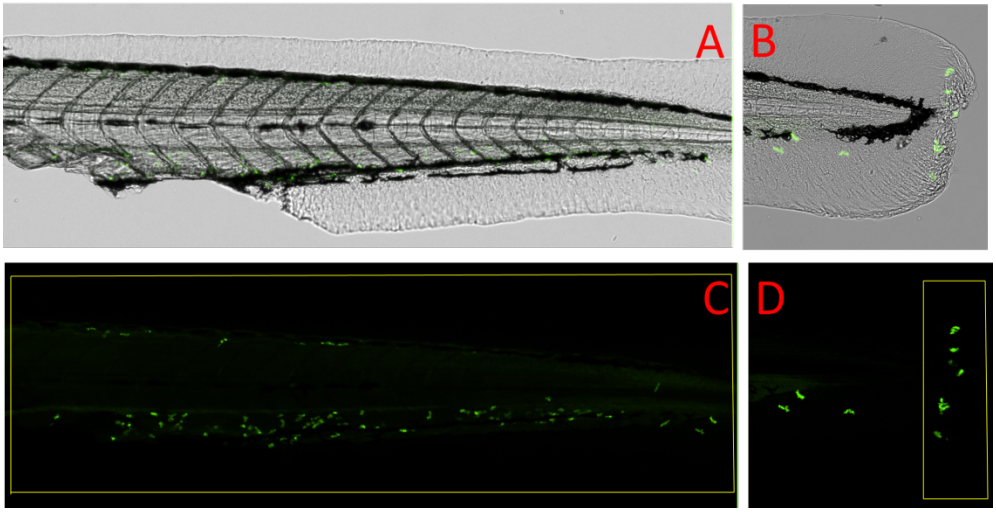

Figure 3: Zebrafish Neutrophils Migrate to a Tail Fin Wound. Panels A&B show brightfield and fluorescent images of a 3 dpf zebrafish with a cut tail and panels C&D show the same zebrafish with fluorescent neutrophils. The yellow boxes indicate the areas in which neutrophils were counted. Total Neutrophil Count = trunk neutrophils + tail neutrophils; Normalized Neutrophil migration = tail neutrophils / (trunk neutrophils + tail neutrophils) X 100.

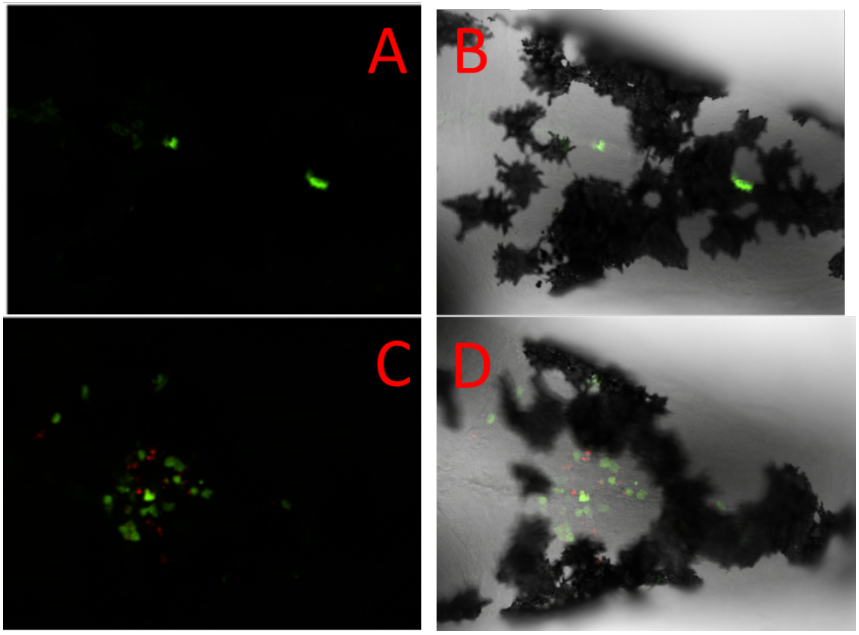

Figure 4: Zebrafish Neutrophils Migrate to *P. aeruginosa* Infection in The Hindbrain Ventricle. Panel A shows fluorescent neutrophils in the head of a zebrafish injected with PBS and panel B shows the same zebrafish in a brightfield and fluorescent image. Panel C shows fluorescent neutrophils (green) and *P. aeruginosa* (red) in a fluorescent image, and panel D shows the same fish in a brightfield and fluorescent image. Total Neutrophil Count = trunk neutrophils + head neutrophils; Normalized Neutrophil migration = head neutrophils/(trunk neutrophils + head neutrophils) X 100.

Statistics:

The total neutrophil counts per embryo were compiled from 2-4 independent experiments and averaged for each treatment that was performed. A normalized neutrophil migration value was obtained for each individual fish using the following equation: normalized neutrophil migration = head/tail neutrophils/(trunk neutrophils + head/tail neutrophils) X 100. The normalized neutrophil migration values per embryo were compiled for 2-3 independent experiments and averaged for each treatment that was performed. The standard error of the mean was calculated for both the normalized migration value and the total number of neutrophils. An unpaired, two-tailed T-Test was performed on the data to find levels of significance.

Respiratory Burst Assay:

1.0 mg of H2DCFDA was weighed out and then mixed into 1 mL of dimethyl sulfoxide (DMSO). This 1 mg/mL stock solution was aliquoted and stored in a -20℃ freezer until used. Next the phorbol myristate acetate (PMA) was weighed out to 1 mg and dissolved into 1 mL of DMSO, which produces a 1 mg/mL stock. This stock was aliquoted and stored at -80℃ until needed. A working solution of H2DCFDA was then made up by adding 20 µL of H2DCFDA to 20 µL of DMSO in a 1.7 mL microcentrifuge tube wrapped in aluminum foil. A working solution of PMA was also created by adding 10 µL of PMA to 490 µL of nuclease free water in a 1.7 mL microcentrifuge tube.

