THESIS

THE PSEUDO PELGER-HUËT ANOMOLY AS A POTENTIAL BIOMARKER FOR CHRONIC LOW-DOSE RADIATION EXPOSURES OF SUS SCROFA LEUCOMYSTAX AND APODEMUS SPECIOSUS

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ABSTRACT

THE PSEUDO PELGER-HUËT ANOMOLY AS A POTENTIAL BIOMARKER FOR CHRONIC LOW-DOSE RADIATION EXPOSURES OF SUS SCROFA LEUCOMYSTAX AND APODEMUS SPECIOSUS

On March 11, 2011 a 9.0 earthquake struck off the east coast of Japan, resulting in a near 20-foot Tsunami that devastated the coastline. Among the damage was the Fukushima-Daiichi nuclear reactor which over pressurized, due to failed cooling systems, leading to the release of a plume of radionuclides into the surrounding environment that included Iodine-131, Cesium-134, and Cesium-137. The people of the region were immediately evacuated, many of whom have still not returned to the exclusion zone, leaving nature to take over. Many wildlife populations, including the Large Japanese Field Mouse (Apodemus speciosus) and Wild Boar (Sus scrofa leucomystax) have begun to thrive in the region largely due to the absence of human influence. The contaminated environment in which these animals live provides a unique opportunity for radiobiological research involving chronic low dose exposures, similar to those that human inhabitants of Fukushima Prefecture and radiation workers are likely to experience. Here, quantitative bio-dosimetry was employed to evaluate environmental radiation exposure in two wildlife species. Specifically, frequencies of abnormal neutrophils referred to as pseudo Pelger-Huët anomalies (PPHAs) in peripheral blood of the large Japanese field mouse and wild boar living in exclusion zone and control zones. PPHAs have been shown to be informative biomarkers of radiation exposure in several scenarios, including archived slides from the 1958 Y-12 criticality accident, the radium dial painters from the first half of the 20th century, and

chronically exposed bats in South African caves containing high levels of thorium. The PPHA morphology was indeed confirmed in the blood of exposed wild boars, however PPHAs did not occur in the large Japanese field mouse. In the future, this PPHA approach needs to be compared to other quantitative methods of estimating dose to wildlife, e.g., dicentric chromosome analysis. The potential impacts of this study include influencing the time frame in which the people of Fukushima can return to their homes, as well as reducing the cost incurred for bio-dosimetric analyses in the event of accidental or occupational radiation exposure.

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INTRODUCTION

Ionizing Radiation and Biological Effects

Radiation at its simplest differentiation is broken into two distinct categories, ionizing radiation and non-ionizing radiation. Generally, a quantum energy of 12 electron-Volts or higher is referred to as ionizing radiation (Johnson, 2017). This indicates that the energy deposition when there is an interaction with matter is capable of ionization within that matter. This ionization is the basis for biological impacts from ionizing radiation. Within the category of ionizing radiation there are charged particles, neutrons, and electromagnetic waves. Charged particle radiation is comprised of beta(β) particles, alpha(α) particles, protons, and heavy charged particles. Electromagnetic waves are comprised of x-rays and gamma(γ) rays that can penetrate easily through biological material, and sometimes pass through without interacting with matter (Johnson, 2017).

Alpha particles are a product of a form of radioactive decay that results when the nucleus ejects 2 protons and 2 neutrons. Examples of α emitters include thorium, radium, uranium, and radon gas (Health Physics Society, 2016). Beta-particles are a product of a form of radioactive decay that results when the decaying atom ejects a free electron or a positron. Examples of β emitters include tritium, sulfur-35, strontium-90, and carbon-14 (Health Physics Society, 2016). Heavy charged particles are positively charged nuclei of elements including iron and carbon that have been stripped of some or all of their orbital electrons. These are most commonly encountered in space my astronauts. X-rays and γ-rays are high energy electromagnetic waves with wavelengths less than 200 nanometers. Both x-rays and γ-rays frequently accompany α and

β particles as they are emitted from a decaying radioactive atom. Wave radiation differs from particle radiation in its effects on the human body because they have an extremely high penetration capability. Examples of wave radiation emitters include radium-226, cesium-137, cobalt-60, iodine-131, and technetium-99 (Health Physics Society, 2017). Lastly, neutrons are commonly a product of a fission reaction in the core of a nuclear reactor. Fission inside of a reactor occurs when a neutron impacts the nucleus of a uranium-235 or plutonium-239 atom and the nucleus splits into two fission products generally with an atomic number around 92 and 135 (Johnson, 2017). Examples of long lived fission fragments include cesium-134, cesium-137, and strontium-90. Cesium is in fact the primary radionuclide of concern in the Fukushima exclusion zone.

The basis for biological impacts of ionizing radiation as stated previously is the ionization of matter. On a cellular and molecular level, ionizing radiation affects the functionality of the cell by damaging the Nuclear deoxyribonucleic acid (DNA) within the nucleus or other cellular functions. Damage can be incurred by either direct action or indirect action. Direct action is responsible for approximately 30% of ionizing events damaging DNA. Damage from either particulate or electromagnetic waves can directly ionize the DNA strand and cause breakage. Indirect action, which is responsible for approximately 70% of DNA ionizing events, results in the generation of reactive oxygen species (ROS) as the tracks of radiation ionize the water molecules in the cytosol. Reactive oxygen species include hydrogen radicals (H•), hydroxyl radicals (OH•), and free electrons (e-) that can diffuse through the cytosol and cause ionizing events that will damage the DNA strand (Hall & Amato, 2012).

Tissue damage is directly proportional to the dose that is absorbed by the tissue at low doses, and directly proportional to the square of the dose at high doses. This is known as a linear-quadratic relationship. Low doses generally result in sub-lethal damage that can be repaired with endogenous DNA repair mechanisms. Higher doses however, can result in double strand breaks of DNA from clustered ionizing events that can result in mitotic cell death or apoptotic cell death. If this cell death is widespread due to a large dose or a concentrated field of irradiation it can result in deterministic effects like acute radiation syndrome and/or stochastic effects such as cancers.

Acute radiation syndrome has been witnessed in a several accidents in recent history. For example, in Goiania, Brazil in 1985 a radio teletherapy institute in the city relocated and took their cobalt-60 source with them while leaving behind a newly orphaned cesium-137 source. The source was roughly 1,375 Curies in activity and administered a dose rate of 4.6 Gy h^{-1} at 1 meter (Ramalho, Nascimento, & Bellido, 1990). The facility was partially destroyed and two individuals entered the premises finding the radiography source. In an attempt to scrap the source capsule, the two individuals broke it open in their homes and were able to remove its contents. The source which was in the form of a cesium chloride salt that gave off a faint blue glow in the dark. Ultimately, within a few days several people began feeling gastrointestinal symptoms and the wife of the junkyard owner took the source capsule to the public health office. As a result parts of the city were evacuated and residents were sequestered in the local stadium for monitoring. The human impact of this accident was the hospitalization of 20 people that all required chelation with Prussian blue. Of those 20 people, 6 of them received doses between 4.5 Gy to over 6 Gy, and 4 of those individuals succumb to their injuries (IAEA, 1988).

Another, far better known, accident occurred in 1986 outside of current day Pripyat, Ukraine (at that time the Soviet Union). The Chernobyl nuclear power plant was operating experimental procedures that bypassed several safety precautions, and ultimately lead to a reactor meltdown and a series of explosions that jettisoned the lid of the reactor. The control rods in the reactor were made of graphite and began to burn at the high temperatures of the core that exceed 30 Gigawatts of thermal energy. As a result nuclear fuel and fission fragments were disseminated throughout the surrounding area in present day Ukraine and Belarus. The human impact of the reactor accident was 134 cases of confirmed acute radiation syndrome, and the short term deaths of 28 individuals that experienced full body doses of 6.5 Gy or higher (Mettler, Gus'Kova, & Gusev, 2007). Many of these individuals were firefighters that were attempting to extinguish fire at the facility. As a result of these two accidents many lessons were learned and put into practice the world over. Twenty-five years later another reactor accident occurred at the Fukushima Daiichi nuclear power plant following the Eastern Japan Great Earthquake on March 11, 2011. Fortunately, there were no cases of acute radiation syndrome at this event; however, an exclusion zone exists around the reactor site to this day.

Eastern Japan Great Earthquake & Fukushima Daiichi Nuclear Power Plant Accident

On March 11, 2011 the Tohoku region of Japan's Honshu Island was struck with a 9.0 magnitude earthquake roughly 77 miles off the eastern coast. There was much debate by the Japan Meteorological Agency in the first several days following the quake, but the final determination of 9.0 on the Richter scale was decided on March 14th, 2011. Following the quake there were several aftershocks recorded and eventually numbered in the thousands by April 7, 2011 (Chai 2011: CBS News 2011). The main earthquake resulted in a Tsunami that devastated

the Pacific coast line of Japan and triggered tsunami warnings in more than 20 countries all around the Pacific rim. The worst of the damage was sustained on the east coast of Tohoku where the wave was estimated in some places to be as high as 24 meters. As the water receded the unfathomable damaged began to be tallied up. By April 13th, 2011 the death toll in Japan had risen to over 13,000, with over 15,000 people missing. Once the damaged infrastructure was fully analyzed the economic impact of the disaster was estimated to be as high as 183 billion U.S. dollars (Norio, Ye, Kajitani, Shi, & Tatano, 2011).

In the aftermath of the Tsunami along the coast line of Fukushima prefecture the Fukushima Daiichi nuclear power plant was left without power or means to cool the reactor core. Fukushima Daiichi was primarily powered by an offsite source and the power generated by the reactor itself. If these power sources failed, like in the case of the earthquake, there were back-up generators and back up batteries that were designed to take over and allow the reactor to have a continuous flow of coolant to removed residual heat. Unfortunately, overheating of the reactors led to a release of radionuclides to the environment.

Initially, a 20 km exclusion zone was established, which was further expanded to 30km. Roughly 100,000 people were removed from their homes and placed in temporary living facilities. The estimated death toll due to the evacuation was set at a minimum of 50 individuals due primarily to limited health care for hospital evacuees and the elderly. As mentioned previously, there are to this day 0 reported cases of ARS from the meltdown. An exclusion zone still exists around the reactor, although it is much smaller than the original 30km radius. Today's exclusion zone contains the towns of Futaba, Okuma, and the southern half of Namie. As of

April of 2017, the northern half that contains much of the urban area of Namie was open to inhabitance once again.

Radionuclides Prominent in the Environment Following the Nuclear Accident

In the event of a nuclear meltdown releases are generally in accordance to with the volatility: fission gases and volatile fission fragments, semi-volatile fission fragments, low-volatile fission fragments, and non-volatile fission fragments (Bentaib & Jacquemain, 2015). The releases of most concern for the Fukushima accident are iodine-131, cesium-134, and cesium-137. Iodine-131 has a half-life of 8.04 days, and thus is only a short-term hazard. Cesium-134 has a half-life of 2.06 years, undergoes beta minus decay to stable barium 134, and it is a soft tissue seeker as a potassium analog. Lastly, cesium-137 has a half-life of 30.08 years, undergoes beta minus decay to barium-137m, and is a soft tissue seeker in the same manner as cesium-134. Barium-137m which has a half –life of 2.5 minutes and undergoes a gamma decay releasing a photon of 662keV, the characteristic energy of cesium-137. Since its half-life is significantly longer than barium-137m the two exist in secular equilibrium with one another (Johnson & Birky, 2012).

