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IOD IN RHINOS—IMMUNITY GROUP REPORT: REPORT FROM THE IMMUNITY, GENETICS AND TOXICOLOGY WORKING GROUP OF THE INTERNATIONAL WORKSHOP ON IRON OVERLOAD DISORDER IN BROWSING RHINOCEROS (FEBRUARY 2011)

Tomas Ganz, Ph.D., M.D., Jesse Goff, Ph.D., D.V.M., Kirk Klasing, Ph.D., Elizabeta Nemeth, Ph.D., and Terri Roth, Ph.D., M.S.

INTRODUCTION

This report provides recommendations for research to understand the basis of iron overload disorder (IOD) in browsing rhinos maintained under the care of humans. This work represents the cumulative efforts of the Immunity, Genetics and Toxicology Working Group of the International Workshop on Iron Overload Disorder in Browsing Rhinoceros, held at Disney's Animal Kingdom in February 2011. The overarching question is the following: Is IOD in browsing rhinos due to compensated hemolysis or is it due to primary dysregulation of iron absorption vs. excretion? Below is the list of important questions and the proposed actions. Priority actions based on feasibility and importance are indicated with an asterisk (*).

INDICATIONS OF COMPENSATED HEMOLYSIS

1) Question: Is red blood cell (RBC) recycling accelerated?

*Action: Compare the life span of RBCs in peripheral blood of white (*Ceratotherium simum*) and black rhinos (*Diceros bicornis*). Measure RBC lifespan by biotinylation of RBCs. The method requires 1) drawing blood, 2) labeling RBCs with biotin, 3) infusing biotinylated RBCs, and 4) sampling the blood for the proportion of biotinylated RBCs at multiple time points over a period of 1 mo. This may restrict the number of animals that could be studied. The reference shows the methodology applied to horses: http://www.ncbi.nlm.nih.gov/pubmed/20673097.

If life span is shortened in black rhinos, examine if this is the consequence of alterations in RBCs or macrophage abnormalities (questions 2–6).

2) Question: Are there any morphologic differences in the RBC values of black vs. white rhinos?

*Action: Compare complete blood count and blood smears of black and white rhinos.

a) Is there reticulocytosis? This is indicative of compensated hemolysis.
b) Are there Heinz bodies in RBCs? Heinz bodies are dark inclusions of denatured hemoglobin (Hb) and are indicative of oxidative damage to Hb.
c) Is there a difference in mean corpuscular volume (MCV)? High MCV in the absence of reticulocytosis reflects iron overload in some species.

3) Question: Is there a diet-related hemolysis?

Antibody-mediated hemolytic problems do not seem apparent, but diet-related hemolysis has not been ruled out. Alfalfa and other forages contain hemolysins.

Action: Take extracts from diet and examine hemolysis and agglutination in vitro comparing white and black rhinos.[1]

4) Question: Could clostridium or other bacterial species that produce hemolysins have a role in RBC hemolysis in black rhinos?

Many diets are high in starch and simple sugars, which may facilitate clostridia overgrowth.

Action: Sequence microbiomes (cecal droppings in particular) and compare in wild and captive as well as whites and blacks. In addition, examine if a switch to low-starch diets lowers levels of hemolysins.

From the Departments of Medicine and Pathology, David Geffen School of Medicine, University of California, Los Angeles, California 90095, USA (Ganz, Nemeth); The College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011, USA (Goff); The Department of Animal Science, University of California, Davis, California 95616, USA (Klasing); and the Center for Conservation Research of Endangered Wildlife, Cincinnati Zoo and Botanical Gardens, Cincinnati, Ohio 45220, USA (Roth). Correspondence should be directed to Dr. Klasing (kcklasing@ucdavis.edu).

5) Question: Are RBCs of black rhinos more fragile and easily damaged by oxidative stress? Is oxidative stress higher in black rhinos?

*Action: Measure antioxidants and indices of oxidative damage to lipids in RBCs, urine, and plasma. Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde (MDA). This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein adducts. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism. MDA and other "thiobarbituric reactive substances" (TBARS) condense with two equivalents of thiobarbituric acid to give a fluorescent red derivative that can be assayed spectrophotometrically. 1-Methyl-2-phenylindole is an alternative, more selective reagent. TBARS levels in black vs. white RBCs, urine, and plasma should be measured. Additionally, RBCs should be subjected to oxidative stress in vitro,[1] and the resulting effects on RBC lysis and TBARS should be examined.

6) Question: Is erythrophagocytic function of macrophages of black rhinos increased in comparison to that of white rhinos?

*Action: Conduct comparative in vitro study of RBC uptake by macrophages (white vs. black, young vs. old) as well as staphylococcus uptake studies as an indication of general phagocytic ability.

7) Question: Why are macrophages laden with iron in black rhinos? Is the intracellular iron derived purely from RBC erythrophagocytosis or are macrophages taking up other iron sources (e.g., iron-transferrin, lactoferrin)?

Action: In vitro studies could be done using macrophages from black and white rhinos. Iron sources would be added (iron citrate, transferrin, lactoferrin, and heme) to determine what is taken up and how much is taken up by the macrophages.