A dosing solution of H2DCFDA was created by adding 4990 µL of egg water and 10 µL of H2DCFDA working solution to a 15 mL conical tube labeled “H”. A dosing solution of H2DCFDA and PMA was then created by adding 4890 µL of egg water, 10

μL of H2DCFDA working solution, and 100 μL of PMA working solution into a 15 mL conical tube labeled “H+P”. These dosing solutions were wrapped in foil and kept on ice until used.

If the zebrafish were part of the arsenic or glucose treatment groups, they were washed out of their treatment solution and into egg water three times, because the presence of certain compounds has been known to skew the results. Zebrafish were then individually added to a 96 well microplate along with 100 μL of egg water. Columns 1-4 held control zebrafish, 6-9 held the experimental group of zebrafish, and 5 was left blank. The H2DCFDA dosing solution was poured into a 25 mL reservoir. A multichannel pipettor with eight tips was used to pipette 100 μL of the H2DCFDA dosing solution into columns 1-2 and 6-7, one column at a time. The H2DCFDA and PMA dosing solution was then poured into another 25 mL reservoir. A multichannel p200 pipettor with eight tips was used to pipette 100 μL of the H2DCFDA and PMA solution into columns 3-4 and 8-9, one column at a time.

The microplate was then covered with aluminum foil and placed on a shaker for about 20 seconds at 150 rpm to ensure a homogeneous mixture. The microplate was then read at time = 0 hr in a microplate reader set to read fluorescence: Excitation – 485 nm, Emission – 528 nm, Optics position – top 510 nm, Sensitivity – 65, and a 5 second shaking before the read. The microplate was also read at time = 4 hr.

To analyze the data, the average un-induced control group’s fluorescence was subtracted from the individual PMA-induced control group’s florescence values. This calculation was also done from the experimental group with and without PMA. The data

were compiled from individual experiments, organized into two columns, the control + PMA group and the experimental + PMA group, and the mean and standard deviations were calculated from the normalized fluorescence values of the two columns. Next, the normalized fluorescence values were compared using an unpaired, two-tailed t-test. The means of the two groups, control + PMA and experimental + PMA, were graphed with error bars using the respective standard deviation (Goody et al., 2013); (Hermann et al., 2004).

Results

130 ppb Arsenic Has No Significant Effect on Total Neutrophil Number in Response to Infection in 2 Day Old Zebrafish: Previous work from the Kim lab showed that zebrafish exposed to 2 and 10 ppb arsenic for 4-9 days displayed a significantly reduced respiratory burst response and increased viral and bacterial burden (Nayak et al., 2007). Therefore, arsenic dampens the innate immune response in zebrafish. We wanted to determine the effect of arsenic on innate immunity involved neutrophils by assessing total neutrophil numbers and neutrophil migration to a wound or site of localized infection with and without arsenic exposure. Because a standard protocol in the Kim lab involves infecting zebrafish with *Pseudomonas aeruginosa* bacteria in the hindbrain ventricle at 2 dpf and assessing neutrophil migration at 3 hours post infection (Phennicie et al., 2010), we chose to infect zebrafish at 2 dpf. Because this means that the zebrafish (exposed to arsenic from fertilization) were only exposed to arsenic for 2 days (rather than 4-9 days as per Nayak et al., 2007) and because another group found more consistent changes in zebrafish innate immunity-related gene expression at 100 ppb arsenic exposure compared to 10 ppb arsenic (Mattingly et al., 2009), we exposed zebrafish to 130 ppb arsenic in these experiments. We hypothesized that an infection into the hindbrain ventricle of the zebrafish would result in an increased total neutrophil count and that arsenic may reduce neutrophil number because arsenic is known to cause neutrophil apoptosis. The zebrafish were grown from the single cell stage to 2 dpf in either 130 ppb or 0 ppb or arsenic. On day 2 post fertilization, the zebrafish were injected with either a subculture of *P. aeruginosa* (PA) with an OD600 target of $9X10^3$ CFU or PBS. At 3 hours post injection, the zebrafish were fixed overnight and then imaged.

The data were obtained by counting the total number of neutrophils in each zebrafish by using the following equation: total neutrophil count = trunk neutrophils + head neutrophils. The results from Figure 5 show that neither arsenic exposure nor bacterial infection into the hindbrain at 2 dpf produce a significant change in the total number of neutrophils. These findings prompted us to ask our second overall question: does either compound affect the migration of neutrophils in response to bacterial infection?