Cesium is a potassium analog and so can bio-accumulate in soft tissues. If the individual does not know how much they ingested or what they have been exposed to methods of bio-dosimetry can be employed. Dicentric chromosomal aberration assay (DCAA) is the current accepted standard for determining dose to a living individual. This assay entails drawing peripheral blood, stimulating the division of lymphocytes using mitogens, arresting them in metaphase with cell cycle blocks, and counting the number of dicentric chromosomes. The

DCAA procedure is costly and can take up four days or more to have definitive results. Since the hematopoietic system is the first system affected by acute radiation syndrome the PPHA assay was investigated as an alternative method of bio-dosimetry.

The Hematopoietic System

Hematopoietic describes a process in the human body that contributes to the formation of blood. The process of hematopoiesis, the generation of blood, begins in the bone marrow with the hematopoietic stem cell (HSC) and the stromal support cells. The can differentiate into erythrocytes (red blood cells), leukocytes (white blood cells), and platelets. The differentiation of the HSC into one of these components is dependent upon which growth factors it receives. For example, if the body requires more red blood cells the kidney will release a molecule known as erythropoietin and when it binds to an HSC it will develop into a proerythrocyte and at that point it is destined to become an erythrocyte.

Following centrifugation of the blood it separates into three distinct layers of tissue. The bottom layer is known as the hematocrit and comprises roughly 45% of blood volume. This layer is comprised of red blood cells that are small bi-concave discs containing no nucleus, numerous iron rich molecules known as hemoglobin, but no protein or lipid synthesis machinery. The purpose of a red blood cells is to carry oxygen molecules to the tissues to be use in the production of adenosine triphosphate (ATP) in mitochondria. the life span of these cells is normally 90-120 days. These are the most abundant cell type in peripheral blood and can be seen in any image of peripheral blood smears in this document. The upper layer of centrifuged blood is known as the blood plasma and comprises roughly 55% of the blood volume and is the liquid

solution that both white and red blood cells are suspended in. The plasma is comprised of water with dissolved salts called electrolytes and proteins. The middle layer of the centrifuged blood is known as the buffy coat and comprises less than 1% of the total blood volume (Walrond, 2003). This layer contains all of the white blood cells and platelets. The plasma and buffy coat contain the components of the immune system. While white blood cells are a term that encompasses many different types of cells, platelets are the product of a cell known as a megakaryocyte that fractures into hundreds to thousands membrane bound bags containing granules and cytoplasm (McKenzie, 1996).

The HSC is a pluripotent stem cell and it further differentiates into two stem cell progeny that ultimately further differentiate into all cellular components of the blood. These cells are the myeloid stem cells and the lymphoid stem cells. First, the lymphoid stem cells undergo a process called leukopoiesis to differentiate into B-lymphocytes and T-lymphocytes. These cells are what are responsible for a person's adaptive immunity. A lymphocyte is one of many cells that can be seen in a peripheral blood smear. When stained with geimsa stain the lymphocytes appear to have a large round nucleus that takes up most of the cell's cytoplasm. A lymphocyte from a peripheral blood smear in this study can be seen in figure 1.

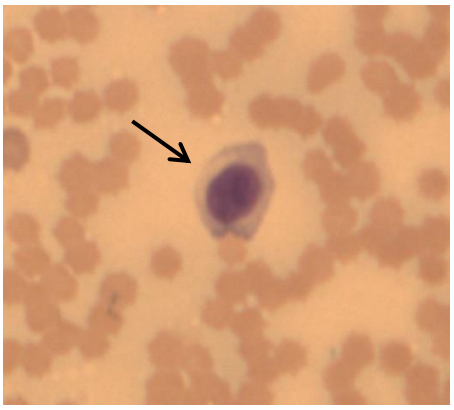

Figure 1: Lymphocyte from boar slide Ba20180109

The myeloid stem cell undergoes one of three processes, erythropoiesis to generate red blood cells, thrombopoiesis to generate platelets, or leukopoiesis to generate cells of innate immunity. The myeloid stem cell gives rise to several cells of importance that must be differentiated in the performance of a peripheral blood smear evaluation. The first of which is called an eosinophil; it is a polymorphonucleated granulocyte that's function is to neutralize parasitic organisms within the body. They can be distinguished from other cells in a peripheral blood smear by the many red granules present in the cell's cytoplasm and a multi-lobed nucleus. An eosinophil can be seen in figure 2.

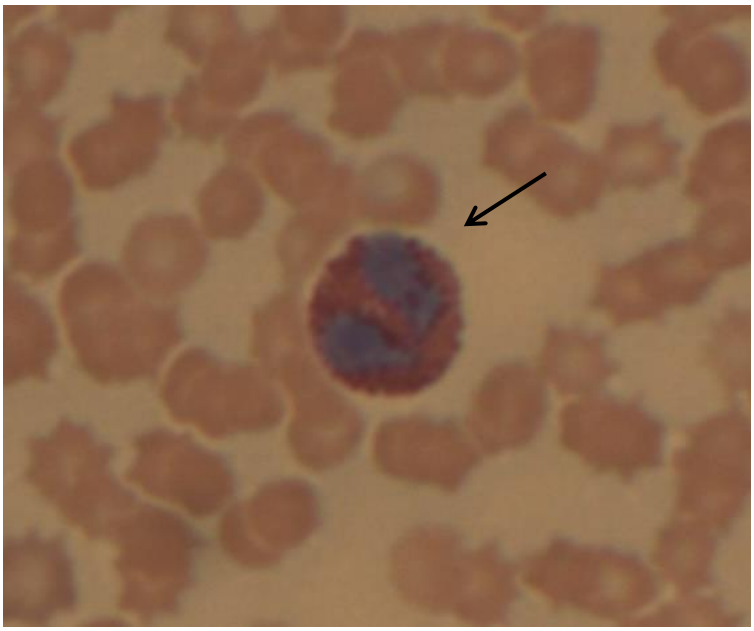

Figure 2: Eosinophil from boar slide Ba20180109

The next type of cell that the myeloid stem cell can differentiate into is a monocyte. Monocytes have a large nucleus that when stained appears bean-like in shape. Monocytes are the largest of the cells in a peripheral blood smear, but it can be difficult to differentiate from lymphocytes when the nucleus does not have the characteristic shape. Monocytes will leave the blood vascular compartment through a process called diapedesis and within the tissues they

mature into a macrophage. Macrophages are essential to an inflammatory response. A monocyte can be seen on a peripheral blood smear in figure 3.

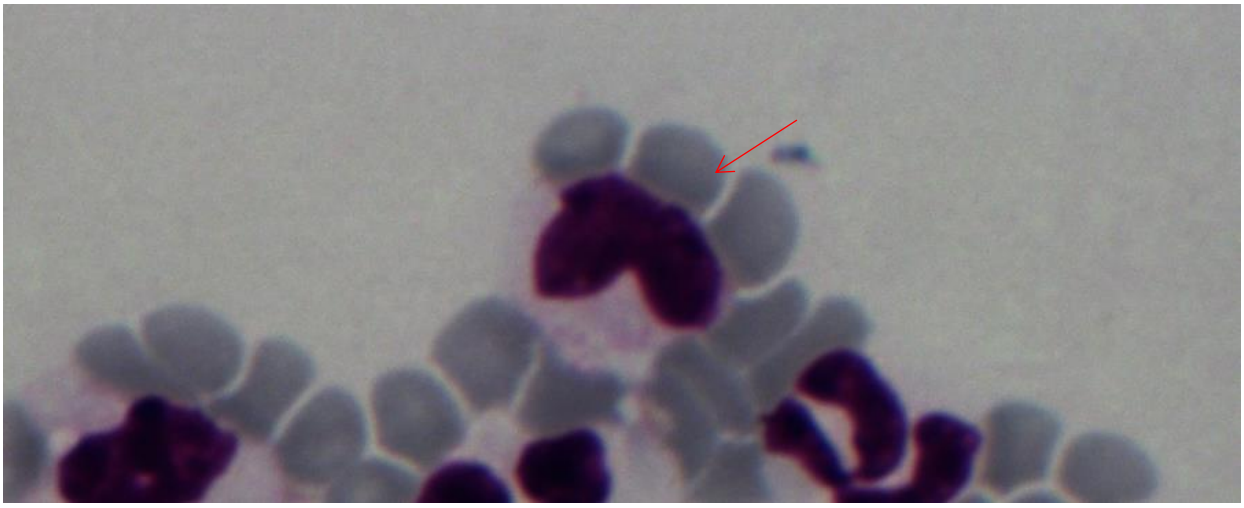

Figure 3. Monocyte from boar slide Ba20170724

The next cell type that the myeloid stem cell can differentiate into is the basophil. A basophil, along with a histaminergic cell called a mast cell, help initiate the inflammatory response. The basophils specifically will secret molecules that are chemotactic towards eosinophils in particular. Basophils create a chemical gradient that draws the eosinophils to the site of inflammation. The basophils also secrete molecules that are broncho-constrictive. It is thought that the basophils are partly responsible for initiating and maintaining an allergic response (McKenzie, 1996). The basophil is the rarest cell type in a peripheral blood smear and can be distinguished by its lobular nucleus and a multitude of darkly colored secretory granules. A basophil can be seen in a peripheral blood smear in figure 3.

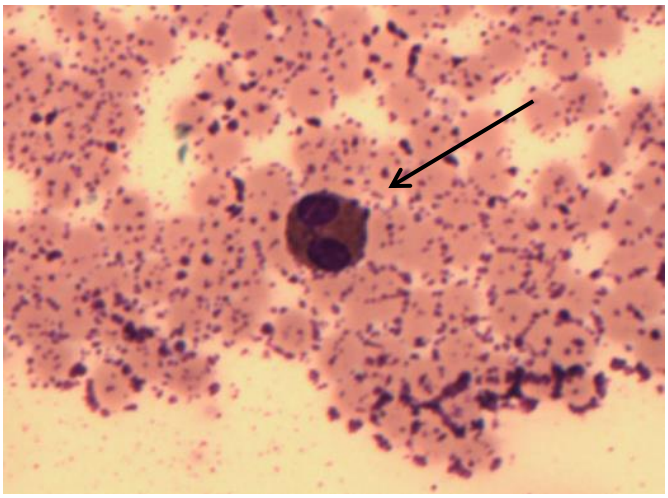

Figure 4. Basophil from boar slide Ba20170724.

The last cell type that the myeloid stem cell can differentiate into is the neutrophil. The neutrophil is a granulocyte and is the most abundant nucleated cell in the peripheral blood. It migrates from the bone marrow and circulates in the blood where it eventually leaves the blood vascular compartment and enters damaged tissue. Neutrophils will phagocytose foreign bodies and secrete oxidative materials such as hydrogen peroxide and free electrons. Neutrophils are polymorphonucleated and generally have between 4 and 5 lobes to their nucleus. Neutrophils with less than 3 lobes are considered hypo-segmented and those with more than 5 are considered hyper-segmented. Both hypo- and hyper- segmented nuclei are abnormalities. The neutrophil can be distinguished from the other granulocytes by its lack of darkly colored or reddish granules in the cytoplasm. An immature form of neutrophil can be normally viewed in the peripheral blood called a band cell. The band cell has the beginning of the indentation that will lead to segmentation and has a horseshoe like appearance. A normal neutrophil can be seen in figure 5.

