REFERENCES

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REVIEW OF LABORATORY AND NECROPSY EVIDENCE FOR IRON STORAGE DISEASE ACQUIRED BY BROWSER RHINOCEROSES

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Abstract: Necropsies of two browser rhinoceroses, African black (*Diceros bicornis*) and Sumatran (*Dicerorhinus sumatrensis*), often reveal extensive iron-pigment deposition in various tissues. This condition (hemosiderosis) has not been observed in species that are natural grazers, African white (*Ceratotherium simum*) and Asian greater one-horned (Indian; *Rhinoceros unicornis*), nor in any species free ranging in the wild. The causes, clinical significance, and consequences of captivity-acquired hemosiderosis have remained controversial despite two decades of compelling evidence that iron tends to accumulate logarithmically in *all* members of affected species in proportion to periods of expatriation; total-body iron loads can reach 10-fold in less than 3 yr and eventually exceed reference ranges by two to three orders of magnitude; iron overburdens are accompanied by laboratory and histopathologic evidence of cellular injury, necrosis and other clinical consequences characteristic of chronic pathologic iron overload disorders (ISD) in humans and other species (hemochromatosis); and that ISD develops in many other exotic wildlife species displaced from their natural habitats. The historical evolution of evidence establishing the development of pathologic ISD in browser (but not in grazer) rhinoceroses and the possible relevance of ISD to other conditions affecting these two species will be reviewed. Evidence reviewed includes new as well as published data derived from quantitative measurements of iron analytes in sera and necropsy tissues and histopathologic evaluations of current and past necropsies of captive and free-ranging rhinoceroses of all four available species. The evolutionary, husbandry, and conservation implications of ISD in rhinoceroses are relevant to understanding ISD acquired by many other species of exotic wildlife when displaced from their natural environments.

Key words: Ferritin, hemochromatosis, hemosiderosis, iron, rhinoceros species.

INTRODUCTION

Progressive loss of wilderness by human intrusion has forced numerous wildlife species to the brink of extinction and beyond. Some have been further decimated by avaricious poaching for the perceived medicinal, cultural (and actual black-market) value of their various body parts. In response to these threats, concerned conservationists have translocated animals from the wild into various sanctuaries for protection, for captive breeding programs, and for potential reintroduction into their original habitats. This has allowed closer scrutiny of otherwise reclusive animals, revealing disorders that have rarely, if ever, been observed in the wild.

Over the past several decades, four of the five extant species of rhinoceroses have been well studied in captivity, and each appears to be susceptible to characteristic sets of clinical conditions. African black rhinoceroses (*Diceros bicornis*) in particular have been affected by a number of disorders of high morbidity and mortality.[31,35,39] Episodes of acute intravascular hemolysis and chronic necrolytic dematopathy (mucocutaneous ulcerative disease) became recognized as the two most common causes of death.[34–38,43] Additional conditions included high susceptibilities to common and exotic microorganisms, such as *Aspergillus, Leptospira, Mycobacteria,* and *Salmonella*; scattered cases of leukoencephalomalacia, an apparently congenital, central nervous system (CNS) degenerative disorder; primary idiopathic or toxic hepatopathies; idiopathic hemorrhagic vasculopathy, possibly an autoimmune disorder targeting microvasculature; nonhemolytic anemia, sometimes with cachexia, resembling the anemia of chronic or inflammatory disease; stress-related sudden death, and generalized hemosiderosis. Virtually none of these conditions has been observed in African white *(Ceratotherium simum),* Asian greater one-horned (Indian; *Rhinoceros unicornis*), or Sumatran (*Dicerorhinus sumatrensis*) rhinoceroses, with the pertinent and important exception of extensive hemosiderosis and susceptibility to infections in the latter.

The prevalence of so many severe and clinically disparate disorders in any individual species suggested that some might share common etiologic or pathogenetic factors.[61] That possibility

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focused attention first on the extraordinary departures from normal mammalian cellular metabolism revealed by studies of rhinoceros erythrocytes and second, on the significance of generalized hemosiderosis, a condition detected to varying degrees in virtually all captive African black and Sumatran rhinoceros necropsies. Here, the quantitative and qualitative evidence for pathologic iron overload disorders (ISD) developing under non-native conditions in two browser species but not in the two grazer species is reviewed.

It should also be noted that *hemosiderosis* (or *siderosis*) is defined as localized or generalized accumulation of hemoglobin degradation products, a purely morphologic finding that may or may not be pathologic. As a general term, *hemochromatosis* refers to specific iron-overload syndromes such as human hereditary hemochromatosis due to human hemochromatosis protein (HFE) gene mutations. The term is also applied to conditions in which hemosiderosis is associated with pathologic changes such as cell injury or necrosis.

