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IRON METABOLISM IN CAPTIVE BLACK (*DICEROS BICORNIS*) AND WHITE (*CERATOTHERIUM SIMUM*) RHINOCEROSES

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Abstract: Black rhinoceroses that are kept in captivity have increased amounts of hemosiderosis as determined microscopically. The objective of the present study was to measure various iron analytes in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. Nonheme iron was measured in liver samples, and iron, total iron binding capacity, percentage of transferrin saturation, and haptoglobin were measured in serum samples. Black rhinoceroses ($n = 16$), but not white rhinoceroses ($n = 9$), accumulated iron in the liver during captivity. Serum iron concentration and percentage of transferrin saturation were increased in black ($n = 40$) compared to white rhinoceroses ($n = 13$). An enzyme-linked immunoabsorbent assay used to measure ferritin in equine sera was adapted to measure ferritin in rhinoceros sera. Serum ferritin concentration of black rhinoceroses was significantly higher ($t = 4.75$, $P < 0.001$) than that of white rhinoceroses and increased significantly ($r = 0.43$, $P = 0.0134$) with time in captivity. Because haptoglobin levels did not differ significantly between black ($n = 20$) and white ($n = 10$) rhinoceroses, the hypothesis that iron accumulates as a result of a hemolytic anemia is less likely. Alternatively, the hypothesis that a dietary change increases iron absorption may explain these results.

Key words: Black rhinoceros, *Diceros bicornis, Ceratotherium simum,* iron, haptoglobin.

INTRODUCTION

Black rhinoceroses (*Diceros bicornis*) are disappearing rapidly under free-ranging conditions. They are being killed by poachers for their horns, which are mistakenly believed to have medical properties. In an effort to protect the species from extinction, some black rhinoceroses have been translocated to large ranches and zoos. Unfortunately, when animals are moved from their natural habitat, they can be affected with disorders that do not occur normally. In the case of black rhinoceroses, an acute hemolytic anemia, with a mortality of greater than 70%, has been devastating.[11] The incidence of this hemolytic syndrome in free-ranging rhinoceroses is unknown. Furthermore, hemosiderosis has been seen in zoo-exhibited and translocated (but not in recently captured or caught) black rhinoceroses.[7] This observation suggests that aberrant iron metabolism occurs when black rhinoceroses are maintained in captive environments. The relationship of hemosiderosis to the acute hemolytic anemia syndrome is also unknown. The absence of hemosiderosis suggests that the syndrome does not occur in free-ranging black rhinoceroses.[7] Iron accumulates when the amount absorbed is greater than the amount that is lost from the body. Because most mammals do not have an effective method for excreting iron, iron accumulation is almost always a result of increased absorption.[15]

Monitoring the amount of stored iron in captive black rhinoceroses may be important. Limited methods are available to measure iron stores. Direct methods include measuring nonheme iron concentration in the liver, bone marrow, and spleen; quantitating iron mobilized by phlebotomy; or assessing chelator-induced urinary iron excretion.[22] The difficulty of obtaining appropriate samples from rhinoceroses precludes the use of these methods. A logical indirect

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analyte of iron stores should be serum iron. Unfortunately, serum iron level is influenced by factors unrelated to stored iron.[15] For example, serum iron concentration increases in dogs and horses when they are administered corticosteroids.[16]

Although ferritin is the iron-storage molecule in tissues, some ferritin occurs in serum and is correlated positively with stored iron levels in man,[8,14] pigs,[18] dogs,[24] cats,[1] and horses.[19] Thus, serum ferritin concentration can be used to estimate total stored iron in those species.

Antibodies to ferritin are usually species-specific (e.g., an antibody to human ferritin does not cross-react with canine ferritin).[25] Therefore, in order to quantitate serum ferratin by immunological techniques, ferritin must be isolated, and a specific antibody made for each new species. Unexpectedly, the antibody to equine ferritin was found to bind to ferritin from rhinoceroses, and the test used to measure ferritin from rhinoceroses, and the test used to measure ferritin in equine sera could be used for rhinoceros sera.

The present study investigated iron metabolism in black and white (*Ceratotherium simum*) rhinoceroses that have been maintained in captivity.