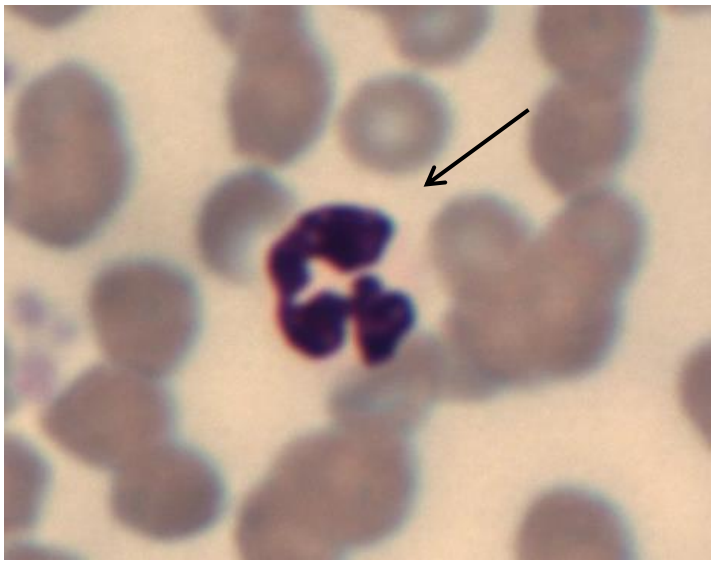

Figure 5. Neutrophil from boar slide

The Pseudo Pelger-Huët Anomaly

The Pelger-Huët Anomaly (PHA) was first described in 1928 by the German physician, Karl Pelger, as a biomarker for a poor prognosis in patients that had contracted tuberculosis (Pelger, 1928). However, in 1932 G.J Huët disagreed when he discovered the anomaly in a young girl that recovered from her tuberculosis. Under further investigation he was able to determine that the anomaly was linked to an autosomal dominant mutation on the long arm of chromosome one (Huët, 1932). The mutation existed in a gene that encodes for the lamin B receptor, which is a membrane associated protein that is embedded in the inner nuclear membrane of the nuclear envelope. The lamin B receptor has two functions that can be described by their location on the protein. The carboxyl terminus contains 8 transmembrane sections that portray C14 sterol reductase activity which involves the breaking of carbon double bonds. Many believe that this aspect of the protein is integral to the production of cholesterol in human cells (Nikolakaki, Mylonis, & Giannakouros, 2017). The amine terminus of the lamin B receptor binds to an intermediate filament called lamin B. Lamin B in turn binds to the

chromatin and provides structure to both them and the nucleoplasm (Holmer, Pezham, & Worman, 1998). The structure of the nucleus is highly important to granulocytes, and in this case neutrophils, because a hyper segmented nucleus makes it much easier for these cells to exit the blood vascular compartment via diapedesis. It also allows for ease in diapedesis throughout tissues as the cell is migrating to damaged areas undergoing the inflammation response (Colella, 2012). An image of the lamin B receptor with the sterol reductase carboxyl terminus and the chromatin tethering amine terminus can be seen in figure 5.

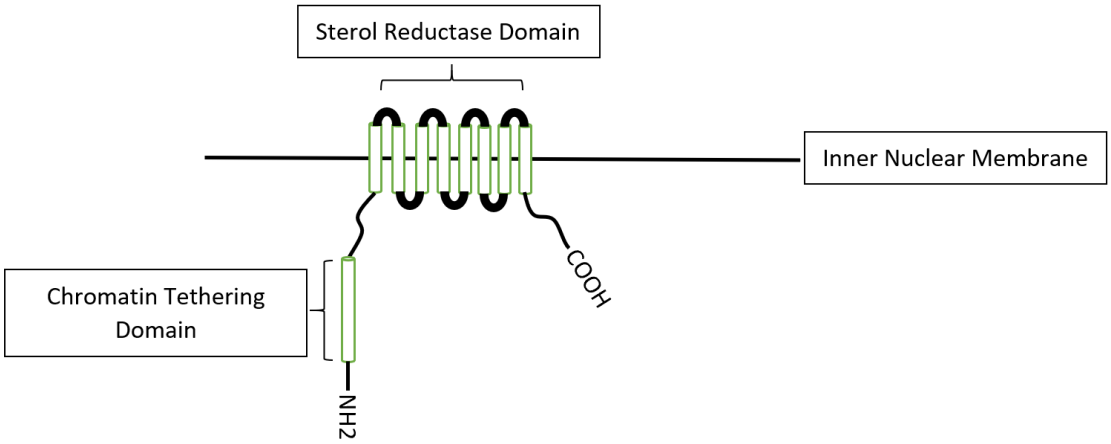

Figure 6. Structure of the lamin B receptor..

While the Pelger-Huët anomaly can be seen in any granulocyte the anomaly is most commonly observed in neutrophils, which is the most abundant leukocyte in peripheral blood. In the event that the cell morphology is induced from an outside stimulus like a toxin or radiation the induced cells become known as pseudo Pelger-Huët anomalies. These cells are distinguishable from other neutrophils in a peripheral blood smear by two factors. The nucleus is bilobular with a round to bean like shape and the two segments are connected by a very thin mitotic bridge (Goans, Iddins, Ossetrova, Ney, & Daniak, 2015). Whether or not this morphology is still caused by a decrease in the lamin B receptor is open for debate. A pseudo

Pelger-Huët anomaly with the thin mitotic bridge and the most common morphology for a neutrophil can be seen in figure 7.

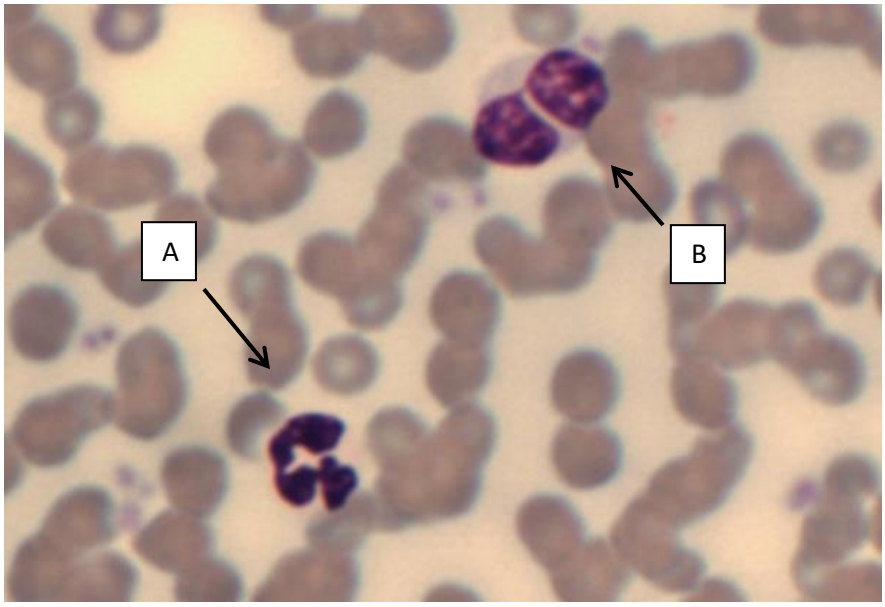

Figure 7. Example of a normal neutrophil (A) next to a Pseudo Pelger-Huët anomaly (B) from boar slide Bd20170717

In addition to toxins PPHAs have been shown as a biomarker for radiation exposure in animals as well as humans. A study conducted in South Africa utilized a population of bats (Chiroptera) that were a resident population in an abandoned mine. The background radiation within the mine was orders of magnitude above normal background because the ore inside contained high concentrations of a mineral known as monazite. Monazite contains an abundance of thorium-232 and its decay daughters. The bats were divided into 3 populations: controls that were exposed to normal terrestrial background radiation, a low dose area of the monazite mine with dose rates of 20 micro Sieverts per hour (μSv/hr), and a high dose area of the mine with dose rates of 100 μSv/hr. Peripheral blood smears were taken from the animals and the neutrophil counts were taken. The percentage of neutrophils that displayed the PPHA morphology was 0.27% in the controls, 12.67% in the low dose areas, and 22.66% in the high

dose areas (Meehan, 2001). This introduced a dose response that could potentially yield an efficient means of estimating dose from this biomarker.

Further research into the viability of the PPHA as a bio-dosimeter have been conducted at the Radiation Emergency Assistance Center/Training Site (REAC/TS). A retrospective study was conducted using victims of the 1958 Y-12 criticality accident. The accident occurred when a group of radiation workers were pouring uranyl nitrate solution that was used in the extraction of enriched uranium from solid waste, into a 55 gallon drum that they believed to be empty. The barrel unfortunately contained water, and the uranyl nitrate containing approximately 2.1kg of uranium 235 went critical. The worker closest to the barrel experienced a blue flash shortly after beginning to fill the barre l (Mclaughlin, Monahan, Pruvost, Frolov, Ryazanov, & Sviridox, 2000). A criticality alarm sounded and the building was evacuated. 8 workers received significant doses ranging from 0.288 and 4.61 (28.8 to 461 rem). Archived slides that were taken from the victims at the time of the accident and several years following were obtained by REAC/TS and were evaluated in the same manner as the bats from the monazite mines in South Africa. The exposures that occurred from the Y-12 criticality accident were separated into 2 cohorts, high dose cohort with n=5 and a low dose cohort with n=3. The archived peripheral blood smears from the victims were evaluated by counting the percentage of neutrophils that presented with the PPHA morphology. The high dose cohort on average presented 13% PPHAs, the low dose cohort presented 6.8% PPHAs, and a control group presented a 3.6% PPHA background count (Goans, Iddins, Ossetrova, Ney, & Daniak, 2015).

The same research group conducted another retrospective study looking at radium watch dial painters from the first half of the 20th century. Radium-226 and radium-228 were commonly used materials for creating illuminated dials for watch faces and airplane instrumentation during this time period. Radium is a long-lived radionuclide that is a biological analog for calcium, which results in heavy deposition of the nuclide in bone. Prior to the abolishment of this practice in 1955 the women that painted the dials would maintain a fine tip on paint brushes by wetting the brush with their mouths and they would in turn introduce the radium to their body. Archived blood slides from 166 dial painters with bone marrow doses ranging from 1.5-6,750 mGy were evaluated by REAC/TS (Goans, Toohey, Iddins, Mccomish, Tolmachev, & Dainiak, 2018). The resulting data displayed a sigmoidal relationship between bone marrow dose and PPHAs as a percentage of neutrophil population. These studies have worked to lay the groundwork for future studies inquiring to the validity of the PPHA as a cheap and effective means of estimating radiation dose. However, prior to implementing this approach in an operational standing the method must be compared to the dicentric chromosome assay which is the current gold standard in bio-dosimetry. In this study Wild Boar (Sus scrofa leucomystax) and large Japanese field mice (Apodemus speciosus) are sentinel species of opportunity with in the exclusion zone of the Fukushima Daiichi nuclear disaster area.

Wild Boar (Sus scrofa leucomystax) Information

Following the evacuation and the establishment of the exclusion zone around the Fukushima Daiichi nuclear power plant, the wild boar populations has grown drastically (Tanoi, 2016). The animals are remarkably destructive to both to the ecosystem and the infrastructure of urban areas that were left behind in the evacuation. The Japanese prefectural government has

implemented a hunting program to aid in the control of the wild boar population in the exclusion zone. Swine are a large terrestrial mammal and have a relative biological equivalence in both anatomy and physiology to humans (Kobayashi et al. 2012). Biological equivalence allows for the boar to be used in translational studies that could potentially have human implications in the future.

The wild boar in the Fukushima prefecture are a moderately sized coastal sub species of wild boar (Sus scrofa leucomystax). The animals travel in family groups generally led by an older sow, or in smaller bands of male boars. Boar litters generally range in size from 3-8.4 piglets, and sows can produce more than one litter per year (Mayer, 2009). These large litter sizes are theorized to be a contributor to the population's boom that occurred following the evacuation of the exclusion zone. Boar are omnivores that will dig with their snout and enlarged canine teeth in search of roots and rodents to eat, which can result in an intake of Cs-134 and Cs-137, both potassium analogs. The Cesium bio-accumulates in the soft tissue of the animal. Wild boar cesium concentration is assumed to be at equilibrium with the environment from the time of birth. The oldest boar that was captured during this study was born at least 2 years after the accident occurred.