Comparative studies on rhinoceros erythrocytes

In the 1980s, intensive investigation of a hemolytic syndrome in black rhinoceroses was initiated because of its high incidence and 75% mortality rate. Common causes of intravascular hemolysis such as hemoglobinopathies, red cell membrane defects, and autoimmune reactions, were ruled out, but biochemical and enzymologic studies revealed a vast array of extraordinary deviations from other mammalian red cells.[32,64] Many of these were so different and of such magnitude that they would have been considered incompatible with normal red cell function and viability were they found in virtually any other vertebrate. Adenosine triphosphate (ATP), essential for maintenance of cation gradients and for initiating crucial metabolic reactions, was only 2–5% of the concentrations found in most mammalian erythrocytes.[19,50,51,62–65,67,70,80,81] The ubiquitous presence of multiple Heinz bodies and absent or extremely low activities of certain enzymes such as catalase, glutathione S-transferase, several kinases, and others,[50,63–65,70] were equally perplexing, because comparable deficiencies were well known to cause hemolytic anemia or immunodeficiency in humans and canines. In rhinoceroses, however, there were no major differences in these enzyme activities between unaffected individuals and those experiencing hemolytic episodes. Additionally, these highly anomalous, even bizarre, biochemical features were shared to varying degrees by all four species, as was the presence of a novel ancillary system for oxidant neutralization via highly elevated (>50×) intraerythrocytic concentrations of the amino acid tyrosine.[18,80,81] This necessitated a reappraisal of what constituted "normality" in rhinoceroses.

One serious metabolic consequence of these red cell characteristics was impairment of the hexosemonophosphate shunt,[50,58,63–65,71,80] the glycolytic pathway responsible for neutralization of ambient oxidative byproducts of many physiologic and pathologic processes. This predisposes black rhinoceroses to a hemolytic syndrome clinically identical to individuals with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency.[50,58,63–65,71] This highly common human disorder then provided a paradigm for developing preventative and therapeutic strategies, such as phosphate supplementation (to stimulate ATP generation) and avoidance of compounds and conditions known to initiate hemolysis in G-6-PD deficient patients.[50–53,62,64,71] These, in turn, have been credited at least partially for the subsequent rarity (one in the last decade) of lethal hemolytic episodes among captive black rhinoceroses.

These studies established that the entire *Rhinocerotidae* family possesses erythrocytes with significantly reduced capacities to neutralize exogenous and endogenous oxidants, with black rhinoceroses being the most significantly impaired and white rhinoceroses the least. Evidence also suggests that cells in other organ systems may be similarly affected, possibly contributing to some of the multiple disorders affecting rhinoceroses.[61,68] Unlike G-6-PD deficiency, diminished antioxidant capacity is not due to individual genetic mutations coding for aberrant enzyme proteins but instead represents species-wide characteristics that must be considered "normal" for them despite contrasting radically with other animals.

These multiple, uniquely restrictive features of rhinoceros red cells have now been confirmed independently by three laboratories with internationally acknowledged expertise.[2,18,19,80] Despite this corroboration, a recent review of black rhinoceros health issues inexplicably and incorrectly concluded that "no metabolic abnormalities" were found in these earlier studies to explain hemolytic tendencies. In particular, that review contained erroneous interpretations and specious conclusions regarding metabolic mechanisms for oxidant neutralization via the hexose monophos-

phate shunt and additionally proffered arguably sophistic appraisals of other clinical conditions affecting captive *D. bicornis.*[10]

Significance of hemosiderosis in African black and Sumatran rhinoceroses

The serious threat imposed by hemolytic episodes in black rhinoceroses had an unfortunate unintended consequence: for many years, it diverted attention and obscured the true nature of hemosiderosis. Over the past half century, hemosiderosis has been a consistent necropsy finding in both black and Sumatran rhinoceroses dying in U.S. and European zoos and reserves.[7,27,35,39,75] Because red cell lysis releases hemoglobin into the circulation for eventual degradation, hemosiderosis was often logically misinterpreted as residual evidence of past hemolytic events, even though few of these *D. bicornis* and only one of the *D. sumatrensis* had histories of hemolytic signs or symptoms. Some necropsy reports simply dismissed hemosiderosis as an incidental finding or viewed it as a characteristic of rhinoceroses in general with no obvious clinical implications.

Dr. Smith introduced the first definitive evidence that hepatic, splenic, or generalized hemosiderosis might reflect an authentic ISD. In 1987, he and his associates began studying iron analytes (serum iron, transferrin, and ferritin) in rhinoceroses compared to horses and other animals. They measured serum ferritin with a species-specific enzyme-linked immunosorbent assay (ELISA) system developed in their laboratory that even today remains the gold standard for quantitative assays of this highly important storage form of iron.[77,78]