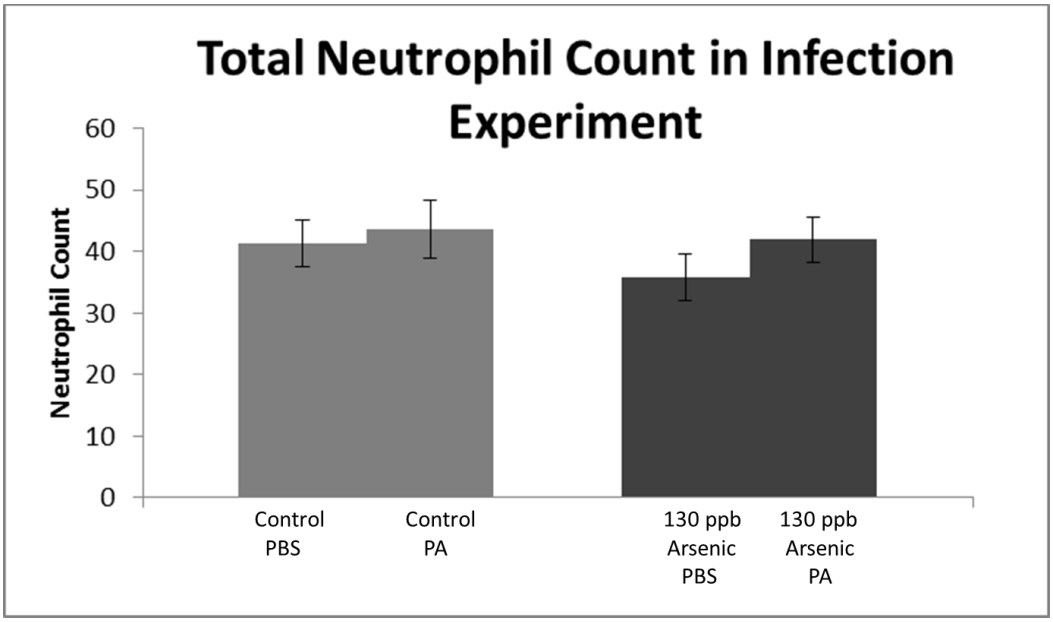

Figure 5: 130 ppb Arsenic Does Not Change Total Neutrophil Number. *P. aeruginosa* does not change the total neutrophil number in control or arsenic treated zebrafish embryos. This experiment was repeated twice and the data were compiled. 10 zebrafish were used in each treatment per replicate. A two-tailed TTEST was used to determine significance and Standard Error of the Mean (SEM) was used to determine error.

The Migration of Neutrophils in 130 ppb Arsenic Treated Zebrafish is Not Significantly Effected in Response to Bacterial Infection in 2 dpf Zebrafish. We hypothesized that bacterial infection in 130 ppb arsenic treated 2 dpf zebrafish will

decrease the migration of neutrophils to the hindbrain ventricle because arsenic has been shown to increase both the viral and bacterial load in zebrafish. The data was calculated by using the following equation: normalized neutrophil migration = head neutrophils/(trunk neutrophils + head neutrophils) X 100. There was a significant increase in the migration of neutrophils in response to infection in the control and arsenic treated zebrafish (Figure 6). This result was expected because neutrophils migrate to sites of infection. The hindbrain does not normally contain neutrophils, thus their migration there is only in response to the infection. There was no significant change in migration between arsenic treated and control zebrafish, which suggests that under these parameters arsenic does not affect neutrophil migration to a hindbrain bacterial infection.

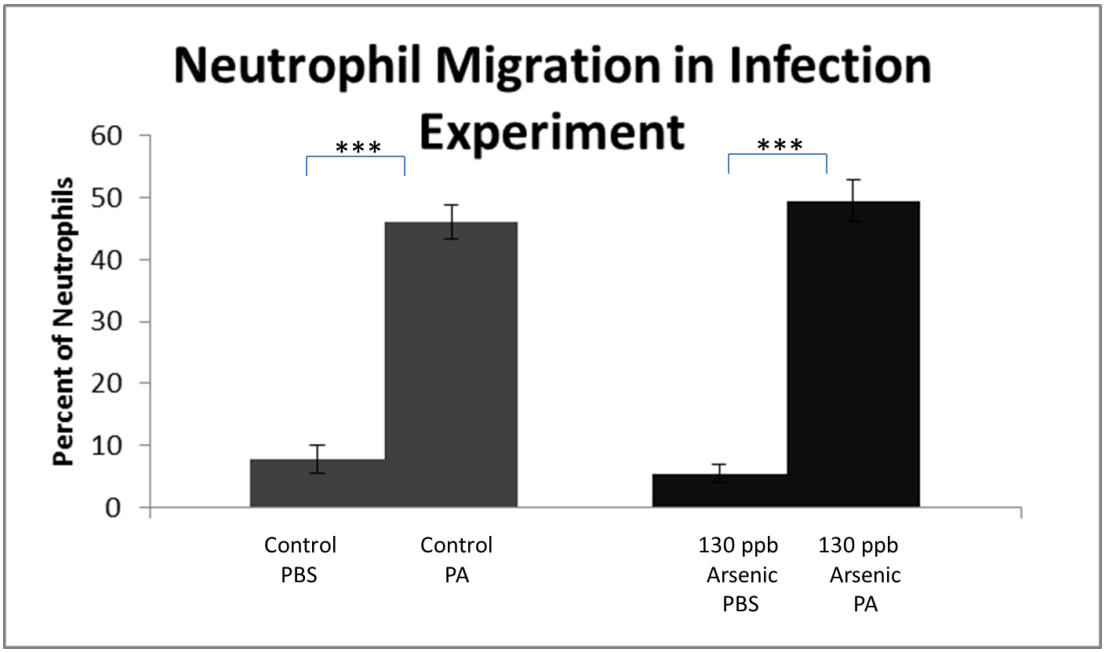

Figure 6: 130 ppb Arsenic Does Not Effect Neutrophil Migration to an Infection. *P. aeruginosa* causes significantly more neutrophil migration to the hindbrain ventricle, but arsenic has no effect on the neutrophil migration. This experiment was repeated twice and the data were compiled. 10 zebrafish were used in each treatment per replicate. A two-tailed TTEST was used to determine significance and SEM was used to determine error.