MATERIALS AND METHODS

Aliquots (50 g) of liver were obtained from black and white rhinoceroses that died or were euthanized in U.S. zoos under the auspices of the Black Rhinoceros Species Survival Plan (SSP) and the Rhinoceros Taxon Advisory Group. Serum samples were obtained opportunistically from rhinoceroses that were phlebotomized as part of husbandry programs or medical procedures. Serum was handled in a manner that avoided contamination with exogenous iron. Samples were shipped frozen to Kansas State University by overnight carrier and remained frozen until analysis.

Serum iron and total iron binding capacity were measured coulometrically.[20] The percentage of transferrin saturation was calculated by dividing the serum iron value by the serum total iron binding capacity and multiplying by 100. Hepatic nonheme iron was measured in triplicate by the method of Torrance and Bothwell,[23] except iron was determined coulometrically. Iron content was calculated from the supernatant iron concentration assuming a water content of 75%.[2] Serum haptoglobin was measured spectrophotometrically.[9]

Ferritin was isolated from rhinoceros liver by a previously described method,[17] and its concentration was determined by bicinchoninic acid protein assay with a bovine serum albumin standard. A double diffusion assay was performed by the Ouchterlony method with 1.0% agarose. Anti-equine ferritin antibody was placed in the center well, and ferritin purified from liver of horses, pigs, and black and white rhinoceroses was put in the outer wells. Precipitin lines were visualized and photographed without staining.

Serum ferritin concentration was measured by an enzyme-linked immunoabsorbent assay (ELISA) developed for equine sera, except that ferritin isolated from black rhinoceros liver was used as the standard.[19] Anti-equine ferritin (affinity-isolated) antibody (F-6146) was purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

The results of iron analytes for black and white rhinoceroses were compared with a student's t-test. Hepatic nonheme iron was correlated to the rhinoceros's age with a Pearson product-moment correlation coefficient.[21]

RESULTS

Hepatic nonheme iron was significantly higher ($t = 2.17$, $P < 0.05$) in black rhinoceroses (2.96 mg/g $\pm$ 0.661 [SE], $n = 23$) than in white rhinoceroses (0.597 $\pm$ 0.113 mg/g, $n = 9$). When hepatic nonheme iron was correlated with the animal's age, a significant age relationship ($r = 0.543$, $n = 18$, $P < 0.05$) was seen in black (Fig. 1) but not white rhinoceroses ($r = 0.276$, $n = 9$, $P > 0.05$). The black rhinoceroses died or were