In August 1993 at White Oak Conservation Center, their preliminary data were presented to the First International Workshop on Diseases of Black Rhinos[76] and eventually expanded into their seminal publication regarding iron metabolism in rhinoceroses.[77] Quantitative measurements of hepatic nonheme iron were five times higher in 16 captive black compared to nine white rhinoceroses, confirming many previous histopathologic impressions of elevated storage iron in the former. Lesser, but still significant, elevations were found for serum iron (3.3-fold, $P < 0.05$) and transferrin saturation (67.5% vs. 43.4%, $P < 0.01$) in 40 black vs. 13 white rhinoceroses, respectively, all in long-term U.S. captivity. Serum ferritin differed even more significantly ($P < 0.001$) with 15- to 25-fold greater concentrations in captive *D. bicornis.* With the caveat that ferritin can also increase as an acute-phase inflammatory reactant, it is widely accepted that serum ferritin concentrations reflect total-body iron stores with an accuracy exceeded only by direct quantitative analyses of hepatic tissue samples.[10,23,24,30]

These were the first definitive data demonstrating that iron accumulated in black but not white rhinoceroses in a manner that was directly dependent on time in captivity ($r = 0.43$). Because haptoglobin concentrations were comparable between the two populations, chronic hemolysis could not account for these discrepancies, and the authors presciently suggested that differences in bioavailability of enteric iron might be responsible. Over the past two decades, this has become the nidus for accretion of a large body of research comparing native and captive diets of browser vs. grazer rhinoceroses.[7,21]

Simultaneously, histopathologic observations of *D. bicornis* captured from the wild in Zimbabwe were cited at the White Oak Workshop by Dr. Kock and codified into another milestone paper.[26,27] The absence of hemosiderosis in one free-ranging and six recently captured animals was interpreted as evidence that the hemolytic syndrome so common in captivity did not likely occur in the wild. Three-quarters of 20 others, however, exhibited hemosiderosis involving multiple organs that "seemed to worsen with time" during boma confinement for periods up to 2 yr.[26,27] These animals subsisted on local browse that was selected by human keepers, however, not by ad libitum foraging. Taken together, these two classical papers by Smith et al.[77] and Kock et al.[27] presented compelling evidence that pathologic ISD is acquired by African black rhinoceroses when living under non-native conditions.

Corroborative evidence for ISD in African black and Sumatran rhinoceroses

In 1999, an Ad hoc Conference on Iron Disorders in Rhinos was convened at the St. Louis Zoo (St. Louis, Missouri, USA) to reconsider implications of these studies in light of additional data gathered by the University of California, Los Angeles (UCLA) Hematology Research Laboratory (Los Angeles, California 90095, USA). At that time, specimens were available from 47 captive rhinoceroses of four species and from 21 *D. bicornis* actively or recently free ranging in the wild.[53] These were subsequently expanded to include all those shown in Table 1.

Our assay results confirmed and extended those of Smith et al.,[77] showing greater than twofold elevations of serum iron and transferrin saturation and marked (one to two orders of magnitude)

Table 1. Serum analyses in captive and wild rhinoceroses. Values are means ±1 SD Juveniles were >1 mo and <4 yr of age. Serum iron, total iron binding capacity, and transferrin saturation were determined at the University of California, Los Angeles, Hematology Research Laboratory by quantitative colorimetry (Kit No. 565, Sigma Diagnostics, St. Louis, Missouri 63103, USA). Ferritin was measured by the Veterinary Diagnostic Laboratory at Kansas State University as described in the text. Specimens taken during acute illnesses, on day of death, or postmortem were excluded.

Species	No.	Iron (μg/dl)	Transferrin saturation (%)	Ferritin (ng/ml)
Captive:				
Adult *D. bicornis*	69–70	227 ± 107	66 ± 21	7,160 ± 9,784
Juvenile *D. bicornis*	26–30	236 ± 135	60 ± 21	1,187 ± 1,189
D. sumatrensis	13–14	131 ± 47	62 ± 22	856 ± 667
C. simum	21–24	121 ± 43	37 ± 11	59 ± 38
R. unicornis	6–11	122 ± 31	36 ± 8	62 ± 31
Free-ranging in Southern Africa:				
D. bicornis	21–26	83 ± 32	34 ± 8	180 ± 83
C. simum	15–20	64 ± 11	24 ± 3	73 ± 44
Reference ranges:				
U.S. Equine		50–198	22–44	43–261
University of California, Los Angeles, Hematology Research Lab		65–165	20–45	40–160

ferritin elevations in captive *D. bicornis*. Other studies provided further confirmation.[13,38] We also found similarly significant increases in transferrin saturation and serum ferritin in several *D. sumatrensis*, documenting that this species was also susceptible to development of ISD.[53,54,56,60,68,69] By contrast, captive white and Indian rhinoceroses had iron analyte values indistinguishable from human and equine reference ranges or from African black or white rhinoceroses that were free ranging in the wild.[53,54,59,60,69] These studies further confirmed that both transferrin saturation and serum ferritin concentrations in the two browser species were dependent variables of time in captivity.