Neither 130 ppb Arsenic nor 1% D-Glucose Has Any Significant Effect on Total Neutrophil Number in Response to Wounding in 3 Day Old Zebrafish: Another way to stimulate neutrophil migration is to induce a tissue wound. A caudal tail fin amputation is routinely done in zebrafish to assay neutrophil recruitment (Renshaw et al., 2006); (Yoo et al., 2012). We originally amputated zebrafish tail fins at 2 dpf to be consistent with our bacterial infection experiments, but wounding was too variable and too severe at 2 dpf due to the small amount of tail fin tissue present, so we conducted tail fin amputation experiments at 3 dpf. Additionally, since we didn't observe any changes in neutrophil number or migration in response to 130 ppb arsenic and bacterial infection at 2 dpf, we wanted to increase the duration of arsenic exposure to 3 days. In this new set of experiments, we also tested an additional compound, glucose. The maximum recruitment of neutrophils to a wound in 3-5 day old zebrafish has been shown to occur at 5 or 6 hours post wounding (Renshaw et al., 2006); (Yoo et al., 2012); however, we assayed neutrophil recruitment at 3 hours post wounding to be consistent with our bacterial infection experiments. The zebrafish were immersed in arsenic at 130 ppb and glucose at a concentration of 1%. After 3 days in arsenic or glucose, a portion of the zebrafish had their caudal tail fin amputated. All the zebrafish were fixed at three hours after the amputation, and then imaged.

We first assessed total neutrophil number in response to wounding and/or 130 ppb arsenic or 1% glucose in 3 day old zebrafish. We hypothesized that the total neutrophil number would increase in response to 1% glucose due to the recent study showing that glucose increased HSCs and red blood cells (Harris et al., 2013). We also hypotheisized that arsenic would drecrease the total number of neutrophils since arsenic used medically

to cause neutrophil apoptosis in APL patients. The data were obtained by using the following equation: total neutrophil count = trunk neutrophils + tail neutrophils. The data in Figure 7 show that neither 1% D-glucose treated zebrafish nor 130 ppb arsenic treated zebrafish showed significant changes in the total number of neutrophils when compared to control zebrafish. The amputation caused a slight, but not significant increase in neutrophils in the control and 1% glucose treated zebrafish , which suggests that a significant amount of new neutrophils are not made in response to this type of wounding. In addition, it can be concluded that 130 ppb arsenic for this duration does not induce a significant level of neutropenia, because we did not see a reduced total neutrophil number in the arsenic treated group compared to the control. There was a slight increase in total neutrophil number in the 1% glucose treated zebrafish in comparison to controls, but this was not significant. Next, we wanted to know if the migration of neutrophils to a wound was affected in response to arsenic or glucose in 3 dpf zebrafish.

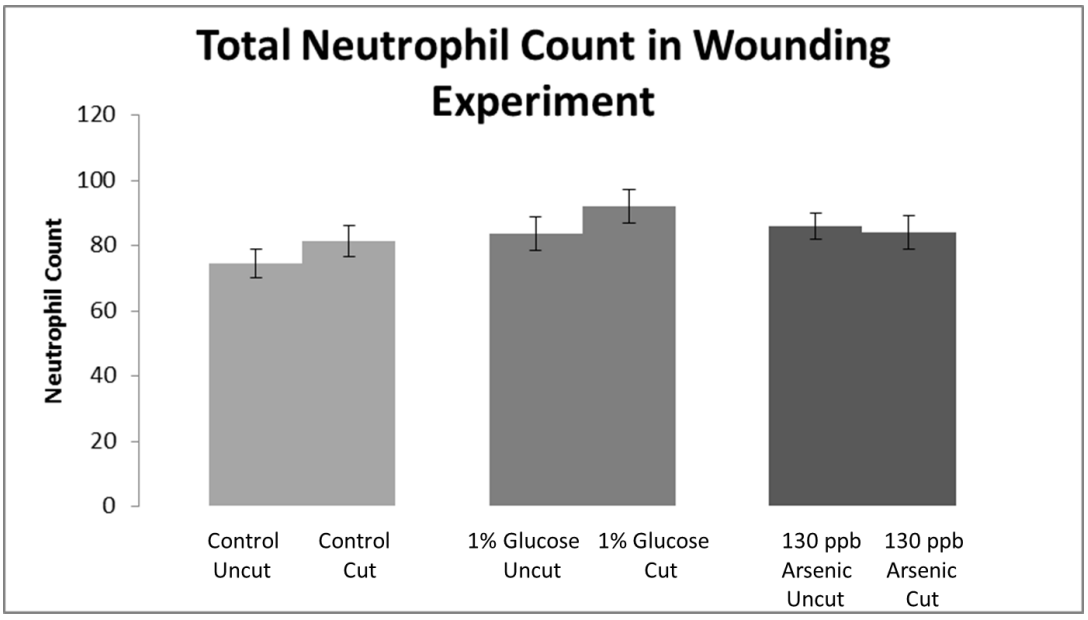

Figure 7: Neither 130 ppb Arsenic Nor 1% Glucose Changes Total Neutrophil Number. Neither wounding, 130 ppb arsenic, or 1% glucose significantly change the total neutrophil number in 3 dpf zebrafish embryos. This experiment was repeated once and the data were compiled. 10 zebrafish were used in each treatment per replicate. A two-tailed TTEST was used to determine significance and SEM was used to determine error.

The Migration of Neutrophils in 130 ppb Arsenic Treated Zebrafish is Significantly Decreased in Response to Wounding in 3 Day Old Zebrafish. We hypothesize that there will be an increased number of neutrophils in the tail in the wounded zebrafish compared to the non-wounded zebrafish, because a normal innate immune response is to mobilize neutrophils to the site of wounding. We also hypothesize that the arsenic and glucose will cause a decrease in neutrophil migration to a wound, because arsenic increases pathogen burden in zebrafish (Nayak et al., 2007) possibly through impaired neutrophil migration and glucose has been shown to impair neutrophil migration in human blood samples (Reviewed in Guo et al., 2010). The data were obtained by calculating the normalized migration value using the following equation: normalized

neutrophil migration = tail neutrophils/(trunk neutrophils + tail neutrophils) X 100. The caudal fin amputation was done to stimulate an innate immune response in 3 dpf zebrafish. At 3 hours post wounding, the zebrafish were fixed overnight, and then mounted and imaged.

