IOD IN RHINOS—EPIDEMIOLOGY GROUP REPORT: REPORT FROM THE EPIDEMIOLOGY WORKING GROUP OF THE INTERNATIONAL WORKSHOP ON IRON OVERLOAD DISORDER IN BROWSING RHINOCEROS (FEBRUARY 2011)

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INTRODUCTION

The Epidemiology Working Group, a subgroup of the participants of the International Workshop on Iron Storage Disease (renamed iron overload disorder, IOD), identified several areas in which information is lacking regarding IOD in browser rhinoceros. One of the first steps necessary in understanding iron overload disorder (IOD) is to define the parameters by which to identify IOD. Some of the parameters that have been used to evaluate IOD include serum iron, serum iron binding capacity, transferrin saturation, and serum ferritin. Clarification of whether a single parameter or a combination of these parameters is best for evaluating IOD is needed. Once IOD can more clearly be defined using these parameters, it will be necessary to identify whether clinical signs of disease are associated with the presence of IOD. This working group feels that it is necessary to define what is "normal" and what constitutes "abnormal" with regard to IOD. Are all browsing rhinos in captivity affected by IOD, and if not, how many are affected? Is IOD associated with an increased risk of developing disease?

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PRIORITIES

The Epidemiology Working Group has identified four priorities for gaining a better understanding of the IOD of browser rhinos.

1) Define the current and historic health issues affecting the captive black rhino population.
2) Define what constitutes an "affected" animal, in terms of IOD, and if IOD is associated with known health issues.

To do this,

1) Establish historic and current status on black rhinos in zoos by:
   a) Review of necropsy reports;
   b) Review of medical and husbandry records of current animals;
   c) Blood values (complete blood count (CBC), etc.);
   d) Ferritin levels; and
   e) Transferrin saturation.
2) Conduct an epidemiologic case-control study.
3) Define "normal" (prospective study of free-ranging black rhinos).
4) Conduct genetics studies to understand the genetic basis for the predisposition of black rhinos to store iron in excess.

To address the first and second priorities, it will be necessary to conduct a retrospective review of health and husbandry records of browser rhinos housed in zoos on the United States. This working group plans to work in collaboration with zoos to obtain data from the years 2000 until 2011 and then to evaluate and critically assess the content of this data. Initially, the clinical and pathologic criteria used to define an IOD "case" will be evaluated and compared among different rhinos and different zoos and over time. The aims of this work are to assess the quantity and quality of the information available regarding captive browser rhinos and IOD and to identify areas in which the current zoo management practices lack the ability

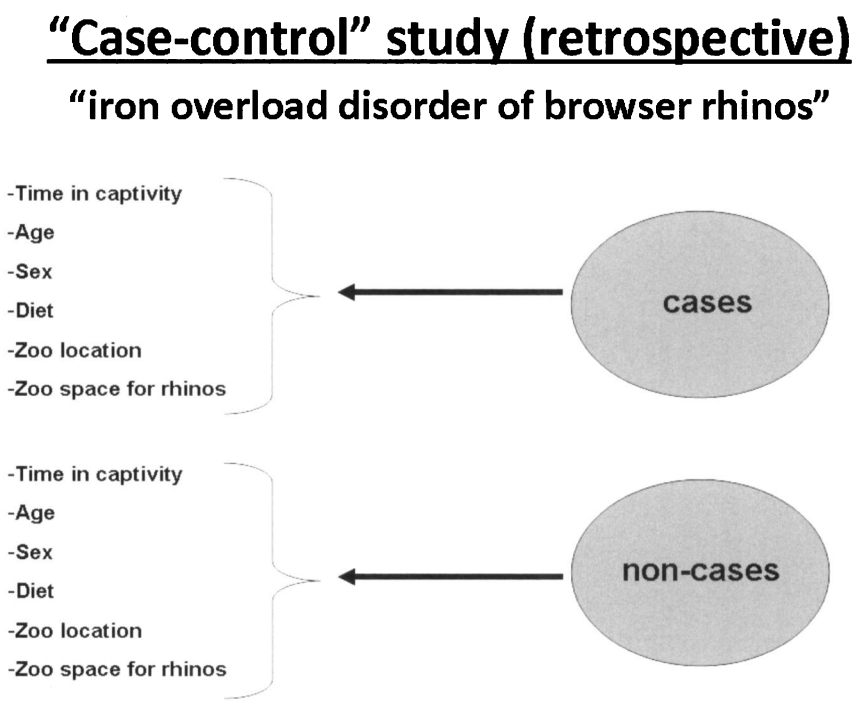

Figure 1. Diagram showing the basic format of a case control study and the risk factors we plan to evaluate in this study.

to properly obtain and store information regarding IOD in browser rhinos (as well as procedures that ensure proper data collection). This working group is aware that information related to IOD will not be available for all captive browser rhinos.