In the decade following the St. Louis conference, the UCLA Hematology Research Laboratory database has been expanded to include serum iron analyte assays on multiple specimens from over 200 captive rhinoceroses of all four available species, along with 46 black and white rhinoceroses free ranging in natural habitats of southern Africa and nine *D. sumatrensis* living in Southeast Asian reserves.[7] Results of additional serum ferritin assays presented in Figure 1 dramatically illustrate the iron load magnitudes and disparities among these various populations. In the absence of ferritin assays, transferrin saturation measurements (ratios of serum iron to total iron binding capacities) provide less accurate but still reliable reflections of body iron status.

Necropsy studies have provided additional corroboration. Quantitative tissue assays in black rhinoceroses demonstrated logarithmic increases in hepatic nonheme iron concentrations as a function of age or time in captivity.[13,53,54,56,59–61] Histopathology revealed moderate, marked, and even massive deposits of ferric iron in multiple organs, particularly the liver, spleen, bone marrow, and lungs (Fig. 2) but also in small and large intestines, lymph nodes, endocrines, and other organs.

In the authors' experience, rhinoceros ISD appears initially to target the reticuloendothelial system. As the condition progresses, hyperplasia and heavy siderosis of interstitial macrophages produce masses of hemosiderin-laden cells that progressively distort the histologic anatomy of affected organs, including displacement of fat and hematopoietic elements from the bone marrow (Fig. 2). Mucosal and transmural infiltrates of iron-loaded macrophages can be seen from duodenum to colon. Eventually, parenchymal cells (particularly hepatocytes and endocrine epithelia) also exhibit iron loading. In the most severe cases, hepatic histopathology has included bridging fibrosis, regenerative nodularity, bile duct proliferation, and rare instances of frank cirrhosis and hepatocarcinoma.[53,54,56,57,59–61] Cytologic evidence of cell injury and necrosis is often prominent, and the resultant release of intracellular ferritin is reflected by serum ferritin concentrations that