For each experimental treatment of wounded vs. unwounded zebrafish, there was a significant increase in the migration of neutrophils to the wound (Figure 8). This result was expected, because neutrophil migration to a site of wounding is an appropriate innate immune response. We observed a non-significant reduction in neutrophil migration to a wound in 1% glucose treated zebrafish. Interestingly, we found 130 ppb arsenic to significantly decrease the migration of neutrophils to a wound in 3 day old zebrafish. This result was not surprising since Nayak et al. have shown that zebrafish from 4 dpf through 9 dpf have a reduced respiratory burst in response to 10 ppb and 2 ppb arsenic. In addition, the data show that the unwounded 1% glucose treated zebrafish have a trend toward increased neutrophil migration to the tail fin compared to both arsenic treated and control unwounded zebrafish. These results are similar to diabetic patients, who have prolonged high blood glucose levels and chronic inflammation and neutrophil residence in their extremities.

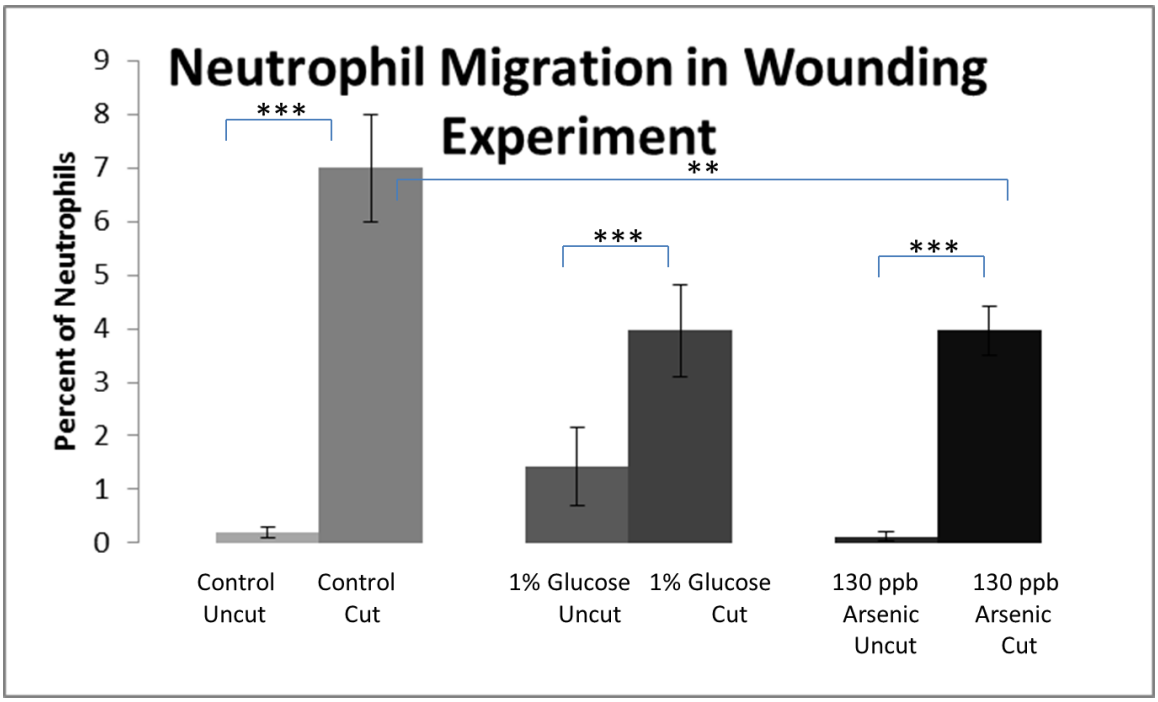

Figure 8: 130 ppb Arsenic Significantly Decreases Neutrophil Migration to a Wound, and 1% Glucose Trends Toward Less Neutrophil Migration to a Wound and More Migration to an Unwounded Zebrafish tail. Wounding of the caudal tail fin causes significantly more neutrophil migration to the wound in each treatment. Neutrophil migration to a tail fin wound is reduced in both glucose and arsenic treated zebrafish, but the reduction is only significant in arsenic treated zebrafish. In glucose treated zebrafish without a wound, there is a greater percentage of neutrophils in the tail fin than in controls or arsenic treated zebrafish. This experiment was repeated once and the data were compiled. 10 zebrafish were used in each treatment per replicate. A two-tailed TTEST was used to determine significance and SEM was used to determine error.

Respiatory Burst Assay Of Glucose Treated 3 Day Old Zebrafish Showed No Significant Change in Respiatory Burst Levels: We hypothesized that the RBA of 1% glucose treated 3 dpf zebrafish would be increased since recent publications have shown that neutrophils from diabetics produce more ROS (Karima et al., 2005); (Hand et al., 2007). The respiratory burst procedure as described above was completed and fluorescence was read in a microplate reader at times 0 and 4 hours. The 4 hour time point data was analyzed and the results show that there is no significant change in the

levels of ROS between the control and the 1% D-glucose treatments (Figure 9). This result was unexpected and did not support our hypothesis.