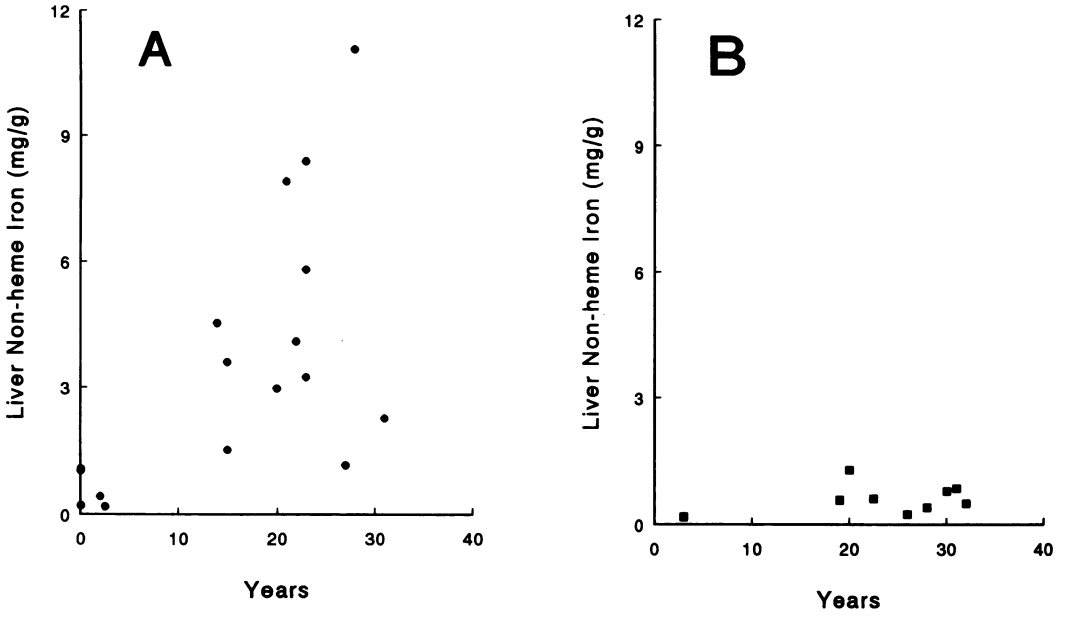

Figure 1. The relationship of liver nonheme iron to age or time in captivity in black **(A)** and white **(B)** rhinoceroses. (N = 18 for black rhinoceroses, N = 9 for white rhinoceroses.

euthanized with a variety of conditions, including hemolytic crisis, blood loss, surgery, tuberculosis, traumatic brain injury, strangulation, sepsis with *Escherichia coli,* traumatic rupture of the liver, liver failure, and unknown causes. The white rhinoceroses died or were euthanized with tuberculosis, neoplasia, surgery, and unknown causes. Livers of three black rhinoceroses of unknown age that had been recently translocated from Zimbabwe as adults were available for assay. The nonheme iron content for these livers was 0.474 ± 0.142 mg/g.

Serum iron concentration and the percentage of transferrin saturation were significantly higher in black than in white rhinoceroses (Table 1). Serum iron levels did not correlate significantly with age for either black or white rhinoceroses. Serum total iron binding capacity and haptoglobin levels did not differ significantly between black and white rhinoceroses.

The lines of precipitate were seen between anti-equine ferritin antibody and the ferritin isolated from the liver of horses, white rhinoceroses and black rhinoceroses but not porcine ferritin (Fig. 2). Ferritin isolated from dogs and cats also did not cross-react with anti-equine ferritin (data not shown). Precipitate lines from black and white rhinoceroses were confluent, indicating that the two antigens were identical. The precipitate lines for equine ferritin and both types of rhinoceros merged with a spur, indicating

Table 1. Serum iron analytes in black and white rhinoceroses.

Serum analyte	Rhinoceros		P
	Black	White	
Iron (μg/dl)	614 ± 200[a] (40)[b]	187 ± 37.2 (13)	<0.05
TIBC[c] (μg/dl)	731 ± 183 (40)	401 ± 45.6 (13)	NS
Saturation[d] (%)	67.5 ± 3.7 (40)	43.4 ± 3.4 (13)	<0.01
Haptoglobin (mg/dl)	52.9 ± 7.21 (26)	48.4 ± 8.80 (10)	NS

[a] Mean ± standard deviation.
[b] Number in parentheses indicates the number of animals studied.
[c] TIBC = total iron binding capacity.
[d] Saturation = (Iron/TIBC) × 100.

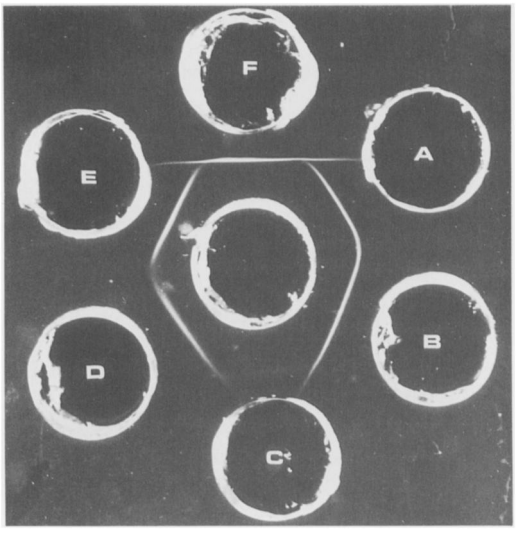

Figure 2. Ouchterlony tests of anti-equine ferritin antibody (center well) with purified ferritin from the liver of a horse (F), white rhinoceros (B & E), black rhinoceros (A & D), and pig (C).