8) Question: Do black rhinos have less effective erythropoiesis (more apoptosis in the bone marrow) than white rhinos?

Ineffective erythropoiesis can cause iron overload, likely through suppression of hepcidin.

*Action: Review bone marrow sections from both species for expansion of erythrocyte precursor pools. Add this procedure to all postmortem examinations.

INDICATIONS OF DYSREGULATED IRON METABOLISM

1) Question: Is there a difference in iron excretion between captive and wild rhinos?

There may be chelators in native browse and also siderophores produced by bacteria, yeast, or fungus and released in the intestines. If these molecules are absorbed, they would bind iron and could be excreted in urine or feces, thereby accelerating iron excretion. These could be absent in captive rhinos. Alternately, bacteria in cecum could chelate the iron and then excrete it instead of allowing it to be absorbed.

*Action: Examine fractional urinary iron excretion in the wild vs. captive rhinos to determine if dietary or microbial iron chelators are present. Stool iron excretion studies are more difficult but also should be considered.

2) Question: Is there a difference in hepcidin levels between black and white rhinos or between captive and wild rhinos?

Hepcidin is the main regulator of iron absorption and distribution. Hepcidin deficiency is the cause of iron overload in humans.

*Action: Measure hepcidin in urine and serum (black and white rhinos; captive and wild rhinos) by mass spectrometry. If hepcidin levels are lower in black rhinos, proceed to item 3.

3) Question: Is there a difference in the expression or exon sequences of iron regulatory genes (e.g., High Iron Fe (HFE), transferrin receptor 2, hemojuvelin, hepcidin) in the liver between black and white rhinos?

These differences could cause differences in hepcidin concentrations and iron absorption.

*Action: White and black rhinos should be compared for messenger RNA (mRNA) expression and exon sequences in the liver of HFE, hemojuvelin, transferrin receptor 2, hepcidin, ...).

A critical aspect of this approach is obtaining fresh samples suitable for analysis. We need to develop a protocol (including appropriate timeline, sample size, storage preservative, and storage temperature) with which to obtain quality RNA from tissues. Rhinos are occasionally euthanatized, and we should opportunistically secure samples of liver (for this question), spleen, cecum, colon, duodenum, jejunum, ileum, and bone marrow (for future use) for subsequent RNA and protein quantification. Possibly start with a rabbit and horse to optimize the protocol. Qiagen and other companies are developing buffer solu-

[1] Given the large amount of blood that is routinely collected by Disney, the research group advises that this resource be utilized fully. Consider getting interns or a MS student involved conducting this research.

tions for preserving DNA, RNA, and protein in the same sample, and these new products should be examined for applicability. Once techniques are validated, tubes with the proper preservative should be placed with various zoos along with collection protocols so that samples can be taken quickly after death of rhinos.

4) Question: Is there a difference in iron absorption between black and white rhinos related to the expression of iron transporters in different regions of the gastrointestinal (GI) tract?

Both the level of transporter expression and the total amount of tissue capable of absorption could influence uptake. Understanding the location of absorption could also affect nutrition or pharmacologic approaches to reducing absorption.

*Action: Once techniques for obtaining fresh tissues are validated, white and black rhinos should be compared for mRNA expression of divalent metal transporter, duodenal cytochrome B, ferritin, ferroportin-1 and hephaestin in samples from different regions of the entire GI tract.

5) Question: Could changes in intestinal bacterial species alter utilization of dietary iron?

Action: Examine ileal and cecal microbiome in wild vs. captive black rhinos.

6) Question: What forms of iron are in plasma?

Action: Measure transferrin saturation and detect other iron binding proteins by adding trace radioactive iron to plasma and by analyzing protein by nondenaturing electrophoresis and autoradiography. Determine by labeling and gel filtration chromatography if there are other molecules that bind appreciable iron. These molecules might be of endogenous, microbial, or dietary origin.

7) Question: Are black rhinos marginally deficient in copper, which could affect the activity of ceruloplasmin and interfere with iron mobilization from macrophages?

Action: Ensure that plasma or serum ceruloplasmin activity is being examined (not just protein concentration). The rationale is that the concentration of ceruloplasmin protein does not decrease during a moderate copper deficiency, but the enzyme may lack activity as a result of missing copper co-factor. Lower activity of ceruloplasmin could entrap iron in macrophages.

8) Question: What about the role of other minerals?

Action: Examine plasma and hepatic levels of zinc, copper, and molybdenum.

9) Question: Could stress (psychological, physical, immunologic) have an effect on iron-related acute phase proteins and alter iron absorption-distribution?

Action: Examine hepcidin, hemopexin, haptoglobin, lactoferrin, and transferrin (normally a negative acute phase protien [APP]). Compare black and white rhinos. Rationale: There is some indication in birds that IOD is exacerbated by transferrin acting as a positive instead of a negative APP.

10) Rule out vitamin D intoxication as a cause of hypercalcemia.

Hypercalcium has been observed in rhinos with IOD. In the horse, renal failure is typified by a rise in plasma calcium (Ca) and phosphorus (P), as well as creatinine.

Action: Measure serum 25OH-D3, blood urea nitrogen, creatinine, Ca, and P and determine if changes are associated with changes in iron status.