Studies conducted in 2012 collected a spectrum of organs from 24 wild boars within the exclusion zone and counted each organ for radio cesium concentration. It was determined that the highest radio cesium concentration was contained in the muscle of the animals (Tanoi et al, 2016). In a separate study conducted at the Savannah River Site (SRS), in South Carolina the body composition of wild boars was characterized. It was found that on average the muscle

mass contributed to 45.1% of the total body mass of the animal, 39.01% in females and 51.52% in males (Mayer, 2009). The large percentage of total body mass that the muscle contributes and the high cesium deposition led to the practice of removing a portion of muscle from the hind leg of the animal and counting the sample in a sodium iodide detector to estimate the body burden. The resulting data can be used to extrapolate the lifetime dose due to internal deposition of the cesium in the animals.

The hematopoietic system in the swine is not unlike that of the humans, further contributing to the translatable nature of the animal as a model. It was observed in the samples taken in this study that the white blood cells are comprised of mostly lymphocytes and neutrophils, with a small percentage of monocytes, eosinophils, and basophils. Prior to this study the PPHA morphology has not been studied in wild boar.

Large Japanese Field Mouse (Apodemus speciosus) Information

The large Japanese field mouse is an endemic species to the island of Honshu in which Fukushima prefecture sits. The field mice are easily captured and have a much smaller home range than the wild boar. The large Japanese field mouse is not to be confused with the small Japanese field mouse (*Apodemus argenteus*) which has very similar body morphology apart from the size difference that is annotated in their name. Both mice species are present in the entire Japanese archipelago, but they have a differing preference in terrain. The small Japanese field mouse prefers a dense canopy while the large Japanese field mouse has a preference for open forests (Shioya, Shiraishi, & Uchida, 1990). Both animals are characterized as semi-subterranean

foragers that often consume seeds. The animals have an almost isotropic exposure to radio cesium depending on the depth of the burrow and the sedimentation rate of the cesium in the soil.

Mice have been used recently to describe chromosomal aberrations in the higher dose areas of the contaminated exclusion zone. Both the small and the large Japanese field mouse displayed dicentric chromosomes that increase in frequency with exposure to elevated dose rates (Kawagoshi et al, 2017). This demonstrates that if the PPHA morphology is present in the animals the method can be compared to DCAA. As a mammal the hematopoietic system in the mice is not unlike that of the humans, further contributing to the translatable nature of the animal. It was observed in the samples taken in this study that the white blood cells are comprised of mostly lymphocytes and neutrophils, with a small percentage of monocytes, eosinophils, and basophils. Prior to this study the PPHA morphology has not been studied in the large Japanese field mouse.

Goals for This Study

The purpose of this study is to evaluate the pseudo Pelger-Huët anomaly as a potential bio-dosimeter for the large Japanese field mouse and wild boar living in the contaminated exclusion zone around the Fukushima Daiichi nuclear power station.

The objectives of this study are as follows:

1. Determine the presence or absence of the pseudo Pelger-Huët anomaly in contaminated Japanese wild boar.
2. Determine the presence or absence of the pseudo Pelger-Huët anomaly in contaminated large Japanese field mice.

3. Compare contaminated populations of each animal with a control population and fit a dose response curve if the morphology is observed.

Contaminated boars were collected from the town of Namie, Fukushima, Japan with the aid of prefectural hunters. Control boars were collected from the SRS near Aiken, South Carolina with the help of the Dr. Jim Beasley at the Savannah River Ecology Laboratory (SREL). Contaminated mice were collected from various locations in Okuma, Fukushima, Japan and Tekasa Gorge in Namie, Fukushima, Japan. Control mice were collected from Soma, Fukushima, Japan and the region of Aizu in west Fukushima.

All animals were captured in cooperation with a team of ophthalmology researchers. Therefore, as part of the animal capture and sample collection procedures ocular tissue was a priority due to the sensitivity of the tissue and the time required to perform ophthalmic exams. After ophthalmic procedures were performed using a slit lamp on the anesthetized animals, blood was drawn from an aortic puncture prior to euthanasia and bilateral enucleation of the eyes. The hypothesis for this study is that the pseudo Pelger-Huët anomaly, which has been shown to be radiation induced in humans in retrospective studies and bats in environmental studies, will be present in peripheral blood smears of radionuclide contaminated Japanese wild boar and large Japanese field mice and a dose response curve can be constructed. Upon confirmation that the PPHA is present in these species, future studies will need to be done to validate the technique against dicentric chromosome assays.

METHODS AND MATERIALS

IACUC Approval

This study was approved by the IACUC of Colorado State University on May 17, 2017 (See Appendix A).

Wild Boar Capture and Field Necropsy

In Japan the capture of the contaminated wild boar in the Fukushima exclusion zone was a collaborative effort between a team of scientists from the Institute of Environmental Radioactivity (IER) and a team of prefectural hunters. The prefectural hunters would travel to each boar trap on a near daily basis to check for an animal and reset the trap if need be. The traps were baited using a powdered soy product and sake that was boiled down into a paste. The traps were triggered to close by a physical interaction of the animal with a trip wire strung through the food. If there was an animal in the trap the team from the IER would block the animal's view in an effort to minimize visual stimulations and subsequent sympathetic overdrive. All contaminated animals were captured as a single animal except for one sow with three piglets. The animals were sedated using a pole dart containing Telazol or Zoletil at a concentration of 5 milligrams per kilogram of body mass. Following the confirmed anesthetization of the animal it was removed from the cage and an inspection of the animal's physical characteristics was conducted and the following data was recorded:

1. Body measurements(cm)
    a. Body length (snout to vent)
    b. Hock length

c. Ear length

d. Auxiliary girth

e. Tail length

2. Body mass (Kg)
3. Ophthalmology exam
    a. Lens inspection using a slit lamp
    b. Pressure testing
    c. Tear production
4. Blood samples drawn from the Aorta via the thoracic inlet.
    a. Blood smears done as soon as possible after blood draw and fixed with methanol(10µl)(see appendix D)
    b. Sodium heparin tube for blood chemistry (5ml)
    c. EDTA blood tube for blood chemistry (5ml)

It was necessary to keep the animal alive until the conclusion of the ophthalmic examination because cataracts begin to form rapidly post mortem. Once the procedure was completed and all data was recorded appropriately the animal was euthanized by exsanguination. The following samples were taken from the boar immediately following euthanasia for their corresponding purposes:

1. Biceps femoris muscle for cesium body burden determination using a sodium iodide detector.
2. Mandible for aging the animal.
3. Single tooth extraction for electron spin resonance used to estimate lifetime dose.

4. Thyroid for iodine-129 analysis

At the culmination of the procedures the boar was placed in a large bag and transported by the prefectural hunters to the designated burial site.

The capturing of control boars at the Savannah River Site (SRS) were conducted in cooperation with the Savannah River Ecology Lab (SREL) which is a satellite campus of the University of Georgia Athens (UGA). All procedures remained unchanged from the boars collected in Fukushima with the exception of the traps used, the sedation method, and disposal method. The traps used in Fukushima were designed for a single boar while the traps used in SRS were designed to capture a family group in one event. The cages were baited daily using corn and had a remote trigger that was accessed and controlled by the hunter's cellular phone. Once the animals were captured they were sedated one by one with the use of a dart gun containing Telazol or Zoletil at a concentration of 5 milligrams per kilogram of body mass. At the culmination of the field necropsy the animals were taken by the wildlife biologist from SREL and placed in the view of a trail cam and used to study scavenger behavior.

Wild Large Japanese Field Mouse Capture and Field Necropsy

All mice were captured exclusively by the team of scientists from the IER in various locations within Fukushima prefecture. Animals were live-captured using a Sherman trap that was baited with a variety of different nuts and seeds. It was found that peanuts were the most effective form of bait. The traps were set up in trap lines of 10-15 traps per line and 4-5 lines per location. The traps were secured to the ground using rubber bands and chopsticks, and a brightly colored piece of tape was tied to a tree above its location to minimize traps being lost in the

underbrush. A Sherman trap that is secured to the ground with chopsticks and a rubber band can be seen in figure 8.

Figure 8. Sherman trap secured to the ground using chopsticks and a rubber band.

Traps were checked daily and reset if needed, and in the event that there was an animal in the trap they were coaxed from the trap into a mesh bag. Undesired species such as the small Japanese field mouse and voles were released. In the first several weeks of capturing the mice were collared using an improvised dosimeter collar made out of an optically stimulated luminescence (OSL) dosimeter chip that were waterproofed with parafilm and secured to a cable tie with electrical tape. Animals were released on the spot and traps were reset in an attempt to recapture the collared mice. If the animals were recaptured the collar was recovered from the animal and the OSL chip was read in an attempt to estimate a dose rate the animal was receiving close to and under the ground. An improvised collar for a mouse can be seen in figure 9.

Figure 9. Large Japanese field mouse with improvised dosimeter collar.

Following recapture in the first couple of week and the initial capture there after the mouse was secured by grasping the scruff of the animal's neck and Telazol or Zoletil at a concentration of 20 milligrams per kilogram of body mass was administered via an intraperitoneal injection. Following the confirmed anesthetization of the animal it was removed from the cage and an inspection of the animal's physical characteristics was conducted and the following data was recorded:

1. Body mass(g)
2. Ophthalmology exam
    a. Lens inspection using a slit lamp
    b. Pressure testing
    c. Tear production
3. Blood samples drawn directly from cardiac stick.
    a. Blood smears done as soon as possible after blood draw and fixed with methanol(10µL)(see appendix D)

Just as in the case of the boars, it was necessary to keep the animal alive until the conclusion of the ophthalmic examination because cataracts begin to form rapidly post mortem. Once the portion of the procedure is completed that requires the animal to be alive and all data is recorded appropriately the animal was euthanized via cervical dislocation. Immediately following confirmation of expiration from the veterinarian a bilateral enucleation of the eyes was conducted, and lenses were dissected out. The lenses were photographed under a slit lamp in a clandestine dark room built using cardboard, tarps, and the cargo bay of a van. While the eyes were being photographed the following samples were taken from the mouse for their corresponding purposes:

1. Stomach for contents study conducted by mouse biologist at IER.
2. Testes for sperm function conducted by same individual.

At the culmination of the procedures the mouse was placed in a zip lock bag and taken to the lab. The entire body was used to conduct cesium burden analysis using a sodium iodide detector. Unlike a boar it was possible to fit the entire animal inside the well of the detector.

Pseudo Pelger-Huët Anomaly Quantification with Light Microscope

All blood smears were stained using Geimsa stain the day of the collection when it was possible (see appendix E). Geimsa stain was not available at SREL so slides were fixed using methanol and transported back to Colorado State University where they were stained promptly upon delivery. The delay in staining does not alter the effectiveness of the protocol. Slides from Fukushima were required to be coverslipped to be in compliance with the USDA permit to import samples, and letters were drafted in both English and Japanese to declare to customs the contents of the shipment (See appendix A &B). The slides were analyzed in cooperation with Dr.

Ronald Goans at the Radiation Emergency Assistance Center/Training Site (REAC/TS) in Oak Ridge, Tennessee. Slides were manually inspected under a light microscope at 40x magnification. Neutrophils were counted systematically as to ensure cells were not counted more than once. The number of PPHAs observed were compared to the total number of neutrophils counted to determine a percentage that showed the morphology. Boars had at least 2 blood smears, and a desired count of 1000 minimum cells were quantified to minimize the standard error of the mean (SEM). Upon generation of the percentage of PPHAs occurred in a population of neutrophils it was plotted against the estimated lifetime dose to the animals from electron spin resonance data (Harshman et al, 2018). The mice had a minimum of 2 slides per animal, but the desired cell count was set at 200 per animal unless a PPHA was found and then all slides that were already counted would be recounted until the desired 1000 minimum cells was achieved. This decision was made to minimize time wasted following a discussion with researchers at the Armed Forces Radiobiology Research Institute (AFRRI). AFRRI had reportedly irradiated laboratory mice and did not observe the PPHA morphology. The large Japanese field mouse is a different subspecies so the analysis was done to confirm the presence or absence of the morphology.