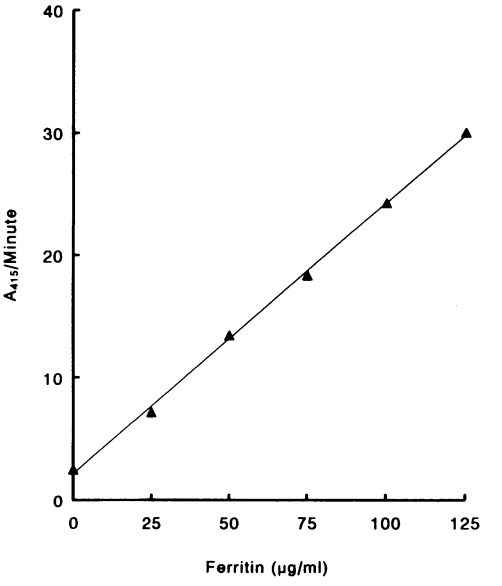

Figure 3. Standard curve for hepatic ferritin isolated from black rhinoceroses assayed with an anti-equine ferritin antibody.[19]

partial identity but suggesting that some epitopes present in the equine ferritin were not present in the rhinoceros ferritin.

Absorbance increased linearly with increased ferritin concentration (Fig. 3) when rhinoceros ferritin was used in an ELISA. Considerable variation was seen in the level of ferritin measured in sera of rhinoceroses (Table 2). Two samples had obviously aberrant results compared to other results in the respective species. Serum ferritin values were 762 µg/dl in one black rhinoceros and 28 µg/dl in one white rhinoceros. These samples were retested several times to determine the appropriate dilution. The values were classified as extreme outliers[12] because they were 79 and 39 trimmed standard deviations above the nearest value for the respective species. When the data from those two animals were removed, serum ferritin concentration was significantly higher ($t = 4.75$, $P < 0.001$) in black than in white rhinoceroses (Table 2). Serum ferritin levels in black rhinoceroses increased significantly ($r = 0.43$, $n = 32$, $P < 0.05$) with time in captivity (Fig. 4).

All of the individual results are available from Dr. R. Eric Miller as part of the SSP program.

DISCUSSION

Iron is essential for all living cells. Although it is the second most abundant metal in the Earth's crust, its availability to most organisms is limited because it cannot be extracted easily from its most common form, insoluble ferric oxide. As a consequence, most organisms have developed systems to efficiently extract iron from their environment, to protect themselves from adverse effects of the free cation, and to tenaciously conserve it. In contrast, mammals do not have significant mechanisms to eliminate excess iron.[15]

Both histologic studies[7] and chemical analysis (this study) indicate that in black rhinoceroses the amount of iron stored increases when they are kept in captive environments. The accumulation of iron (Table 1 and Fig. 1) and the acute hemolytic anemia observed in black rhinoceroses do not seem to occur in white rhinoceroses. However, that does not indicate a cause-

Table 2. Serum ferritin values (mean ± SEM) in black and white rhinoceroses measured with an ELISA using an anti-equine ferritin antibody.

	Black rhinoceroses (μg/ml)	White rhinoceroses (μg/ml)	*P*
All samples	29.7 ± 21.0[a] (36)[b]	3.39 ± 2.80 (10)	NS
Minus highest samples	8.70 ± 1.70 (35)	0.590 ± 0.242 (9)	<0.001
Living animals	7.75 ± 1.56 (20)	0.308 ± 0.076 (4)	<0.001

[a] Mean ± standard deviation.
[b] Number in parentheses indicates number of animals studied.

and-effect relationship between these two abnormalities.

Cross-reactivity of the anti-equine ferritin antibody with rhinoceros is usually species-specific.[24] Cross-reactivity allowed us to measure serum ferritin in rhinoceroses with an assay developed for equine sera by substituting rhinoceros ferritin for equine ferritin as the standard. It was not determined if serum ferritin in the rhinoceroses was correlated with body stores of iron. A limited number of samples were available, and most of the rhinoceroses had medical disorders that could have altered serum ferritin or liver iron concentrations. Because serum ferritin level is correlated with the amount of iron stored in the liver and spleen of several species,[1,8,14,18,19,24] including the equine assay used in this study, serum ferritin concentration should be an indicator of total body stores of iron in rhinoceroses.

