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EVALUATION OF SERUM FERRITIN AND SERUM IRON IN FREE-RANGING BLACK RHINOCEROS (*DICEROS BICORNIS*) AS A TOOL TO UNDERSTAND FACTORS AFFECTING IRON-OVERLOAD DISORDER

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Abstract: Iron overload disorder (IOD) is a significant health issue for captive black rhinoceros (*Diceros bicornis*). Measurement of serum ferritin with a validated rhinoceros ferritin ELISA has been used extensively to detect animals in U.S. zoos that are at risk of developing IOD. However, there is limited information on serum ferritin levels in free-ranging black rhinoceros using this same assay. Serum ferritin, iron, and gamma-glutamyl transpeptidase (GGT) were determined in 194 black rhinoceros from southern Africa. Mean ferritin in free-ranging black rhinoceros (290.54 ±247.4 ng/ml) was significantly higher than in free-ranging white rhinoceros (64.0 ± 102.4 ng/ml) sampled in this study from Kruger National Park, South Africa. However, there were no significant differences between genders or age groups. Ferritin values varied with geographical location of the black rhinoceros, although this was not clinically significant. Serum iron values were also higher in black rhinoceros (40.4 ± 19.1 μmol/L) compared to white rhinoceros (29.7 ± 10.7 μmol/L). There was no association between ferritin and GGT. This study provides serum ferritin, iron, and GGT values from free-ranging black rhinoceros that can be used for as comparative target values for captive animals.

Key words: Black rhinoceros, *Diceros bicornis*, iron overload disorder, serum ferritin, serum iron

INTRODUCTION

The role of iron in diseases of black rhinoceros (*Diceros bicornis*) has been the subject of research for several decades.[5,19,23] Iron metabolic imbalances have been implicated in leukoencephalomalacia, hemolytic anemia, hepatopathy, and chronic nonhemolytic anemia.[19] Hemosiderosis is a common postmortem finding in multiple tissues from captive adult black rhinoceroses.[17] Iron overload disorder (IOD, formerly iron storage disease) is considered a significant concern in browsing species of rhinoceros, including the endangered Sumatran rhinoceros (*Dicerorhinus sumatranesis*).[23] This has led to speculation that dietary factors in captivity may play a role in increased iron absorption.[6] An alternative hypothesis is that there is a genetic predisposition to iron accumulation in these species.[1] Despite research into etiology, diagnosis, and treatment, a significant proportion of the captive black rhinoceros population continues to be affected by IOD.

Captive black rhinoceros have significantly elevated serum iron and transferrin levels compared to free-ranging eastern black rhinoceros.[20] Rhinoceros appear to accumulate iron over time in captivity.[25] Although IOD has not routinely been observed in free-ranging rhinoceros, hemosiderosis has been reported in recently captured boma-confined black rhinoceros.[14] This presents an additional health risk to already highly endangered black rhinoceros.[13] Since conservation of black rhinoceros in many range countries is dependent on relocation of animals to private sanctuaries, conservancies, and fenced areas within parks, confinement for transport or placement in bomas for adaptation, protection, or treatment has become more commonplace. However, more information is needed on blood profiles of captive and wild black rhinoceros to assess health status. The aims of this study were to compare serum

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ferritin and serum iron levels in free-ranging black and white rhinoceros and determine whether values varied based on age, gender, and geographic location. This paper reports the results of this evaluation and the potential influence of demographic and geographic factors on these measurements.

MATERIALS AND METHODS

Study population

Banked serum samples were available from 194 black rhinoceros and 32 white rhinoceros immobilized between 1997 and 2013 for translocation, transmitter placement, or other routine field procedures. Among the black rhinoceros population, there were 11 calves (ages 0 up to 2 yr), 26 juveniles (2 up to 5 yr), 62 subadults (5 up to 7 yr), and 88 adults (7 yr and older), with 7 of unknown age. There were 93 female and 100 male black rhinoceros (1 unrecorded gender), and 10 female and 22 male white rhinoceros. White rhinoceros samples were only obtained from subadult ($n = 23$) and adult animals ($n = 9$). All white rhinoceros were immobilized in Kruger National Park (KNP), South Africa. Black rhinoceros were sampled from national parks and private reserves throughout South Africa, Swaziland, and Malawi. Boma-confined black and white rhinoceros were sampled in KNP. Locations were grouped by predominant habitat type and geographical proximity into 10 different groups for analyses.