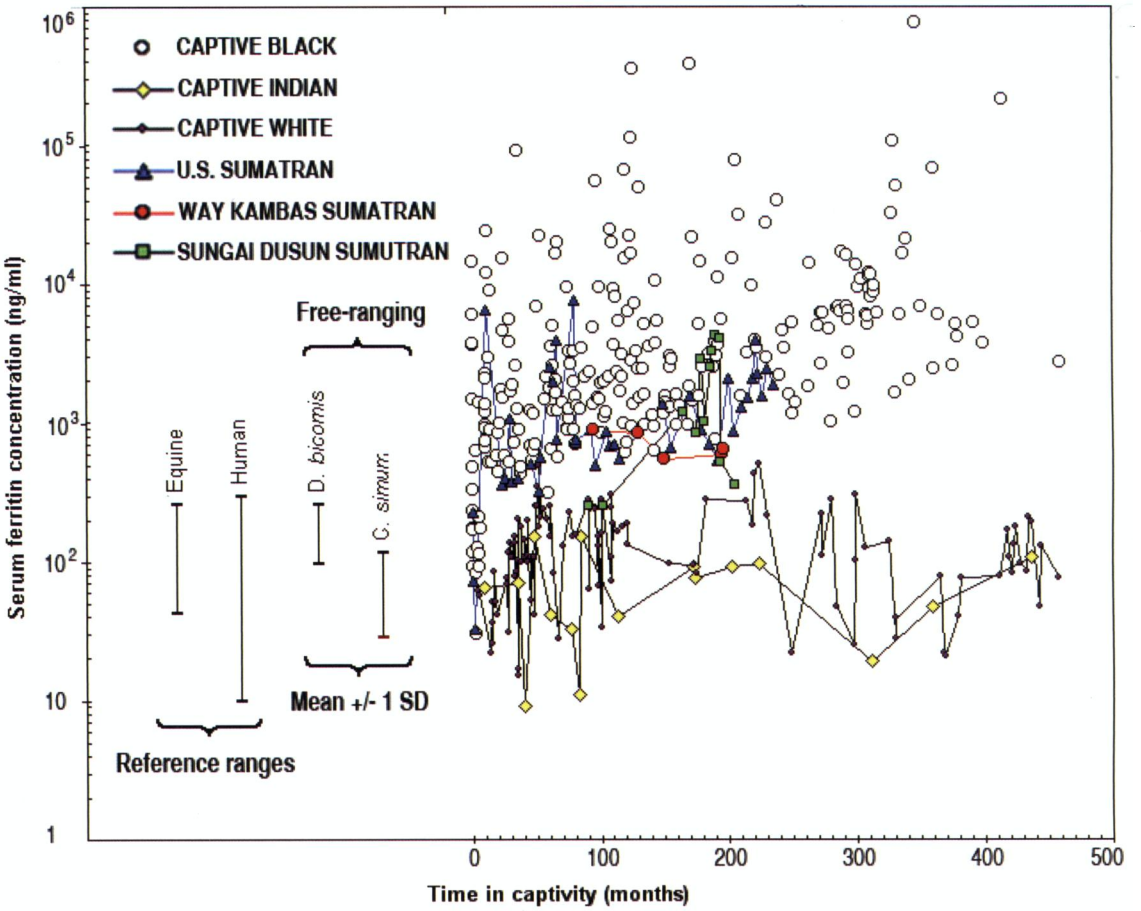

Figure 1. Serum ferritin concentrations in four species of rhinoceroses as a function of time in captivity. Note log scale for ferritin values. Data include multiple assays on 92 *D. bicornis*, 26 *C. simum*, 15 *R. unicornis*, and 14 *D. sumatrensis*. The latter group is divided into separate populations confined under differing conditions.[7] Symbols for three species are connected only for ease of identification. Nine of the 15 highest serum ferritin values were obtained from postmortem or day-of-death specimens when necropsies often revealed extensive hepatocellular degeneration.

often exceed known apoferrritin synthetic rates by several orders of magnitude.

The authors have observed similarly severe histopathologic changes (including overt hemochromatosis with or without cirrhosis) in reviews of Sumatran rhinoceroses necropsied after residing in U.S. and European zoos for periods up to 16 years.[7,54,56,57,59,60,69] In both species, the magnitude and tissue distribution patterns of hemosiderin were similar, but they differed distinctly from those observed in human hereditary hemochomatosis, hemolytic disease, or transfusion overload.[1,3–5,16,22,33,42,87] Some histopathologic features resembled patterns characteristic of two other iron-loading syndromes in humans, African or Bantu siderosis[3,5,16,33] and ferroportin disease,[11,73] as well as the ISD classically described in lemurs.[79]

Necropsy experience now includes over 60 rhinoceroses of all four species, providing more precise definition of the comparative pathology of ISD (Paglia, unpubl. data). The histopathologic examples shown in Figure 2 add to those previously published.[53,56,59,61,69] Hyperferremia with increased serum ferritin concentrations and elevated transferrin saturation also occurs in all three species of tapirs (*Tapirus* spp.) residing in U.S. zoos but not in those free ranging in expansive reserves.[66] ISD remains a dominant problem adversely affecting large numbers of other exotic wildlife species as well.[7,26,31,71]

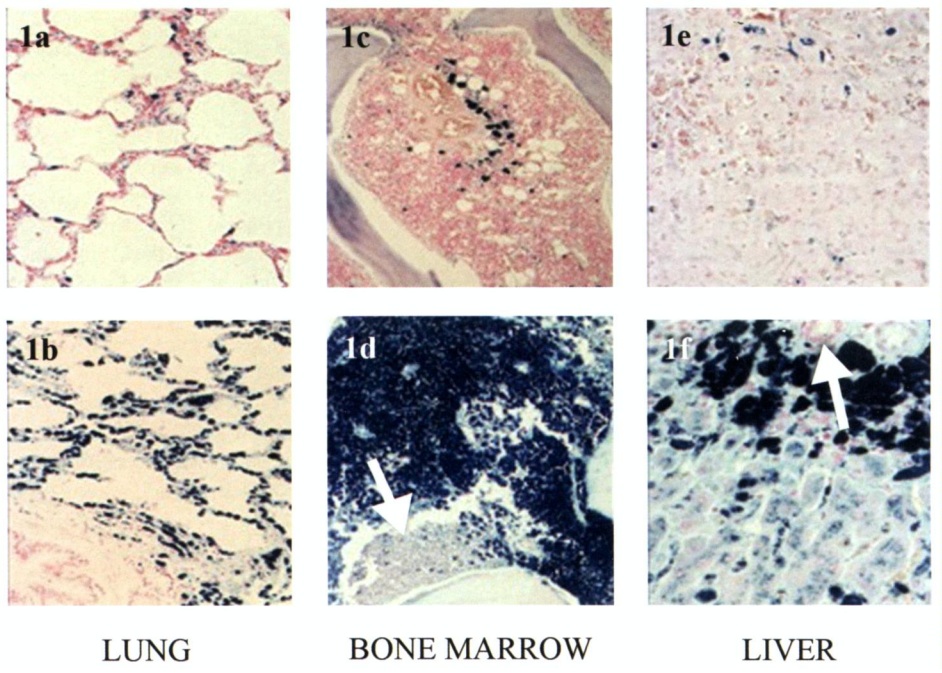

Figure 2. Comparative histopathology of lung (100×), bone marrow (100×), and liver (400×) between *C. simum* (upper row) and *D. bicornis* (lower row) of comparable ages and time in U.S. captivity. Perl's Prussian blue stain for ferric iron. Scattered pulmonary alveolar-wall macrophages contain minimal iron in *C. simum* (1a), whereas *D. bicornis* macrophages are hyperplastic with heavy hemosiderin deposition (1b). Hematopoietic tissue dominates the fatty marrow of *C. simum* (1c) but is replaced in *D. bicornis* (1d) and compressed into a small residual island against a bone spicule (arrow) by confluent sheets of hemosiderin-laden macrophages. In *C. simum* liver (1e), only scattered macrophages and Kupffer cells react positively with Perl's Prussian blue stain, compared to *D. bicornis* liver (1f), which shows excessive ferric iron in parenchymal cells with hepatocellular degeneration and necrosis, in addition to dense hemosiderin deposits in Kupffer cells and portal macrophages. Arrow indicates bile duct.