RESULTS

Pseudo Pelger-Huët Anomaly Dose Response in the Wild Boar

In Table 1, the percentage of neutrophils that displayed the PPHA morphology can be seen for each individual animal. During evaluation of the blood smears, the lifetime dose estimation was not known or accessed by the evaluator in an effort to remain unbiased. Upon the completion of evaluations the lifetime dose estimations were acquired from the third party that conducted the estimations (Harshman et al, 2018). Any cells that displayed a morphology that were suspect PPHAs were imaged and circulated amongst the evaluators and in all cases a consensus was reached. Four contaminated boars were removed from the study because the blood smears were not of a high enough quality to permit evaluation. The cause of the inadequate blood smears was due to ruptured white blood cells causing an inability to differentiate between cell types. Eleven boars had only one viable blood slide leading to several boars falling short of the desired cell count of 1000 cells. These boars that fell short of 1000 cells can be referenced in Table 1. The cell counts acquired, corresponding percentages, and estimated lifetime doses can be seen in Table 1.

Table 1: Percentage of Wild Boar Neutrophils Displaying PPHA Morphology and Lifetime Dose Estimates

Boar ID	PPHAs	Normal Neutrophils	Total cells counted	Lifetime Dose(Gy)	%PPHA	SEM
Ba20170605	3	1507	1510	0.2630	0.1987	±0.0115
Ba20170609	13	526	539	2.4030	2.4119	±0.0669
Bb20170609	5	545	550	0.1460	0.9091	±0.0407
Ba20170615	4	537	541	0.4377	0.7394	±0.0370
Ba20170616	10	1038	1048	0.5430	0.9542	±0.0302
Bb20170616	4	1034	1038	0.8764	0.3854	±0.0193
Ba20170617	4	609	613	0.2171	0.6525	±0.0326
Ba20170623	8	532	540	0.4362	1.4815	±0.0524

Ba20170627	4	511	515	0.5620	0.7767	±0.0388
Ba20170704	2	1037	1039	0.3540	0.1925	±0.0136
Ba20170717	2	516	518	0.0004	0.3861	±0.0273
Bb20170717	2	661	663	0.4380	0.3017	±0.0213
Bc20170717	5	1052	1057	0.4440	0.4730	±0.0212
Ba20179720	12	2122	2134	0.2164	0.5623	±0.0162
Ba20179724	9	1543	1552	0.2430	0.5799	±0.0193
Bb20170724	2	978	980	0.0024	0.2041	±0.0144
Bc20170724	3	1507	1510	0.0024	0.1987	±0.0115
Ba20180108	3	1015	1018	0.0016	0.2947	±0.0170
Ba20180109	3	1029	1032	0.0015	0.2907	±0.0168
Bb20180109	1	1041	1042	0.0005	0.0960	±0.0096
Bc20180109	3	1507	1510	0.0004	0.1987	±0.0115
Ba20180110	1	1004	1005	0.0010	0.0995	±0.0100
Bb20180110	0	1004	1004	0.0005	0.0000	±0.0000
Bc20180110	2	603	605	0.0008	0.3306	±0.0234
Bd20180110	0	1017	1017	0.0011	0.0000	±0.0000
Be20180110	0	1009	1009	0.0010	0.0000	±0.0000
Bf20180110	2	1000	1002	0.0012	0.1996	±0.0141
Bg20180110	0	1004	1004	0.0014	0.0000	±0.0000
Bw20180110	2	1003	1005	0.0008	0.1990	±0.0141
Bx20180110	0	1016	1016	0.0008	0.0000	±0.0000
Bz20190110	0	211	211	0.0008	0.0000	±0.0000
Ba20180111	1	1015	1016	0.0015	0.0984	±0.0098
Bb20180111	1	512	513	0.0015	0.1949	±0.0195
Bc20180111	1	1010	1011	0.0008	0.0989	±0.0099
Bd20180111	2	1007	1009	0.0010	0.1982	±0.0140
Be20180111	1	1018	1019	0.0010	0.0981	±0.0098
B20f180111	2	1004	1006	0.0004	0.1988	±0.0141
Ba20180112	2	1009	1011	0.0018	0.1978	±0.0140

In Figure 10 the dose response can be seen relating the percentage of neutrophils that were viewed with the PPHA morphology as a function of lifetime dose.

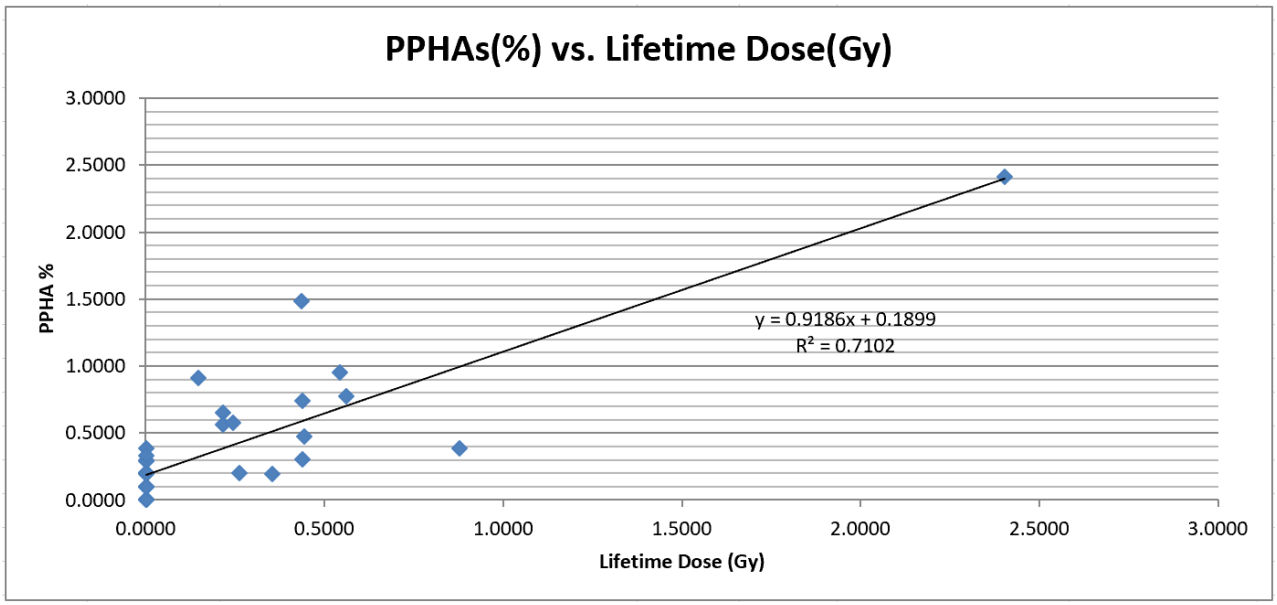

Figure 10: Percentage of Wild Boar Neutrophils with PPHA Morphology as a function of Estimated Lifetime Dose (Gy).

Figure 11 shows the PPHA morphology from a wild boar that was captured in the Fukushima exclusion zone. The thin mitotic bridge that is characteristic of the PPHA morphology is indicated by the red arrow.

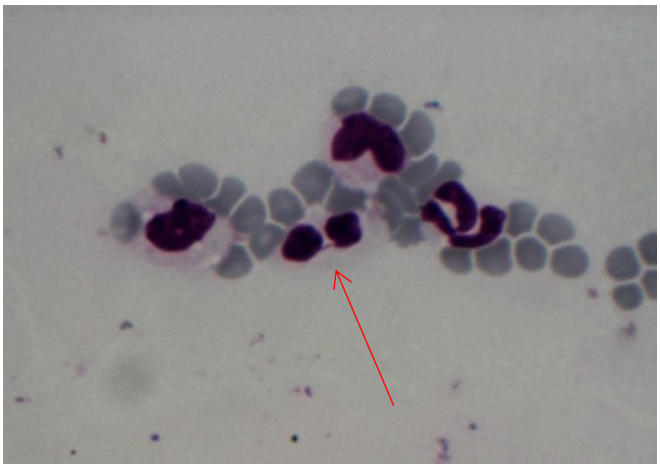

Figure 11: Pelger Huët Anomaly from Peripheral Blood Smear in Boar Bb20170724. Pseudo Pelger-Huët Anomaly Absence in Large Japanese Field Mouse

Twenty mice were evaluated for this study that were captured in dose rates ranging from 0.13 µSv/hr to 45 µSv/hr, and ranging in age from 1 month to 18 months. Two blood smears were evaluated from each mouse and lifetime dose estimates were not accessed by the evaluator until the counts were completed in an effort to remain unbiased. Any cells that displayed a morphology that was not immediately positively identified as PPHAs were imaged and circulated amongst the evaluators and in all cases a consensus was reached. Across the entirety of the data set there were no observed PPHA morphologies within the large Japanese field mouse.

DISCUSSION

Data Analysis

PPHAs were observed and scored in the wild boar and a dose response curve produced from the data. Lifetime dose estimates ranged from less than 1 mGy to 2.4 Gy across 38 boar that had viable slides for the study. A linear regression curve was fit to the data and the reported R^2 was 0.71. The dose response of wild boar PPHAs compares well with the radium watch dial painters study, which had an R^2 of 0.7 with an n of 168 (Goans, Toohey, Iddins, Mccomish, Tolmachev, & Dainiak, 2018). The radium watch dial painter data set was blinded to researchers creating the dose response curve for boar to avoid bias. There is a large gap in the data set for the boars between 0.88 Gy and 2.4 Gy lifetime dose. This gap in the data is due to the research group from the IER failing to gain access to the higher dose rate areas of the exclusion zone in Okuma town and only capturing one elderly boar in a relatively high dose region of Namie known as Takase Gorge. The majority of the boars captured were in the decontaminated urban areas of the exclusion zone. Additional data is currently being collected and in the future it will be evaluated and added to this data set.

No PPHAs were observed in the large Japanese field mice. Twenty mice were evaluated with dose rates ranging across three orders of magnitude, so if PPHAs were expressed, the morphology would be seen in the sample set. Mice will be captured in the future in an effort to look at genetic and proteomic biomarkers, and blood smears will be taken on these animals to further confirm the absence of the PPHA morphology.

Laboratory Work Issues In Japan

The largest hindrances to the success of this project was the language barrier between Japanese administrative workers at the IER and English-speaking students, logistical errors, and a lack of experience. The original goal of the hematological portion of the study was to extract peripheral blood samples and stimulate lymphocytes for division, subsequently administering a colcemid cell cycle block to arrest the cells in metaphase, and ultimately producing chromosome spreads. The chromosome spreads would have served as a means to estimate dose to the animals and compare to any cataracts that were found in the animals. Unfortunately, there were many unsuccessful attempts to generate chromosome spreads while in Fukushima for numerous reasons. First and foremost, logistical errors were the bedrock of issues in the summer of 2017. Prior to leaving for Japan in the end of May a complete supply list was sent to the IER requesting what was necessary for the proper preparation of chromosomal aberration assays. The supply list was compared to an inventory that was taken accurately and professionally from a year prior in the summer of 2016 and items that were on this inventory were not ordered. Upon arrival to Japan it was discovered that the lab had been moved from an old building to a brand new building and was not fully set up, and many of the supplies on the inventory from the year prior had been used or misplaced. Some of the pertinent supplies, to include acetic acid, had been used in the year leading up to arrival and required the ordering of more. The ordering process of chemicals takes a large amount of time at the IER and the acetic acid did not arrive until nearly the end of the summer. In the mean-time some acetic acid was borrowed from a neighboring lab and used to run the first set of boar samples. Mouse samples were put on hold because 1ml sodium heparin tubes were not ordered for their blood extraction, and it was discovered later that they were not available in Japan.