Sample and data collection

All samples were collected by South African National Parks (SANParks) Veterinary Wildlife Services staff according to the Standard Operating Procedures for the Capture, Transportation, and Maintenance in Holding Facilities of Wildlife. This protocol was approved by the SANParks Animal Use and Care Committee. All animals were deemed healthy on physical examination. Immobilization data, gender, age, animal identification, and location were recorded. Blood was collected from the auricular or median radial vein into serum tubes which were transported to the laboratory on ice. Serum was separated by centrifugation at 2,500 rpm for 10 min and pipetted into cryovials for storage at –20 or –80°C until analyses were performed. Ferritin and iron have been shown to be stable for years in frozen sera.[26] Any samples with visible signs of hemolysis were excluded from analyses.

Serum iron analyses

Serum iron analyses were performed by IDEXX Laboratories (Pretoria, South Africa) as previously described.[18] Iron levels were determined with the use of a VITROS 350 Dry Slide Chemistry Analyzer according to the manufacturer's instructions (VITROS Chemistry Reagents by Ortho-Clinical Diagnostics, Johnson-Johnson). The test sample was deposited onto the slide and evenly distributed by the spreading layer to the underlying layers. Iron (as ferric ion) is removed from transferrin at acidic pH and migrates to the reducing layer, where ascorbic acid reduces iron to the ferrous form. The ferrous ion is then bound to the dye and forms a colored complex in the reagent layer. Following the addition of sample, the slide is incubated and the reflection density measured after 1 and 5 min at 600 nm, 37°C. The difference in reflection density is proportional to the iron concentration in the sample.

Serum gamma-glutamyl transpeptidase (GGT) analyses

GGT values were measured using the ABAXIS Large Animal rotor for serum chemistries. Two hundred microliters of serum were placed in the test chamber of the rotor and colorimetric reaction measured by the ABAXIS VetScan2 (ABAXIS, Union City, CA), following manufacturer's instructions. Chemistry values for rhinoceros have been internally validated at ABAXIS (K. Aron, pers. comm.) and published in previous reports.[16–19]

Serum ferritin ELISA

The rhinoceros ferritin assay has been previously described.[25] The procedure is an enzyme-linked immunosorbent assay (ELISA), which measures serum ferritin by the sandwich technique. All laboratory controls and ferritin standards were frozen and transported from Kansas State University to the KNP laboratory to allow appropriate standardization of the assay (required CITES and import/export permits were obtained). All standards and controls were the same as those used in the U.S.-based assay so that direct comparison of values could be made. Conjugated horseradish peroxidase anti-horse ferritin was freshly prepared in the KNP laboratory. Each test plate was incubated with anti-horse ferritin antibody solution. Afterwards, a blocking solution was added and wells washed. Standards, controls, and sample dilutions of sera were tested in