Role of ISD in multiple disorders of affected rhinoceroses

With evidence strongly supporting development of ISD in browser rhinoceroses, a number of hypotheses were proposed for consideration by the St. Louis conference attendees attempting to relate iron overload to other disorders affecting *D. bicornis* and *D. sumatrensis*.[53] These ranged from logical and probable to imaginative and highly speculative but all were based on the known deleterious effects of highly reactive free radicals produced by reduction of oxygen in respiring organisms.

Ions of transition metals such as iron can catalyze hydroxyl-free radical production, via Fenton and Haber–Weiss reactions, or act as free radicals themselves by accepting or donating electrons. This toxicity is reduced or eliminated by complexing free iron to high-affinity apoproteins that have evolved for its uptake, transport, and storage. In reactions with carbohydrates, lipids, proteins, or nucleic acids, additional free radicals may be formed, resulting in chain reactions that amplify their destructive effects. Collectively, these induce injury at multiple cellular and subcellular sites producing membrane lipid peroxidation, protein-bond disruptions, and nucleoprotein alterations among other consequences. This has been extensively and authoritatively reviewed by others.[6,17,21,32]

Infectious diseases: It is axiomatic that excess iron stores compromise an important first-line defense mechanism of vertebrates. So-called "nutritional immunity" relies on sequestration of host iron in protein complexes to deprive potential pathogens of this biologically essential cation.[28,72,82–84] Susceptibility to infections of all kinds is significantly increased in iron-loaded subjects, and most microorganisms thrive and become more virulent in high-iron environments, both in vitro and in vivo. In addition, phagocytic and bactericidal properties of polymorpho- and mononuclear leukocytes are known to be diminished by iron excess in vitro and in vivo, and these

impairments may be caused by, or in addition to, alterations in myeloperoxidase activity. In rhinoceroses, reduced activities of heme enzymes such as erythrocyte catalase[50,63–65,67,70] include leukocyte myeloperoxidase (Lehrer and Paglia, unpubl. data), which would be expected to impair phagosome microbicidal activity and further contribute to reduced resistance to infectious agents.

In view of the enormous body of literature on this subject, it seems inescapable that severe iron overburdens of ISD contribute significantly to the high incidence and virulence of tuberculosis in rhinoceroses as it does in humans,[41] as well as *Aspergillus* and other fungal pneumonias, and infectious diseases such as leptospirosis and salmonellosis, that have been commonly observed in *D. bicornis.*[35]

Hemolytic anemia: The potential for a cause-and-effect relationship between ISD and acute or chronic hemolytic anemia has been extensively explored. Before rhinoceros ISD was accepted as a distinct pathologic entity, hemolytic disease had been widely viewed as the most likely explanation for extensive tissue iron deposition. The converse possibility that excess iron might be the *cause* rather than *the consequence* of premature red cell destruction was suggested by Dennis (pers. comm., 1997),[59,61] but this hypothesis lost credence as preventative measures were instituted and hemolytic episodes abated to rarities, while ISD has continued to increase progressively in both incidence and severity.

Two comprehensively documented bodies of knowledge make it probable that ISD can contribute directly to hemolytic disease: the high susceptibility to oxidant-induced hemolysis characteristic of all rhinoceros erythrocytes, and the biochemical mechanisms responsible for the high toxicity of free iron (vide supra). Mammalian erythrocytes exposed to iron-catalyzed oxidant stress lose both ATP and the sacrificial reductant glutathione, forming increased amounts of membrane lipid peroxides, methemoglobin, and membrane-bound hemichrome degradation products of hemoglobin. The latter are identifiable microscopically as Heinz bodies, which are normally present in 10–20% of rhinoceros erythrocytes but markedly increase in response to oxidant challenge.

The extraordinary dearth of ATP in rhinoceros red cells leaves little reserve to fuel the cation pump and prevent a hemolyzing influx of water. The efficacy of phosphate supplementation to prevent or treat hemolytic episodes in rhinoceroses was based on the well-known secondary effects of hypo- and hyperphosphatemia in humans, conditions that are accompanied, respectively, by decreased and increased intracellular concentrations of erythrocyte ATP. This strategy of induced hyperphosphatemia has effectively interdicted active hemolytic episodes in three black rhinoceroses at different U.S. zoologic institutions.[51,52] (Paglia et al., unpubl. data.)