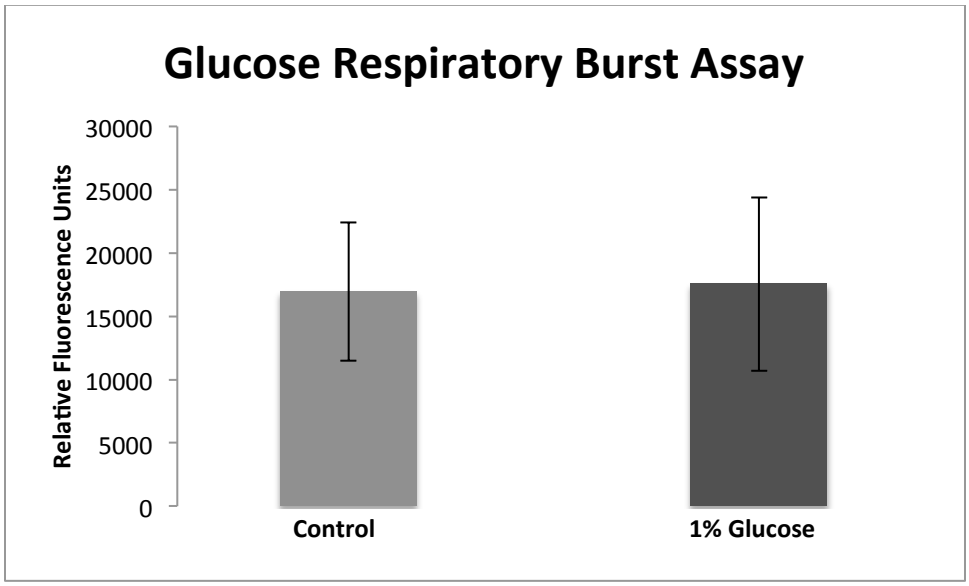

Figure 9: 1% Glucose Has No Effect On The Respiratory Burst in Zebrafish. This figure shows that there is no change between control and glucose treated zebrafish in the amount of reactive oxygen species produced by phagocytes. This experiment was repeated once and the data were compiled. 16 zebrafish were used in each treatment per replicate. A two-tailed TTEST was used to determine significance and SEM was used to determine error.

Discussion

Given the importance of neutrophils in the resolution of wounds and infections and the ability of compounds ingested by humans to affect neutrophil behavior, we undertook these studies to determine the effects of arsenic and glucose on neutrophils using the zebrafish model system. These experiments have shown that control, arsenic, or glucose treated 2-3 dpf zebrafish do not show a change in total neutrophil number in response to wounding or infection. We also observed that infection and wounding significantly increased the migration of neutrophils to the site of infection or wounding, but the addition of arsenic in the infection experiments had no effect on the migration values. Although there was no change observed in arsenic treated zebrafish in response to infection, there was a significant decrease in neutrophil migration to a wound in arsenic treated zebrafish and a trend toward a decrease in neutrophil migration in glucose treated zebrafish in response to wounding. There was also an observed trend in the unwounded zebrafish treated with glucose that showed an increased percentage of neutrophils in the tip of the tail compared to both control and arsenic treated, unwounded zebrafish. All together, these data show that incubation of zebrafish embryos in compounds that are continually ingested into the human body does affect neutrophil behavior. Some interesting, unresolved questions that arose from these experiments as well as future directions for this line of investigation will be discussed below.

The effect of arsenic on neutrophil migration to a wound vs. a localized bacterial infection

We stimulated an innate immune response in order to assay neutrophil migration in two different ways: we injected bacteria into the hindbrain ventricle of 2 dpf zebrafish to establish a localized infection and we amputated the caudal tail fin of 3 dpf zebrafish to cause a wound. We then tested the effect of 130 ppb arsenic on the migration of neutrophils to the hindbrain ventricle or the caudal tail fin. We found that arsenic at 130 ppb only significantly affected neutrophil migration in wounded zebrafish and not infected zebrafish. This discrepancy could be due to multiple reasons, but we think the most likely reason is that one experiment was done in 2 dpf zebrafish and the other was done in 3 dpf zebrafish. In the transgenic MPX zebrafish line that we used to visualize neutrophils, fluorescent neutrophils are first visible around 30 hours post fertilization (Renshaw et al., 2006). Therefore, neutrophils are still in the process of maturing on the second day of zebrafish development. From our data, you can see that the total neutrophil counts almost doubled from approximately 40 to 80 neutrophils per embryo between days 2 and 3 post fertilization (Figures 5 and 7). Additionally, conducting experiments in 3 dpf zebrafish that have been incubated in arsenic since fertilization means that the embryos have been exposed to arsenic for 24 more hours than when experiments were performed in 2 dpf zebrafish. Research in the Kim Lab previously found effects of 2 ppb and 10 ppb arsenic on the innate immune response in zebrafish on days 4-9 post fertilization (Nayak et al., 2007), therefore it may take longer than 2 days of exposure for arsenic to impact zebrafish immunity. To determine if the longer exposure time to arsenic and/or the increased number of neutrophils per embryo could account for the difference in the effect

of arsenic on neutrophil migration in 3 dpf wounded zebrafish compared to 2 dpf infected zebrafish, future experiments could involve infecting and assessing neutrophil migration in 3 dpf zebrafish or wounding and assessing neutrophil migration in 2 dpf zebrafish. We tried wounding and assessing neutrophil migration in 2 dpf zebrafish and found the tail fin tissue too small to consistently amputate. Therefore, we think infecting the zebrafish at 3 dpf to account for the increased neutrophils and longer exposure time to arsenic would be the best future experiment to try to answer this question.

Another possibility is that neutrophils may respond differently to infections and wounds and arsenic only effects neutrophil migration to a wound. Support for this idea can be seen from our data where bacterial infection causes a higher percentage of neutrophils to migrate to the site compared to a wound (approximately 45% vs. 7%, respectively; Figures 6 and 8). Because neutrophil migration to an infection is so significant, any subtle effect of arsenic on that migration may have been drowned out. Because a smaller proportion of neutrophils migrated to a tail fin wound, that may have allowed us to observe differences in neutrophil migration in control and arsenic treated groups. Injecting fewer bacteria into the hindbrain could result in approximately 7% of neutrophils migrating to that site and then the effect of arsenic between these two experiments could be better compared. It is also possible that stimulating the innate immune response and neutrophil migration in the brain is very different than in the tip of the tail. It makes sense that there would be more of a response to an infection or wound in the brain than in an extremity because the brain is critical for life and an extremity is not. To control for this difference, a wound could be induced in the brain or an infection could be established in an extremity and then neutrophil migration and the effect of arsenic on

neutrophil migration could be determined and compared. It would be interesting to perform these experiments to better understand the context in which arsenic significantly effects neutrophil migration.