Following the completion of the first set of blood samples it was determined using light microscopy that the protocol was unsuccessful in generating any chromosome spreads. Following some investigation, it was discovered that the hose between the CO_2 tank and the incubator had been pressurized so the incubator read an appropriate CO_2 pressure on the digital screen, but the tank had been turned off so there was not a flow of gas and the cell samples subsequently died. This problem was rectified with the help of facilities maintenance and the procedure was attempted again, and the samples died due to bacterial contamination. This was due to a lack of experience with sterile technique and the unfortunate rushed nature of returning from the field where wild animals were handled and immediately conducting cell biology procedures. In the future, upon returning from the field, clothes should be changed and a thorough cleaning of oneself should be conducted prior to working in the biological safety cabinet. In addition, the UV light should be used on a daily basis at the end of every session in the biological safety cabinet to kill any residual bacteria that are deposited from the researcher during the procedure.

Research inexperience contributed greatly to the problems with the chromosome preparations. There was an inherent trust in the equipment that was unfortunately a flawed mindset. When the new microbiology lab was opened roughly 3 weeks into the 10 week research trip it lacked a lot of necessary equipment to include a centrifuge, working incubator, in-room deionized water, hot water bath, deep freeze, and a functional biological safety cabinet. Over the following weeks the equipment was located and the problems fixed with the equipment that was already in the laboratory. A lack of experience greatly prolonged this period of set up and resulted in zero successful chromosome preparations at the half way point of the research

trip. In the future all equipment should be function checked prior to the beginning of any protocols. It should not be assumed that equipment has been properly maintained and properly operated in the past.

Laboratory time was not prioritized as high as it should have been. Two different species (wild boar and the large Japanese field mouse) were being captured at multiple different locations along the west coast of Fukushima Prefecture which is extremely time consuming. Most days would begin with following the prefectural boar hunters in the morning, and if there were animals it could take until 5-6pm at night to complete the field necropsies and ophthalmic exams. Following completion of the boar hunting, mouse traps needed to be checked that were up to an hour drive away, and if there were animals in the traps they would need to be processed immediately. This led to extremely long field days and once the group returned to the lab they were exhausted, and corners were cut in proper sterile technique. The corners being cut were not intentionally done, but due to an inherent exhaustion that set in after several weeks of 12-15-hour days in the field. In the future laboratory work needs to be prioritized much higher than it was. The group should ensure the ability to split up so that half completes field work, and the other half returns to the lab to clean up and begin any procedures that need to be completed. In addition, a pride in being present for all of the field work hindered the laboratory productivity. If there was a choice between conducting laboratory work or being done early, and conducting field work the choice to remain in the field and get as much done as possible was chosen every time. Retrospective review of this mentality illustrates the need to set that pride aside for the greater good of the mission at hand. In future work, the long term implications of work habits

needs to be considered over short term self-efficacy due to taking part in every bit of the exhausting physical work that is being done.

Fortunately, blood smears had been taken on the boars since the beginning of the project for veterinary data, and mice capture rates were high enough that blood smears could begin being taken and still obtain a large sample size. The author of the papers that cited the PPHA anomaly as a new permanent biomarker for radiation exposure was contacted and a discourse began that ultimately led to a collaborative evaluation of the blood smears at REAC/TS in summer 2018. The cytogenic bio-dosimetry laboratory that is part of the REAC/TS campus was further able to instruct the proper technique for performing blood smears. In future studies this will aid in maintaining consistency and ensure far less slides have to be excluded due to poor execution of the smear.

Differential Boar Trapping Methods Between The Savannah River Site and Fukushima

As previously stated there were two locations where wild boars were captured for this study. There were key differences in the hunting techniques and tools used that resulted in a drastic difference in efficiency. In Fukushima prefecture hunters employed by the prefectural government were all elderly men that were capturing the animals to collect a bounty. The traps were placed around the town of Namie primarily where residents, whom were returning to the exclusion zone, claimed they saw the animals. Due to this the traps were largely being placed where transient animals were sited and many of the traps that were visited throughout the 10 week research trip were empty. Since wild boars and humans have an antagonistic relationship in Fukushima prefecture the boar population was likely moving up the mountain side more towards the southern

half of Namie, and possibly into the town of Okuma where radiation doses rate were higher. All of the aforementioned reasons contributed to the low number of boars that were captured in the contaminated zone. The total number of boars that were captured was 24 with 17 animals yielding proper blood smears that were viable for evaluation. Possibly the largest contributor of low animal numbers was the traps themselves that were being used in Fukushima. The traps were designed to hold a single boar at a time and required the animal to enter the cage to physically contact a trip wire for the doors to close. A number of the traps had trail cams set up by a wild life biologist at the IER to see how the boars were behaving around them. Several of the images showed the animals going up to the traps in a group with a select few going up to the trap but often not going through the door way. This phenomenon is calling being trap shy and can be a learned behavior from being caught before and released or others in the family group being captured. In figure 12 boar Ba20170617 can be seen in the single boar trap design used in Fukushima.

Figure 12. Typical single boar trap used in Fukushima containing Ba20170617.

At the Savannah River Site the SREL utilized a much different style of trap to capture the boars. The traps took advantage of the family groups that the animals often travel in versus the single boar traps that relied on an animal being physically separated from it's family by a barrier. The cages were of many different building materials, but they all had the same basic design. They were large circular cages all with relatively large open entrances that the whole family group could fit through at once. Some of the more improvised cages had a large 8' wide × 4' tall cage door that lifted vertically along rails into position and was held up by a winch that was released by a trip wire. Most of the cages had a camera trap that was connected to the winch system and could be remotely accessed and controlled by a cellular telephone. This allowed for the hunter to watch from a safe distance and drop the cage door at the most opportune time to maximize the number of animals that were captured. The hunters would bait the trap several times per week but leave the traps open until they were able to get the entirety of the family group accustomed to entering the trap. At this point they would drop the trap and capture several animals at once. During the course of the SREL hunt the largest capture occurred in one of the more sophisticated traps. The trap was remotely controlled by a trail cam, but what made it unique was its dual ring structure. The outer ring was a thin profiled support system that consisted of no more than a dozen legs and diagonal rails to guide the inner ring up into place. The inner ring was the actual fence which remained elevated several feet off the ground until the trap was triggered, and it slid down the diagonal rails into place trapping the animals inside. The total number of animals collected at SREL was 33 pigs in 5 days. The more sophisticated trap can be seen in Figure 13 containing 16 pigs.

Figure 13. A more sophisticated family group trap used in SRS containing 16 pigs.

In Figure 14 an improvised trap with the 8’ wide× 4’ tall door can be seen containing one large boar.

Figure 14. An improvised family group trap used in SRS containing 1 boar.

Result Implications and Areas of Concern

The presence of the PPHA morphology in peripheral blood smears may be useful as an inexpensive and effective biomarker of chronic low dose radiation exposure. However, the relationship between the percentages of neutrophils that display the morphology and lifetime radiation dose needs strengthening to ensure confidence in the boar model. More boars with a more complete range of lifetime dose estimations could potentially do this, and the boars that were captured in summer 2018 could provide this. As done in summer of 2018, the lifetime dose estimations for these animals are unknown to the evaluators of these slides in an effort to remain unbiased.

The lack of chromosomal aberrations analysis in the current sample population is concerning because dicentric chromosome analysis is the current accepted standard of bio-dosimetry and would need to be done to validate the PPHA as a biomarker of dose. Problems that may arise with interpreting chromosomal aberrations on wild boar include incomplete dose estimates from external exposure. In the past dose estimates have been done by taking an air dose rate at the position of capture and while factoring for decay extrapolating backwards to the point of birth and integrating the lifetime external dose. The weathering of environmental contaminants, decontamination efforts, and the inherent heterogeneity of the contamination introduces a significant amount of potential error into dose estimation. More precise methods like electron spin resonance using the teeth of the animal will aid in removing a large amount of this error and increase confidence.

The large Japanese field mouse sample size was 10 control animals and 10 contaminated animals ranging in age from 1-18 months. The animals were living in dose rates that ranged from 0.13 μSv/hr to 45 μSv/hr and it was expected to see the PPHA morphology considering it is

a wider range than the boars. The mice however, did not display the PPHA morphology so given the current data the method is invalid for use as a mouse radiation biomarker.

Future Studies

Work needs to be done to validate the PPHA morphology as a biomarker of radiation exposure. Trends have been illustrated in bats, humans, and now boars but PPHA has not been compared to chromosomal aberrations. A paired study needs to be conducted to compare the dose estimates from dicentrics and the percentage of neutrophils that present the morphology. Unfortunately, a large void exists in regard to biomarkers in the wild boar to include chromosome dicentrics in the literature. The method would need to be calibrated against known exposures to obtain a proper dose estimates for the boars. It cannot be expected for their cells to react to ionizing radiation exactly as a human cell, which are well characterized. To calibrate the boar's cell response, blood can be collected from a population of control animals and irradiated at various degrees using a blood irradiator. The blood can then be processed for chromosomal aberrations and a dose response curve can be generated. After generation of a dose response curve, the contaminated wild type animals can be evaluated with the intent to validate the PPHA.

In addition, the PPHA assays need to be definitively characterized as to what makes it a PPHA. A consensus has been reached on the necessity of a bio-lobed structure to consider the cells a PPHA, however there is still debate a on what comprises the thin mitotic bridge that connects the two lobes. Endogenous Pelger-Huët anomalies are caused by a mutation on chromosome 1 that encodes for the lamin B receptor. This mutation disrupts the integrity of the structural components in the nucleus and results in the abnormal morphology (Collela &

Hollensead, 2012). In the case of the radiation induced morphology studies have been able to observe the morphology in peripheral smears within 6 hours following irradiation in non-human primates (Goans, Iddins, Ossetrova, Ney, & Dainiak, 2017). This rapid manifestation of the morphology is contradictory to the idea that a mutation is likely responsible. Further investigation of what is in the bridge must be conducted. One possible approach is to use electron microscopy to image the bridge, and another could be to use fluorescent probes. If the bridge is comprised of a dicentric chromosome being pulled between two segmentations a centromere probe could help indicate that.

In the nearest future, the boar slides that are currently in Japan (collected summer 2018) will be evaluated to strengthen the PPHA dose response curve created for the boars. The primary goal of the next research trip to Japan is to assay chromosomal aberrations in wild mice and investigate proteomic biomarkers. Although the PPHA morphology was not present in the 20 mice that were used in this study, blood smears will be taken to increase the sample size and to confirm this null result.

CONCLUSIONS

Ionizing radiation has been a consistent stimulator of scientific minds since Wilhelm Conrad Rontgen's discovery of x-rays in 1895 (Hall & Amato, 2012). What would have once been brandished as magic was now an energy that could be harnessed for technological advancements that could exponentially improve the lives of humans, yet at the same time could potentially cause harm. Ionizing radiation has long been a large concern to the public due to an inherent fear based in a low level of knowledge in regards to technical information. There is a large gap between the experts and the public that is seemingly unbridgeable, but if there is a middle ground between them it would not be a fear based in ignorance but a healthy respect based in the need for a responsible utilization of the atom.