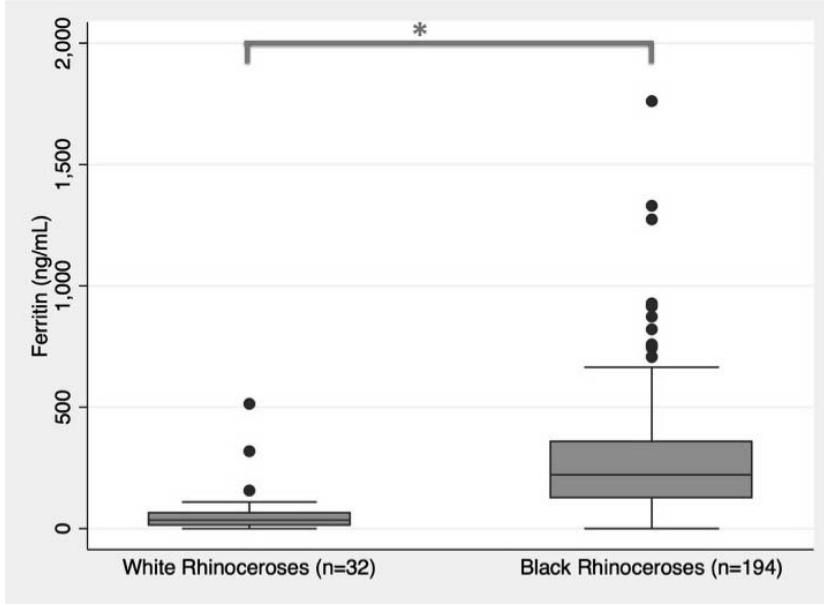

Figure 1. Serum ferritin values (ng/ml) in wild white ($n = 32$) and black ($n = 194$) rhinoceros.

triplicate and incubated at 37°C for 2 hr. After the washing, the anti-horse ferritin conjugated horseradish peroxidase was added and incubated an additional 2 hr at 37°C. Residual conjugate was removed and substrate added. The reaction was read spectrophotometrically at 410 nm. A standard curve was generated with the use of standards and controls with known concentration values. Sample dilutions were compared against the standard curve and values calculated based on formulas generated from the standard curve. Ferritin values are expressed as ng/ml.

Data analyses

The distributions of serum ferritin, serum iron, and GGT values were calculated for black and white rhinoceros sample populations, and then data stratified by age, gender, and geographical location. The T test was used to compare mean ferritin, iron, and GGT values between species and genders, and analysis of variance (ANOVA) for comparison of geographical locations and age categories. Regression analysis with Madikwe-Marakele-Pilansberg as the reference group and pairwise comparison of mean ferritin and iron values were used to evaluate difference between geographical locations The Pearson correlation (rho) coefficient was used to evaluate association between liver enzyme, GGT, and serum ferritin levels.

Records were stored on an Excel sheet and STATA software (StataCorp. 2009. Stata Statistical Software: Release 13. College Station, Texas, USA) was used for the statistical analyses. Data are presented as mean ± standard deviation (SD), unless otherwise stated. Statistical significance was set at $P < 0.05$.

RESULTS

When mean ferritin values were compared between free-ranging African rhinoceros species (*Ceratotherium simum*, *D. bicornis*), black rhinoceros values (290.5 ± 247.4 ng/ml) were significantly higher compared to those from white rhinoceros (64.0 ± 102.4 ng/ml; $P < 0.0001$) (Fig. 1). There was no significant difference in mean serum ferritin values based on age category among black rhinoceros ($P = 0.37$) (Table 1). Among white rhinoceros, only subadult (70.0 ± 119.4 ng/ml) and adult (48.9 ± 32.8 ng/ml) individuals were sampled; however, there was no significant difference in ferritin values between these two age categories ($P = 0.61$).

When mean ferritin values were compared by gender, there was no significant difference observed between female (270.7 ± 242.7 ng/ml) and male black rhinoceros (310.4 ± 252.4 ng/ml; $P = 0.27$). Similar to black rhinoceros, no gender difference was observed in mean ferritin values in white rhinoceros (female 48.2 ± 31.1 ng/ml; male 71.3 ± 122.1 ng/ml; $P = 0.56$).

Table 1. Serum ferritin (ng/ml) and iron (μmol/l) in different age categories of black rhinoceros.

Age category	Ferritin (ng/ml)			Iron (μmol/l)	
	n	Mean	SD	Mean	SD
Calf	11	208.4	181.2	64.6	19.8
Juvenile	26	338.3	340.4	40.9	21.4
Subadult	62	318.3	215.4	36.2	17.4
Adult	88	275.5	248.4	39.7	17.9

The effect of geographical location of sampled rhinoceros on serum ferritin levels was determined. Black rhinoceros were sampled in 10 locations around southern Africa (including South Africa, Swaziland, and Malawi), although white rhinoceros were sampled in only two sites within Kruger National Park (free-ranging, "Kruger" and in bomas, "KNP bomas"). Among black rhinoceros, comparison of mean ferritin values showed a borderline significant difference ($P = 0.074$) between different locations (Fig. 2). Black rhinoceros from Kruger had a lower mean ferritin value (157.8 ± 150.1 ng/ml) compared to black rhinoceros from Madikwe-Marakele-Pilansberg area (281.0 ± 321.8 ng/ml; $P = 0.08$). None of the other locations had significantly different ferritin values in black rhinoceros when compared to animals from the Madikwe-Marakele-Pilansberg area ($P > 0.05$). Black rhinoceros housed in KNP bomas had a mean ferritin of 142.8 ± 93.4 ng/ml, similar to that observed in free-ranging Kruger animals. White rhinoceros ferritin values did not differ between those sampled in bomas or free-ranging in KNP (60.8 ± 117.3 and 68.8 ± 80.2 ng/ml, respectively; $P = 0.83$).