The lack of an effect of 1% glucose on neutrophil number, migration, or respiratory burst

In addition to arsenic, we also determined the effect of another compound, glucose, on neutrophil numbers and migration to a wound. We expected the total number of neutrophils in zebrafish grown in 1% glucose to increase because it has been shown that 1% glucose increases the onset and magnitude of hematopoietic stem cells (Harris et al., 2013), which are precursors to red blood cells, macrophages, and neutrophils. In addition, glucose has been implicated in acting as a protective agent against neutrophil apoptosis (Healy et al., 2002), which further supports the idea that the total neutrophil number in zebrafish treated with glucose should have increased compared to control zebrafish. Reasons why we may not have observed an increase in total neutrophil number in this experiment could be due to its relatively short run time or the concentration of glucose used. It might take more than 3 days of glucose exposure to have enough time to produce a significant increase in total neutrophil number in zebrafish. We suggest that, in future experiments involving glucose, increased exposure duration and higher concentrations of glucose are tested for an effect on total neutrophil number in zebrafish. It is also possible that 1% glucose could increase HSCs and red blood cells without affecting neutrophil numbers. As the migration of neutrophils to a wound in glucose treated zebrafish showed a trend toward reduced migration, repeating this experiment and

re-analyzing the data with a larger sample number may make the effect of 1% glucose on zebrafish neutrophils more clear.

In addition to neither the migration nor total neutrophil number in 1% glucose treated zebrafish being significantly changed, the RBA was not affected. We expected the respiratory burst assay to show an increased respiratory burst response in 1% glucose treated zebrafish compared to controls because glucose is known to increase the respiratory burst in human neutrophils (Karima et al., 2005) (Hand et al., 2007). One reason why we don't see a significant increase in the respiratory burst in our experiments might be due to the lack of specificity of the RBA in whole zebrafish embryos. This zebrafish assay measures all ROS produced in the whole zebrafish in response to the chemical PMA, not just ROS produced by neutrophils. Therefore, a change in the neutrophil respiratory burst in response to glucose could be masked in the RBA in zebrafish because ROS are measured in whole embryos and not just neutrophils. Because zebrafish embryos are so small and have a small blood volume, it is not practical to isolate blood from embryos. However, the effects of increased concentrations of glucose on the respiratory burst could be tested in whole embryos or on blood isolated from older zebrafish.

It is possible that the glucose added to the media may not be well absorbed by zebrafish embryos. However, changes to the expression of glucose metabolism genes were observed in zebrafish incubated in 40 mM glucose (0.73% glucose) from 96 hpf to 144 hpf (Elo et al., 2007) and changes to blood cell development were seen in zebrafish exposed to 1% glucose from fertilization along with increased glucose concentration in zebrafish treated with glucose (Harris et al., 2013). Therefore, we believe that glucose

added to the media is absorbed by zebrafish embryos. Measuring levels of acetylated hemoglobin and/or insulin in glucose exposed zebrafish would allow us to determine if and relatively how much glucose is absorbed by zebrafish embryos. In future experiments involving glucose exposure and zebrafish, a control treatment with a comparable, yet inactive solute (osmotic control) would be beneficial in order to determine if osmotic stress is playing a role in innate immunity and neutrophil migration.

Additional Future Directions:

These experiments were a jumping off point into the investigation of the effects of arsenic and glucose on neutrophil migration to an infection or wound. In future investigations of the effects of certain compounds on neutrophil numbers and migration, we suggest that a few different experimental procedures be used. For streamlining of total neutrophil counts, we suggest using flow cytometry to quantify the percentage of GFP positive cells per sample because manual counting of GFP positive cells in an image is not very efficient or reliable. In addition, the imaging process is time consuming and requires a high level of skill and patience to acquire images that are clear enough to count neutrophils. Also, the images of the trunk of the zebrafish do not account for all of the neutrophils in the entire zebrafish whereas flow cytometry would be able to analyze fluorescent cells in the entire zebrafish embryo. For future analysis of neutrophil migration, we propose using time-lapse microscopy in live embryos instead of acquiring images from fixed embryos. In zebrafish, it has been shown that the peak recruitment of neutrophils to a wound occurs 5-6 hours post wounding (Renshaw et al., 2006); (Yoo et al., 2012) and also that neutrophils can migrate away from the wound site, called reverse migration, and return to circulation (reviewed in Starnes et al., 2012). We analyzed

neutrophils at the site of a wound 3 hours post wounding and saw fewer neutrophils at the wound site in arsenic exposed zebrafish, but we are unable to definitively say whether neutrophil forward migration or reverse migration or both are affected by 130 ppb arsenic. The use of time-lapse microscopy and a transgenic line of zebrafish expressing a photoconvertable fluorescent protein in their neutrophils could be used to answer this question. This experiment would be very technically demanding, but would provide more information on how arsenic affects neutrophil migration. We hope these studies will be followed up on in the future because investigation into how compounds that we ingest impact our health are very important endeavors.

References

Chen, Yau-Hung, and Ching-Yuh Chern. "Evaluation of the Anti-Inflammatory Effect of Chalcone and Chalcone Analogues in a Zebrafish Model." Molecules (2013): 2052-2060. Print.

"Division of Environmental Health." *Well Water Quality*. N.p., 1 Jan. 2013. Web. 20 Apr. 2014. <http://www.maine.gov/dhhs/mecdc/environmental-health/eohp/wells/index.htm>.