The study of sentinel species like the Japanese wild boar and the large Japanese field mouse allow for the safe investigation of new potential biomarkers for radiation without the need of human exposure. The pseudo Pelger-Huët anomaly was investigated in the wild boar and large Japanese field mouse as a potential biodosimeter that could drastically reduce the time to dose estimation in a triage situation for mass casualty incidents. The morphology was not observed in the large Japanese field mouse therefore further investigation of this alternative biomarker is unnecessary The PPHA morphology was observed in the Japanese wild boar and a dose response curve was established. The curve matched trends in retrospective studies that have been conducted using archived blood smears from the Y-12 criticality accident and radium watch dial painters. Further investigation needs to be conducted as to the validity of the biomarker when compared to dicentric chromosome analysis, and the cell morphology needs to be further characterized.

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APPENDIX A: IACUC APPROVAL LETTER

Colorado State University
Knowledge to Go Places

Research Integrity & Compliance Review Office
Office of the Vice President for Research
321 General Services Building - Campus Delivery 2011 eprotocol
TEL: (970) 491-1553
FAX: (970) 491-2293

NOTICE OF APPROVAL FOR ANIMAL RESEARCH
Animal Welfare Assurance Number: A3572-01

DATE:	May 17, 2017	
TO:	Freeman, Katie, College of VetMed & Biomed Sci	
	Pederson, Sami, College of VetMed & Biomed Sci, Johnson, Thomas, Environmntl & Radiologicl Hlth, Jensen, Wayne, College of VetMed & Biomed Sci	
FROM:	Kim, Elaine, CSU IACUC	
PROTOCOL TITLE:	Cataract Prevalence of Wildlife in Fukushima, Japan	
FUNDING SOURCE:	Funding - Grants/Contracts	
PROTOCOL NUMBER:	17-7080A	
APPROVAL PERIOD:	Approval Date: May 17, 2017	Expiration Date: May 16, 2020

Colorado State University's Institutional Animal Care and Use Committee (IACUC) has completed its review of protocol 17-7080A, titled "Cataract Prevalence of Wildlife in Fukushima, Japan ." In accordance with federal and state requirements on the care and use of animals and policies established by Colorado State University, the committee has approved this new protocol. If the committee is placing any special requirements on the approval, they will be included at the bottom of this letter.

This protocol will need to undergo Continuing Review and approval prior to May 16, 2018.

Prior approval to changes in the approved protocol must be obtained before implementation of those changes. If you would like to involve additional personnel or change any aspect of the protocol in the future, please submit an Amendment Request to the Institutional Animal Care and Use Committee for review via eProtocol https://csu.keyusa.net.

Good luck in your research endeavors.

Sincerely,

Elaine K Kim

Kim, Elaine

Page: 1

APPENDIX B: USDA PERMIT

United States Department of Agriculture

Animal and Plant Health Inspection Service

Veterinary Services

National Center for Import and Export

Animal Products

4700 River Road
Unit 40
Riverdale, MD 20737

Telephone:
(301) 851-3300

FAX:
(301) 734-8226

Prof. Thomas E. Johnson / Colorado State University
Environmental and Radiological Health Science Building
1618 Campus Delivery
Fort Collins, CO 80523

Monday, December 12, 2016

Dear Prof. Thomas E. Johnson:

Your USDA Veterinary Permit to import and/or transport controlled materials, organisms, or vectors accompanies this cover letter.

Review this permit carefully, as the statements and language may have changed to reflect the requirements of newly published regulations.

Please note the following:

- USDA Veterinary Permits no longer require a signature. Use of the permit for importation of the described commodity(ies) is acknowledgement that the permittee is legally responsible for complying with the permit conditions.
- Review the import permit for errors. Should you identify any errors, please contact our office immediately
- A copy of the permit must accompany every shipment.

Do Not send the permit back to this office.

Contact our office with any questions or concerns at 301-851-3300, option 1.

Sincerely,

DShangford

Dr. Deborah Langford
Staff Veterinarian
Import Animal Products
National Import Export Services (NIES)

***USER FEES:** New permit application $150.00, Renewal permit $97.00, Amended permit $75.00, FBS inspection $512.00 (all fees are per application) and the Import Compliance fee $565.00 per shipment.

U.S.DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES
RIVERDALE, MARYLAND 20737
file:///D:/Inetpub/wwwroot/Epermits/images/

UNITED STATES VETERINARY PERMIT FOR IMPORTATION AND TRANSPORTATION OF CONTROLLED MATERIALS AND ORGANISMS AND VECTORS

PERMIT NUMBER
132546
Research

DATE ISSUED
12/12/2016

DATE EXPIRES
12/12/2017

NAME AND ADDRESS OF SHIPPER(S)

Various shippers within...
Japan

CC:
Service Center, CO (Lakewood, CA)

NAME AND ADDRESS OF PERMITTEE INCLUDING ZIP CODE AND TELEPHONE NUMBER

Prof. Thomas E. Johnson
Colorado State University
Environmental and Radiological Health Science Building
1618 Campus Delivery
Fort Collins, Colorado 80523
970-491-0563

U.S. PORT(S) OF ARRIVAL
DENVER, CO

MODE OF TRANSPORTATION AIR

AS REQUESTED IN YOUR APPLICATION, YOU ARE AUTHORIZED TO IMPORT OR TRANSPORT THE FOLLOWING MATERIALS

Teeth, hair, and/or blood samples (fixed and/or on slides) from wild boar (porcine origin)

RESTRICTIONS AND PRECAUTIONS FOR TRANSPORTING AND HANDLING MATERIALS AND ALL DERIVATIVES

THIS PERMIT IS ISSUED UNDER AUTHORITY CONTAINED IN 9 CFR CHAPTER 1, PARTS 94,95 AND 122. THE AUTHORIZED MATERIALS OR THEIR DERIVATIVES SHALL BE USED ONLY IN ACCORDANCE WITH THE RESTRICTIONS AND PRECAUTIONS SPECIFIED BELOW (ALTERATIONS OF RESTRICTIONS CAN BE MADE ONLY WHEN AUTHORIZED BY USDA, APHIS, VS).

- **Adequate safety precautions shall be maintained during shipment and handling to prevent dissemination of disease.**

- With the use of this permit I, Prof. Thomas E. Johnson, Permittee, acknowledge that the regulated material(s) will be imported/transported within the United States in accordance with the terms and conditions as are specified in the permit. The Permittee is the legal importer/recipient [as applicable] of regulated article(s) and is responsible for complying with the permit conditions. The Permittee must be at least 18 years of age and have and maintain an address in the United States that is specified on the permit; or if another legal entity, maintain an address or business office in the United States with a designated individual for service of process; and serve as the contact for the purpose of communications associated with the import, transit, or transport of the regulated article(s). **Note: Import/Permit requirements are subject to change at any time during the duration of this permit.

- ***Each shipment shall be accompanied by an ORIGINAL signed document from the producer/manufacturer confirming that: **1)** the exported material was derived from wild boar (porcine) that originated in Japan; **2)** prior to export to the United States, the exported materials were treated as follows: **(a)** the teeth were treated using a solution of bleach in an ultrasonic cleansing system, **(b)** the hair samples were washed with isopropanol and air dried, **(c)** the slides contain blood fixed with methanol and acetic acid then sealed with a fixative and coverslip, and ...[continued on page 2]...

continued on subsequent page(s).....

TO EXPEDITE CLEARANCES AT THE PORT OF ENTRY, BILL OF LADING, AIRBILL OR OTHER DOCUMENTS ACCOMPANYING THE SHIPMENT SHALL BEAR THE PERMIT NUMBER

SIGNATURE Deborah Langford
DLangford

TITLE
Staff Veterinarian
National Import Export Services

NO. LABELS

VS FORM 16-6A (MAR 95) Replaces VS Form 16-3A and 16-28 which are obsolete

RESTRICTIONS AND PRECAUTIONS: (continued from Permit Form VS 16-6)

- ...[continued from page 1]... **(d)** the blood samples were fixed with methanol and acetic acid plus glutaraldehyde to achieve a final concentration of 0.2% glutaraldehyde; and **3)** the exported material was not exposed to or commingled with any other animal origin material.
  [This certification must CLEARLY correspond to the shipment by means of an invoice number or shipping marks or lot number or other identification method. An English translation must be provided.]

- COMMERCIAL DISTRIBUTION OF THE IMPORTED MATERIAL IS PROHIBITED.

- This permit DOES NOT authorize direct or indirect exposure of or inoculation into laboratory and domestic livestock (including but not limited to: birds/poultry, cattle, sheep, goats, swine, and/or horses). Work shall be limited to *in vitro* uses only. No extraction of nucleic acids is to be performed on imported material.

- Packaging, containers, and all equipment in contact with these materials shall be sterilized or considered a biohazard and be disposed of accordingly.

- THIS PERMIT IS VALID ONLY FOR WORK CONDUCTED OR DIRECTED BY YOU OR YOUR DESIGNEE IN YOUR PRESENT U.S. FACILITY OR APPROPRIATELY INSPECTED LABORATORY. THE AUTHORIZED IMPORTED MATERIAL(S) MUST BE SHIPPED/CONSIGNED DIRECTLY TO THE ADDRESS OF THE PERMITTEE OR TO THE ADDRESS OF THE ADDITIONAL PERMITTEE(S) AS IDENTIFIED ON THIS PERMIT. (MATERIALS SHALL NOT BE MOVED TO ANOTHER U.S. LOCATION, OR DISTRIBUTED WITHIN THE U.S., WITHOUT USDA, APHIS, VS, NIES AUTHORIZATION.)

- On completion of your work, all permitted materials and all derivatives therefrom shall be destroyed.

- This permit does not exempt the permittee from responsibility for compliance with any other applicable federal, state, or local laws and regulations.

- Imported material may be subject to regulations enforced by the United States Department of Interior, Fish and Wildlife Service (FWS). Importer must contact FWS, information is available at web pages http://www.FWS.gov/permits/ and/or http://www.FWS.gov/le/travelers.html

- The restrictions on this permit remain in force as long as the material is in the United States.

U.S.DEPARTMENT OF AGRICULTURE
APHIS / VETERINARY SERVICES, RIVERDALE, MARYLAND 20737.

ATTACH TO U.S. VETERINARY PERMIT - 132546

RESTRICTIONS AND PRECAUTIONS: (continued from Permit Form VS 16-6)

- Any person who VIOLATES the terms and conditions of permits, and/or who forge, counterfeit, or deface permits may be subject to criminal and civil penalties in accordance with applicable law. In addition, all current permits may be cancelled and future permit applications denied.

- A copy of this permit must be included with the shipping documents. For imported materials, these documents must be presented to CBP Agricultural Specialists upon arrival at the U.S. port of arrival.

APPENDIX C: SHIPMENT LETTERS IN ENGLISH FOR BLOOD SMEAR SLIDES.

INSTITUTE OF ENVIRONMENTAL RADIOACTIVITY
環境放射能研究所

3 August 2017

To Whom It May Concern:

This shipment EGX72206975P contains hair, ear clippings, and fix blood slides of porcine origin and fixed blood slides from field mice from Japan.