Serum iron values in white rhinoceros (29.7 ± 10.7 μmol/L) were significantly lower than in black rhinoceros (40.4 ± 19.1 μmol/L; $P = 0.0007$). Unlike ferritin, there was a statistically significant difference ($P < 0.0001$) between mean iron values from different age categories of black rhinoceros (between juveniles and calves, subadults and calves, and adult and black rhinoceros calves) (Table 1). Among white rhinoceros, only subadults and adults were sampled and no significant difference in age-related serum iron was observed (subadults 28.6 ± 10.7 μmol/L; adult 32.5 ± 12.6 μmol/L; $P = 0.36$).

Serum iron values were not statistically different between female and male black rhinoceros (42.1 ± 18.4 μmol/L and 38.8 ± 19.9 μmol/L,

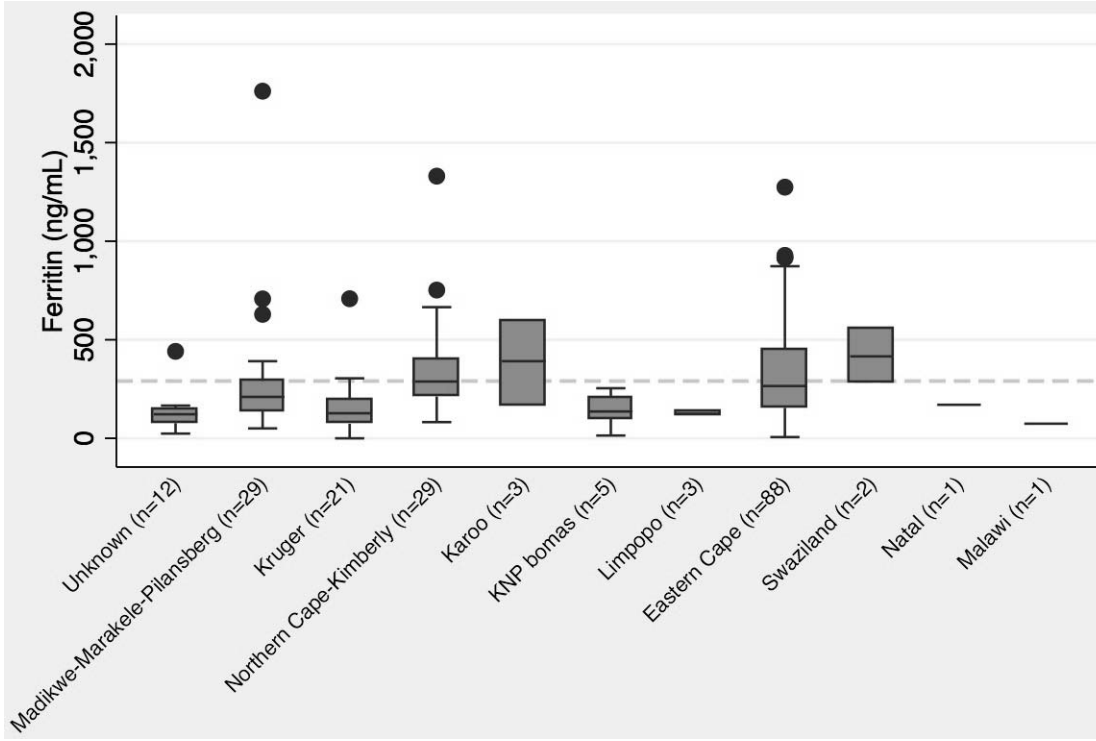

Figure 2. Black rhinoceros serum ferritin values (ng/ml) based on geographical location. Locations are listed with number of samples in parentheses.

respectively; $P = 0.24$). No differences were observed in mean serum iron values between female and male white rhinoceros (31.5 ± 12.3 and 28.9 ± 10.2 μmol/L, respectively; $P = 0.54$).