Dovi, Julia, He Li-Ke, et al. "Accelerated wound closure in neutrophil-depleted mice." *Journal of Leukocyte Biology*. 73. (2003): 448-455.

Elo, B, C Villano, et al. "Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpytuvate carboxykinase as a marker for exposure to anti-diabetic compounds." *Journal of Molecular Endocrinology*. 38. (2007): 433-440.

Goody, M.F., Peterman, E., Sullivan, C., Kim, C.H. Quantification of the Respiratory Burst Response as an Indicator of Innate Immune Health in Zebrafish. J. Vis. Exp. (79), e50667, doi:10.3791/50667 (2013).

Guo, S, and L.A. DiPietro. "Factors Affecting Wound Healing." *Journal of Dental Research*. 89.3 (2010): 219-229.

Guo-Qiang, Chen, Shi Xue-Geng, et al. "Use of Arsenic Trioxide (As2O3) in the Treatment of Acute Promyelocytic Leukemia (APL): I. As2O3 Exerts Dose-Dependent Dual Effects on APL Cells." *Blood*. 89.9 (1997): 3345-3353.

Hand, W, Debra Hand, et al. "Increased polymorphonuclear leukocyte respiratory burst function in type 2 diabetes." *Diabetes Research and Clinical Practice*. 76. (2007): 44-50.

Harris, James, Virginie Esain, et al. "Glucose metabolism impacts the spatiotemporal onset and magnitude of HSC induction in vivo." *Blood*. 121.13 (2013): 2483-2493.

Healy, Declan, R William, et al. "Glucose, but not glutamine, protects against spontaneous and anti-Fas antibody-induced apoptosis in human neutrophils." *Clinical Science*. 103. (2002): 179-189.

Kapaj, Simon, and Prosun Bhattacharya. "Human Health Effects From Chronic Arsenic Poisoning–A Review." Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering 41 (2006) 2399-2428.

Karima, M, A Kantarci, et al. "Enhanced superoxide release and elevated protein kinase C activity in neutrophils from diabetic patients: association with periodontitis." *Journal of Leukocyte Biology*. 78. (2005): 862-870.

Kindt, Thomas, Richard Goldsby, and Barbara Osborne.*Kuby Immunology*. Sixth. New York: W. H. Freeman and Company, 2007.

Kummer, Ursula, Jurgen Zobeley, et al. "Elevated Glucose Concentrations Promote Receptor-Independent Activation of Adherent Human Neutrophils: An Experimental and Computational Approach."*Biophysical Society*. 92.2597-2607 (2007)

Mathias, Jonathan, and Anna Huttenlocher. "Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish." Journal of leukocyte biology 80 (2006): 1281-1288. Print.

Mattingly, Carolyn, and Antonio Planchart. "Perturbation of Defense Pathways by Low-Dose Arsenic Exposure in Zebrafish Embryos." Environmental Health Perspectives 117 (2009): 981-987.

Nayak, AS, CR Lage, and CH Kim. "Effects of low concentrations of arsenic on the innate immune system of the zebrafish (Danio rerio)." *Toxicology Sciences*. 98.1 (2007): 118-124.

Nielsen, Martha. "Assessment of Arsenic Concentrations in Domestic Well Water, by Town, in Maine, 2005–09." Scientific Investigations Report (2010): n. pag. Print.

Niethammer, Philipp, Clemens Hrabher, et al. "A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish." *Nature*. 459. (2009): 996-1000.

Phennicie, R.T., and C. H. Kim. "Specific Resistance to Pseudomonas aeruginosa Infection in Zebrafish Is Mediated by the Cystic Fibrosis Transmembrane Conductance Regulator." infection and immunity 78 (2010): 4542-4550.

Renshaw, Stephen, Catherine Loynes, and Daniel Trushell. "A transgenic zebrafish model of neutrophilic inflammation." Blood. (2006): 3976-3978.

Starnes, Taylor. "Neutrophil ReverseMigration Becomes Transparent with Zebrafish." Advances in Hematology (2012): n. pag. Print.

"Statistics about Diabetes." *American Diabetes Association*. American Diabetes Association, 26 Jan 2011. Web. 6 Apr 2014. <http://www.diabetes.org/diabetes-basics/statistics/>.

Taylor, Harriet, and Margaret Dallman. "P38 and JNK have opposing effects on persistence of in vivo leukocyte migration in zebrafish." Immunology and Cell Biology 91. (2012): 60-69. Web. 8 May 2014.

"The innate and adaptive immune systems."*informedhealthonline.org*. informedhealthonline, 04 Apr 2012. Web. 6 Apr 2014. <http://www.informedhealthonline.org/the-innate-and-adaptive-immune-systems.2255.en.html>.

Wiley, Joanne, Linda Sherwood, and Christopher Woolverton.*Prescott's Microbiology*. Eighth. New York: McGraw Hill, 2007.

Yoo, S., and A. Huttenlocher. "Early redox, Src family kinase, and calcium signaling integrate wound responses and tissue regeneration in zebrafish." The Journal of Cell Biology (2012): 225-234.

Author's Biography

Jacob R. Longfellow was born in Lewiston, Maine on March 31st, 1992. He and his extended family live in Farmingdale, Maine, where he grew up surrounded by family. He graduated from Hall-Dale High School in 2010 in the top ten of his class. In the fall of 2010 Jacob began his higher education at the University of Maine where he would join Beta Theta Pi fraternity and quickly rise to president. He graduated with Honors on May 10th, 2014 with a Bachelor of Science in Microbiology. He was accepted into the Masters of Science program in Microbiology at the University of Maine for the fall of 2014.

Jacob enjoys spending time with his close friends and family. He devotes most of his free time to improving his fraternity and relationship with his fraternal brothers. After graduate school, he hopes to attend medical school in the U.S., whereupon graduation he plans to return to Maine to eventually open his own medical practice.