In the absence of such data it will be necessary to exclude those rhinos from phase 2 of our study, which will assess the impact of selected risk factors on the risk of IOD among captive browser rhinos and compare the distribution of risk factors among IOD cases versus non-IOD cases. However, the quantification and assessment of "which" data are not available and "why" such data are unavailable are included in the primary objectives of the first phase of study. As a component of this study this working group will generate a list of recommendations to enhance the current data collection process among zoos. In a similar manner, we will evaluate the information related to potential risk factors for IOD. The aims of phase 1 are to quantify the amount of available information already available as part of regular zoo management practices currently in place and to identify gaps in the past and current ability of establishments to obtain such information. In addition, we will generate a list of potential risk factors for which sufficient quantity and quality of data are not currently being gathered. These "missing" factors would be the focus of our recommendations for improvements needed by the current rhino management system.

In phase 2 of the planned retrospective study the goal is to assess the impact of selected risk factors on the risk of IOD among captive browser rhinos (see Fig. 1). Analysis will compare the distribution of values-parameters-factors among IOD "cases" and "non-cases" while obtaining corresponding *P*-values with which to assess the significance of associations of risk factors and the outcome (IOD). Statistical significance will be declared at $P < 0.05$. A priori, it is important to note that "clinical-physiologic" relevance will be considered in our study while assessing factors for which small sample sizes will be available. After the univariable analyses, if data quantity permits, we will build a multivariable model (logistic regression) that will control for the effect of multiple factors affecting the probability of an animal being classified as an IOD "case." The Hosmer–Lemeshow statistic will be used to test the overall fit of the model. The logistic regression model results will be transformed to the probability scale to demonstrate the impact of the risk factors on the outcome (IOD case).

The results of phases 1 and 2 of this retrospective study will complement a "prospective" study

in which information on parameters from both zoo and free-ranging browser rhinos will be collected. Parameters will include, but not be limited to, age, season, location, indication of trauma, external and gastrointestinal parasite load, blood work (including CBC, serum chemistry panel with liver enzymes, serum ferritin), and body condition score. The results of the retrospective case-control study (phase 2) will generate data to implement the "prospective" study that will allow us to further evaluate IOD in rhinos.

It is the expectation of this working group that in the next 2 yr we will generate and publish a scientific paper hoping to provide valuable information that can be used to prevent, control, and manage this disorder in the captive browser rhino population. In addition, we will seek funding to implement a study exploring the genetic basis for the predisposition of browser rhinos to IOD.

In order to improve the health and management of browsing rhinos, this working group proposes to make it a priority to understand the genetic basis for the predisposition of the browsing rhinos to store iron in excess. Knowing the basis of increased iron uptake in these animals will provide an educated basis for implementing mechanisms for prevention and intervention of disease development resulting from the increased body iron stores.

IOD in rhinos occurs predominantly in browser rhinos and not in grazers. To identify candidate genes that are associated with iron storage disease it will be necessary to perform comparative transcriptome analyses between browsers and grazers. Essentially, samples from livers, spleen, intestine, and possibly bone marrow will be collected from browsers (Black and Sumatran) and grazers (White and Indian). Next generation sequencing will be performed on reverse transcribed DNA made from RNA isolated from these tissues. Bioinformatic analyses will be performed to identify genetic differences among the species. This working group expects to identify genetic mutations in many genes that are unique to browsers and grazers. The mutations will be annotated using Polyphen, software that examines each mutation relative to the known sequence in other species, the level of conservation at the amino acid position, and the structural perturbation that might result from the mutation. The software then annotates the mutations as "probably damaging," "possibly damaging," "unknown," or "benign." This annotation allows us to prioritize the candidate genes that we identify. Functional studies will then be performed on the candidate genes in order to determine if the mutation is associated with a dysfunction in iron homeostasis. The functional studies will identify the gene that is responsible for predisposing browser rhinos to iron overload, as well as utilize tissue collection for transcriptome analysis to identify the gene responsible for iron IOD in browser rhinos.

Tissues obtained from necropsies are ideally obtained immediately following the demise of the animal. Tissue (1-g pieces) will be placed into empty tubes or tubes containing a preservative solution of RNAlater. The tissues should then be placed immediately into a liquid nitrogen bath or a dry ice–ethanol bath to flash-freeze. The tissues should then be transferred into a −80°C freezer for storage until the sample can be shipped. Samples flash-frozen without preservative will need to be shipped on dry ice by overnight express mail. Samples flash-frozen in preservative can be shipped with a cold pack or at ambient temperature by overnight express mail.