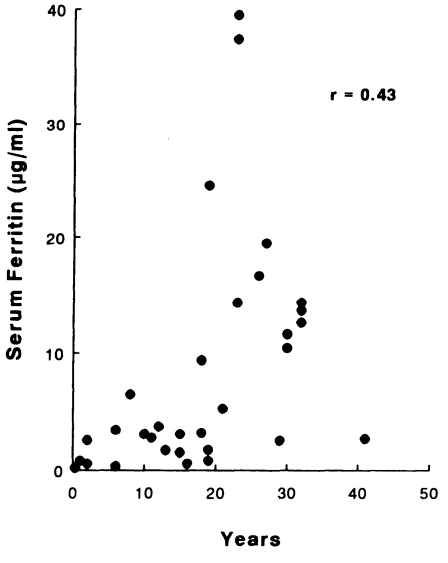

Figure 4. Relationship of serum ferritin to age in black rhinoceroses.

Serum ferritin levels can increase in conditions unrelated to the amount of stored iron; e.g., serum ferritin concentration increases during acute infections as part of the acute-phase response and during sever hepatic disease.[13] These conditions may complicate the interpretation of serum ferritin results from rhinoceroses. The samples were obtained from rhinoceroses that had died or were treated for medical conditions. Nevertheless, if samples from live rhinoceroses are compared, black rhinoceroses have significantly higher levels ($t = 19.2$, $P < 0.001$) of serum ferritin than white rhinoceroses (Table 2), and these levels increased with time in captivity. That change did not occur in white rhinoceroses (data not shown).

Two mechanisms could account for iron accumulation. First, more iron may be available in the vegetation fed in captivity than in that available under free-ranging conditions. White rhinoceroses occupy a grassland savannah, have broad lips, and graze on short grasses. In contrast, black rhinoceroses browse on forbs, shrubs, and trees. The low quality of the black rhinoceroses' forage necessitates that they spend

most of their time gathering food. When black rhinoceroses are translocated, their diet is gradually changed to alfalfa or grass hay.[6] That change may increase the amount of iron available for absorption. The inorganic iron content of the black rhinoceros browse does not differ significantly from that of clover or rye hay.[5] Unfortunately, inorganic iron bioavailability cannot be predicted from iron content because iron absorption is inhibited by tannates, phosphates, and other metals and is enhanced by ascorbate.[4]

Second, iron absorption can increase with increased erythropoietic activity such as that seen with chronic hemolytic anemia. For example, Basenji dogs with pyruvate kinase deficiency accumulate large amounts of iron in the liver and bone marrow. Bone marrow accumulation of iron may cause myelodysplasia.[26] Although iron overload can occur in human patients with chronic anemia treated with numerous transfusions, it can also occur in patients with thalassemia major or congenital dyserythropoietic, pyruvate kinase deficiency, and hemolytic glucose-6-phosphate dehydrogenase anemias who have not received transfusions.[3] This phenomenon has been produced in dogs with experimentally induced hemolytic anemia.[10] If the hemolytic anemia observed in black rhinoceroses occurs in a chronic, less-severe form, iron absorption should be increased.

When erythrocytes are hemolyzed intravascularly, the free hemoglobin is bound to haptoglobin. The haptoglobin–hemoglobin complex is cleared from the blood by hepatocytes. A lower concentration of serum haptoglobin occurs in chronic anemias and would be expected to occur in black rhinoceroses if hemolytic anemia was chronic. Because the serum haptoglobin concentration in black rhinoceroses does not differ from that in white rhinoceroses, this mechanism is less likely. Four black rhinoceroses and one white rhinoceros were found to have no measurable haptoglobin. Thus, haptoglobin can decrease in both black and white rhinoceroses, which opens the question of whether white rhinoceroses also have an undetected hemolytic anemia.

Iron accumulates in the liver of black, but not white, rhinoceroses kept in captivity. Dietary intake of iron may be more important than chronic hemolytic anemia in the pathogenesis of this generalized hemosiderosis.

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