Nonhemolytic anemias: Precipitous drops in circulating red cell mass in the absence of hemolysis led to recognition of another serious condition in captive black rhinoceroses, idiopathic hemorrhagic vasculopathy syndrome (IHVS).[40,45] These cases were clustered temporally and geographically and may have involved cold agglutinins, but their etiology was never confidently established.

Other forms of chronic *non*hemolytic anemia affect both African black and Sumatran rhinoceroses. We have observed dense sheets of hemosiderin-laden macrophages in bone marrow sufficiently extensive to displace hematopoietic elements and induce myelofibrosis, possibly resulting in a form of myelophthisic anemia or pancytopenia. Other cases have been associated with progressive weight loss, cachexia, and necropsy evidence of severe, but clinically occult, necrotizing pneumonia and chronic renal disease or both (Miller, Ball, Meehan, and Paglia, unpubl. data); these findings are similar to those reported by others.[44] It seems highly likely that some of these cases of chronic anemia represent examples of the so-called anemia of inflammation or anemia of chronic disease (vide infra).

Leukoencephalomalacia: Strong circumstantial evidence has been presented linking ISD with leukoencephalomalacia, a lethal CNS myelin degenerative disorder afflicting four female black rhinoceros calves born in U.S. zoos.[53,61] By all assessments, quantitative assays of sera and tissues and evaluation of necropsy histopathology, affected kindreds included many with the most profound levels of iron overburden ever recorded in any rhinoceros species. The dams in all four instances had serum ferritin concentrations ranging from >10,000 to >30,000 ng/ml and transferrin saturations of 90–100%, far above the groups shown in Table 1. Two of the dams and a granddam exhibited hepatocellular degeneration with regenerative nodularity and bile duct proliferation, and one had overt cirrhosis with a low-grade hepatocellular carcinoma, all late-stage hallmarks of classical hemochromatoses.

Evidence supported the transplacental passage of maternal iron analytes, prompting speculation that massive maternal iron loads might play some

pathogenetic role by catalyzing production of highly toxic hydroxyl-free radicals and other reactive oxygen species during crucial periods of CNS development.[53,61] This hypothesis was supported by the known contributions of excess iron to other neurodegenerative disorders such as Friedreich's ataxia in humans.[32]

Other disorders: Additional hypotheses have been proffered regarding potential connections between ISD and other high-incidence disorders in browser rhinoceroses. Because ISD involves the entire reticuloendothelial system, vascular and sinusoidal lining cells of multiple organs are also demonstrably affected. It seems plausible that endothelial cell damage could contribute to IHVS, considered by many to be basically a defect of microvascular integrity, perhaps with an autoimmune component. Similarly, microvascular deficiencies might also contribute to the mucocutaneous ulcerations of chronic necrolytic dermatopathy. As yet, there is no definitive evidence to support such hypotheses for either disorder, but these speculations nonetheless provide intriguing challenges for future research.

Etiology of ISD in browser rhinoceroses

The precise cause of ISD in rhinoceroses remains unknown but continues under intensive investigation. Because the extraordinary biochemical and metabolic features of rhinoceros erythrocytes are also shared by other perissodactyls, they must be viewed as normal characteristics rather than individual pathologic anomalies. This carries the corollary that certain species occasionally select evolutionary alternatives that have been rejected by most other vertebrates. Hypotheses based on such perspectives may have special merit.[8]

Browsing mammals emerged during the Oligocene epoch when bioavailability of many essential metals was low. This may have favored avid physiologic mechanisms for iron uptake without development of counterbalancing excretory pathways. When grasslands became extensive during the subsequent Miocene, grazers began to evolve with their more specialized dietary regimen aided by feedback control mechanisms to prevent excessive iron accumulation. This has led to one current hypothesis, originally suggested by Dr. Smith,[76,77] that browser rhinoceroses regulate iron by selecting forage with natural chelators to reduce its bioavailability. Such animals are consequently dependent on access to certain botanical species in their native habitats to avoid developing iron overload.

The authors' current working hypothesis is that brower rhinoceroses (and other species that acquire ISD in unnatural environments) lack some component of the molecular mechanisms that normally orchestrate iron balance in vertebrates. Because *all* individuals in the two affected species are susceptible (and none in the other two is), it is difficult not to suspect genetic predispositions. A gene defect in *HFE* like those in human hereditary hemochromatosis has been eliminated,[2] but numerous other candidates remain.[15]

It seems fortuitous (and ironic) that a long period of relative indifference to ISD in rhinoceroses has coincided with a renaissance of interest in iron homeostasis. This was engendered by a recent series of elegant investigations revealing the crucial roles of hepcidin and ferroportin in maintaining iron balance.[14,47,48] Hepcidin, a 25-amino acid antimicrobial peptide, has been identified as the principal hormone controlling plasma iron levels by its interaction with ferroportin, the membrane egress channel for intracellular iron. Hepcidin deficiency and abnormalities of its interaction with ferroportin are now recognized as the molecular lesions causing most iron overload states in humans and other animals.