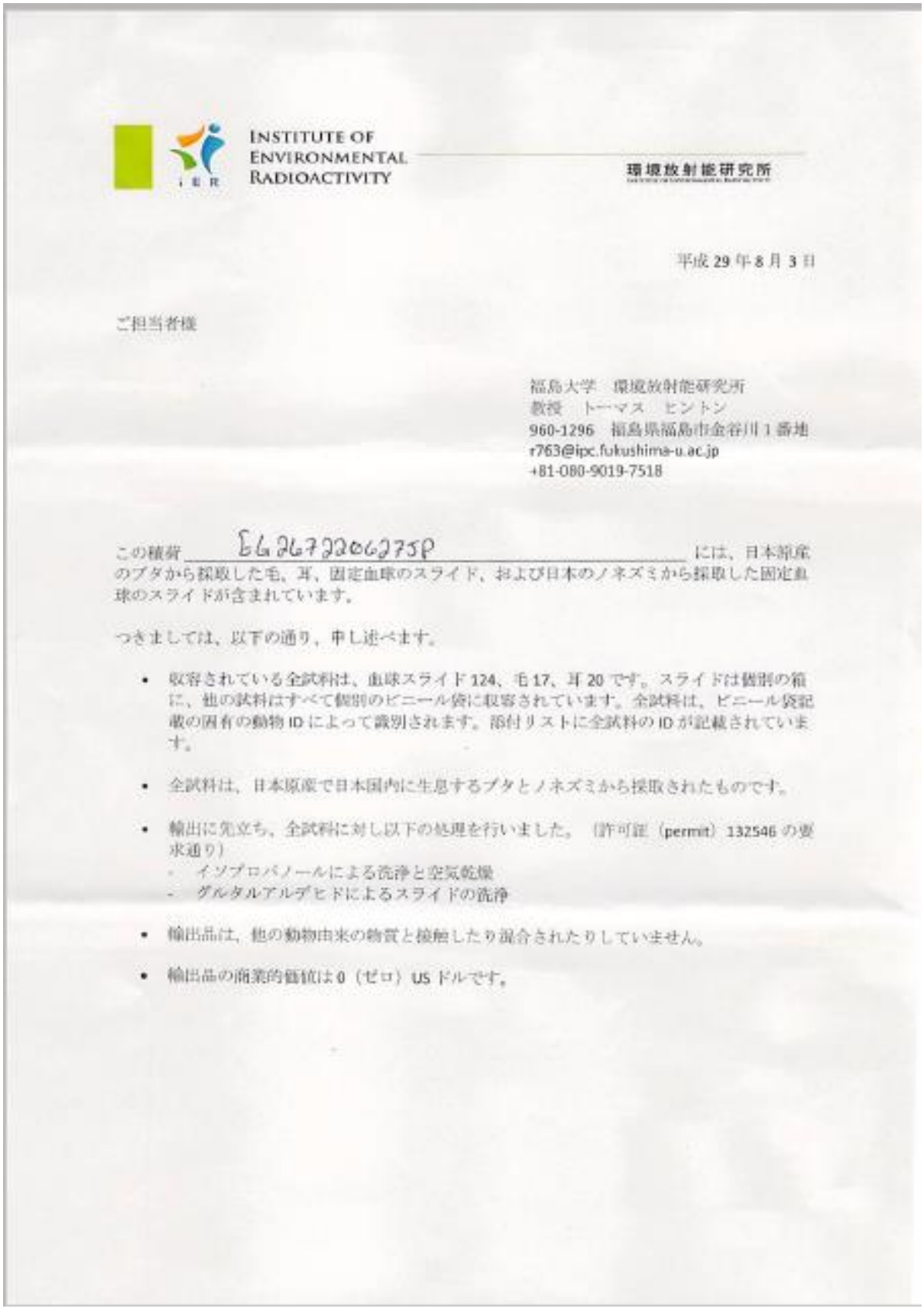
We hereby state the following:

- There are 124 blood slides, 17 hair samples, and 20 ear clippings in total contained in this shipment. Samples are in individual plastic bags except for slides that are contained in an individual box. All samples are identified by their unique animal ID written on the bag. An enclosed list contains all of the sample IDs.
- All samples are derived from pigs and mice that originated in and are housed in Japan.
- Prior to export, all samples were subjected to the following treatment (as required by permit 132546):
  - Washed with isopropanol and air dried
  - Slides were cleaned with glutaraldehyde
- The exported materials have not been exposed to or commingled with any other animal origin material.
- The exported materials have a commercial value of $0 USD.

Thomas Hinton
Professor
Institute of Environmental Radioactivity
Fukushima University
1 Kanayagawa, Fukushima 960-1296, Japan
r763@ipc.fukushima-u.ac.jp

1

APPENDIX D: SHIPMENT LETTERS IN JAPANESE FOR BLOOD SMEAR SLIDES



INSTITUTE OF ENVIRONMENTAL RADIOACTIVITY
環境放射能研究所

平成 29 年 8 月 3 日

ご担当者様

福島大学　環境放射能研究所
教授　トーマス　ヒントン
960-1296　福島県福島市金谷川 1 番地
r763@ipc.fukushima-u.ac.jp
+81-080-9019-7518

この積荷 EL267220627SP には、日本原産のブタから採取した毛、耳、固定血球のスライド、および日本のノネズミから採取した固定血球のスライドが含まれています。

つきましては、以下の通り、申し述べます。

- 収容されている全試料は、血球スライド 124、毛 17、耳 20 です。スライドは個別の箱に、他の試料はすべて個別のビニール袋に収容されています。全試料は、ビニール袋記載の固有の動物 ID によって識別されます。添付リストに全試料の ID が記載されています。
- 全試料は、日本原産で日本国内に生息するブタとノネズミから採取されたものです。
- 輸出に先立ち、全試料に対し以下の処理を行いました。（許可証（permit）132546 の要求通り）
  - イソプロパノールによる洗浄と空気乾燥
  - グルタルアルデヒドによるスライドの洗浄
- 輸出品は、他の動物由来の物質と接触したり混合されたりしていません。
- 輸出品の商業的価値は 0（ゼロ）US ドルです。

APPENDIX E: SAMPLE LIST FOR SHIPMENT FROM JAPAN TO THE UNITED STATES

Sample ID	Fixed Blood Sl	Ear Clippi	Hair Sample
Ma170608	2	0	0
Ma170616	2	0	0
Mb170616	2	0	0
Mc170616	1	0	0
Ma170619	1	0	0
Ma170621	1	0	0
Ma170622	1	0	0
Mb170622	1	0	0
Ma170628	1	0	0
Mb170628	1	0	0
Ma170629	1	0	0
Ma170702	1	0	0
Ma170703	1	0	0
Ma170708	1	0	0
Ma170709	1	0	0
Mb170709	1	0	0
Ma170711	1	0	0
Ma170712	1	0	0
Mb170712	1	0	0
Mc170712	1	0	0
Md170712	1	0	0
Me170712	1	0	0
Mf170712	1	0	0
Mg170712	1	0	0
Mh170712	1	0	0
Ma170713	1	0	0
Ma170714	1	0	0
Mb170714	1	0	0
Ma170718	1	0	0
Mb170718	1	0	0
Ma170720	1	0	0
Mb170720	1	0	0
Ma170721	2	0	0
Mb170721	2	0	0
Mc170721	2	0	0
Md170721	2	0	0
Me170721	2	0	0
Mf170721	2	0	0
Mg170721	2	0	0
Mh170721	2	0	0
Mi170721	2	0	0
Ma170722	2	0	0
Ma170724	2	0	0
Mb170724	2	0	0
Ma170727	2	0	0
Ba20170605	3	0	1
Ba20170608	3	1	1
Ba20170609	3	1	1
Bb20170609	3	1	1
Ba20170615	3	1	1
Ba20170616	3	1	1
Bb20170616	3	1	1

Ba20170617	3	1	1
Ba20170620	3	1	1
Ba20170623	3	1	1
Bb20170623	3	1	1
Ba20170627	3	1	1
Ba20170704	3	1	1
Ba20170717	3	1	1
Bb20170717	3	1	1
Bc20170717	3	1	1
Ba20179720	3	1	1
Ba20170724	3	1	0
Bb20170724	3	1	0
Bc20170724	3	1	0
Bd20170724	3	1	0
Totale	**124**	**20**	**17**

APPENDIX F: MAKING A BLOOD SMEAR

1. Place a drop of blood that is roughly 10µL in volume near one end of a blood slide.
2. quickly take a second slide and while holding it at an angle above the slide containing the drop of blood make contact with the slide as seen in figure 15.
    a. It is important to do this quickly because the blood will coagulate rapidly in air.

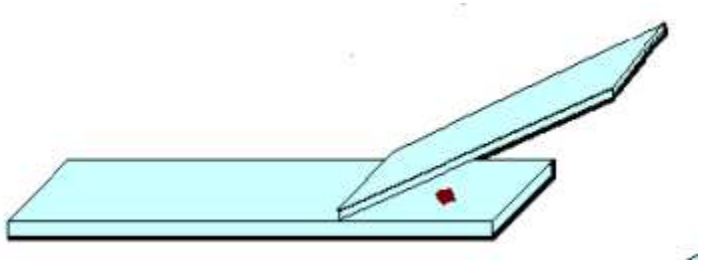


Figure 15. Placement of second slide in the process of creating a blood smear.

3. While ensuring that contact is maintained between the two slides draw the top slide back towards the blood drop slowly. Capillary action will cause the blood to spread out along the glass surface depicting in figure 16.

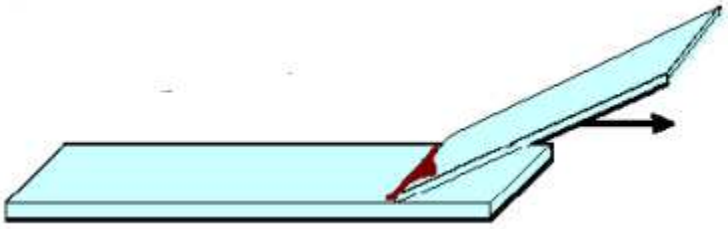


Figure 16. Drawing back the top slide as to make contact with the blood drop

4. In a quick motion push the top slide forward. The blood with smear behind the slide in a uniform fashion as depicted in figure 17. Ideally the resulting smear will have a feathered edge that will be the location of evaluation.

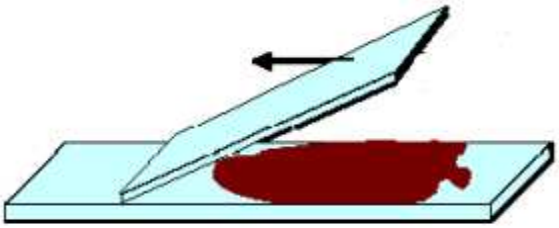


Figure 17. Placement of second slide in the process of creating a blood smear

5. Allow the blood to dry and then using an eye dropper coat the smear in 100% methanol to fix the cells in place.
6. Return to the lab and see to appendix E for Geimsa staining and coverslipping.

APPENDIX G: GEIMSA STAINING BLOOD SMEARS

1. Ensure that blood smears are fixed in methanol and properly dried and labeled.
    a. If an ink pen is used for labeling methanol will cause the label to run. Write label with pencil and write over with a permanent marker once slides are fully prepared.
2. Prepare a 5% Giemsa stain in GURR
    a. GURR buffer solution preparation:
        i. Add 1 tablet to 100mL dH2O → dissolve
        ii. GURR buffer tablets can be seen in figure 18.

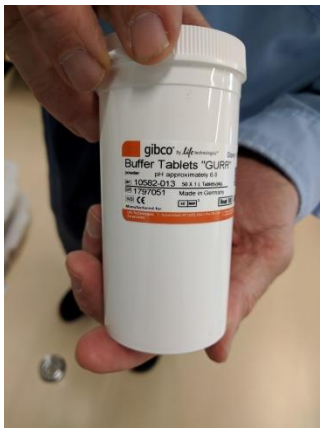


Figure 18. GURR buffer tablets used in preparation of Geimsa stain.

    b. Add 2.5mL Giemsa solution to 47.5mL GURR in a Coplin jar
3. Submerge slides in Giemsa solution for 45 minutes in a Coplin jar.
4. Rinse in fresh deionized H2O. stained and rinsed blood smears can be seen in Figure 19.

Figure 19. Geimsa stained blood smears.

5. Submerge slides in xylene to ensure all oils are removed.
6. Mount coverslip with Permount mounting medium.
    a. Apply pressure to the coverslip to remove any air bubbles.
    b. Clean any excess mounting medium that is forced out from under the coverslip.
7. Store in cool dry place. Avoid keeping them for extended periods of time in hot vehicles if transporting them.

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