Conversely, overproduction of hepcidin blocks dietary iron uptake and mobilization of iron from macrophages and hepatocytes, sequestering iron in the storage pool and impairing erythropoiesis. This effectively produces a form of iron-deficiency anemia, even in the presence of increased iron stores, resulting in the anemia of chronic or inflammatory disease, another disorder frequently seen in rhinoceroses. Collaborative studies are currently testing the hypothesis that ISD and some chronic anemias in rhinoceroses might be caused by anomalous interactions of hepcidin, ferroportin, and their modulators.[15,24]

DISCUSSION

All of the studies reviewed here are mutually supportive in confirmation of a pathologic ISD developing rapidly and inevitably in browser rhinoceroses when displaced from their natural habitats. At birth, calves born in captivity exhibit marked elevations in serum ferritin concentrations that return within days to normal reference ranges. These neonatal spikes may represent acute-phase reactions under duress of birthing, or they may be a reflection of maternal iron loads or both. In as little as 3–4 yr, body burdens of iron in captive *D. bicornis* can elevate 10-fold or more. This occurs even more rapidly in *D. sumatrensis*, and iron continues to accumulate in both species

directly or logarithmically relative to their periods of confinement.[27,53,54,59–61,77]

Development of ISD can be prevented in calves and juveniles (and ameliorated in animals already affected) through the simple, but resource-demanding expedient of serial phlebotomies.[1,55] This preventative and therapeutic procedure has long been the standard of practice for human patients with hereditary hemochromatosis,[1,4,17,33] and it has been attempted and/or instituted successfully at several zoologic institutions in the U.S. Standing phlebotomy of trained rhinoceroses has been developed into a sophisticated and practical procedure by the animal health teams at Disney Animal Kingdom.[46]

Even though rhinoceroses are encumbered by a number of inherent metabolic disadvantages (most importantly, impaired capacities to protect against free-radical redox reactions), they have survived and thrived since the Eocene until imperiled more recently by poaching, habitat encroachment, and now by chronic progressive ISD. Despite uncertainties regarding the relation of ISD to other clinical disorders, the toxic biologic effects of excess iron remain unequivocal in every species or biologic system so far studied. Acquired ISD therefore represents a "clear and present danger" to a multitude of exotic species and deserves the attention of all involved in veterinary medicine and wildlife preservation.

This confronts conservationists with the difficult dilemma of whether it is ethically and practically justifiable to preserve gene pools of endangered species by dislocating them from their natural habitats into protected environments, even if captive conditions induce pathologic disorders of high morbidity and mortality. A companion dilemma has been previously addressed as to whether it is justifiable to recolonize depleted wildlife habitats with offspring of captive breeding programs when they are known to have clinical conditions (e.g., ISD with compromised immunity) that further endanger them or the indigenous populations or both.[49] At present, there is no clear consensus on these questions, but such controversies remain healthy stimulants for further consideration and future research.

In retrospect, it seems remarkable that two full decades have passed since the seminal studies of Smith et al.[76,77] and Kock et al.[26,27] were originally introduced, culminating now in the wealth of data presented in this 2011 International Workshop on ISD in Rhinoceroses. Perhaps such inertia is merely an example of Max Planck's observation that "*A new scientific truth does not triumph by convincing its opponents and making them see the light, but rather its opponents eventually die, and a new generation grows up that is familiar with it.*"[85]

Until recently, ISD research has generated ambivalent interest and restrained grant support from veterinary and wildlife agencies, regardless of its serious implications for a wide range of different species.[9] Despite compelling evidence, independent confirmation, and extensive corroboration, the significance of iron accumulation in rhinoceroses somehow still remains controversial. Some continue to consider ISD an epiphenomenon related to one or more of the many other conditions known to affect rhinoceroses in captivity. Others have viewed it as a hypothetical consequence of "metabolic disturbances" that remain conceptually obscure and ill defined. Burdens of proof now reside with the skeptics.

Shelden, credited with synthesizing the diverse clinical and pathologic findings in human hereditary hemochromatosis into a coherent entity,[74] has perceptively and presciently suggested that an inborn error of metabolism might be the basis of this disorder, simultaneously accepting that his proposal might not have lasting merit: "*... but as with other scientific hypotheses, its justification [would] lie in the acquisition of any new knowledge entailed by its destruction.*"[74] The foregoing observations, conclusions, and speculations on ISD and its potential role in the etiology and pathogenesis of disorders affecting browser rhinoceroses are offered with identical intent. The data and ideas presented in this *September 2012 Supplement Issue of the Journal of Zoo and Wildlife Medicine* should be critically analyzed so that they might serve as an appropriate point of departure for the next quantum leap in related research. Victor Hugo provided the ultimate encouragement: "*Nothing else in the world is so powerful as an idea whose time has come.*"[86]

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