Serum iron values from white rhinoceros housed in KNP bomas were not statistically different than those in free-ranging individuals (30.2 ± 10.0 and 28.9 ± 12.1 μmol/L, respectively; $P = 0.73$). Serum iron values from black rhinoceros varied by geographical location with the highest mean iron occurring in Madikwe-Marakele-Pilansberg (54.2 ± 20.4 μmol/L), and lowest in rhinoceros from the Eastern Cape (33.3 ± 13.9 μmol/L). When compared to Madikwe-Marakele-Pilansberg, mean iron values in black rhinoceros sampled in Kruger (39.0 ± 19.0 μmol/L), Kruger bomas (40.6 ± 13.8 μmol/L), Northern Cape-Kimberly (38.0 ± 21.2 μmol/L), and Eastern Cape were significantly lower ($P < 0.05$).

Mean serum GGT values were significantly different for white rhinoceros (20.7 ± 5.7 U/L) and black rhinoceros (14.5 ± 4.0 U/L) ($P = 0.0001$). There was no significant correlation between the ferritin and GGT values among white ($P = 0.83$) or black ($P = 0.68$) rhinoceros.

DISCUSSION

Results from this study confirm previous observations that black rhinoceros have higher levels of serum ferritin and serum iron than white rhinoceros.[4,18] A potential explanation is that this may be due to differences in foraging behavior and habitat between the two species. Black rhinoceros are browsers found in open woodland and thorny bush habitats, in contrast to white rhinoceros, which are grazers and prefer open savanna.[19] Iron intake may vary because of differences in iron and iron-binding compounds (i.e., tannins and other plant phenolics) in vegetation.[15] There may also be a genetic basis for the difference between species, with black rhinoceros having a more efficient intestinal absorption of iron as an adaptation to a diet rich in tannins. Interestingly, the mean ferritin values in free-ranging black rhinoceros (range of means for juveniles, subadults, adults 275.49–338.27 ng/ml) from this study were considerably lower than those reported in captive black rhinoceros (juveniles 1,187 ± 1,189 ng/ml; adults 7,160 ± 9,784 ng/ml).[24] Ferritin values may be due to different diets, management factors including chronic inflammation or stress associated with captivity, concurrent disease, hemolysis of samples, and environmental exposure to iron (soil, water).[26]

There were no clinically or statistically significant differences in serum iron values between female and male rhinoceros. Similarly, serum ferritin levels were not affected by gender. Unlike captive black rhinoceros, in which serum ferritin levels appear to increase with age, there was no change in serum ferritin levels based on age category in this study of free-ranging black rhinoceros.[25] However, mean serum iron levels were significantly higher in calves as compared to juvenile, subadult, or adult rhinoceros after adjusting for location.

The mammalian fetus acquires iron through transplacental transfer and is born with hepatic iron stores to meet the demands of rapidly dividing cells and erythrocyte production, which may explain the higher serum iron concentration observed in calves.[9] Milk has low iron content, and iron needs to be ingested from other environmental sources (vegetation, soil, water). As animals age, iron stores may be impacted by blood loss associated with parasitism, hemorrhage, or concurrent disease. Free-ranging rhinoceros are typically parasitized with ticks, bots, flukes, and other endoparasites.[19] Acquisition of parasites with age may explain the lower levels of iron found in juvenile, subadult, and adult rhinoceros compared to calves. The lack of change in iron body stores in free-ranging rhinoceros with age may be due to an evolved balance of intake with loss from endemic levels of parasitism. Because captive rhinoceros do not typically carry any significant load of parasites, this may lead to increased body stores over time. Another possible explanation is that iron intake by wild black rhinoceros is lower. Browse species selected by black rhinoceros in Zimbabwe had iron content of 29–215 ppm.[7]

The hypothesis that environment plays a role in IOD was supported by the variation in serum iron and ferritin levels by geographical location of black rhinoceros sampled. A study investigating iron levels in natural black rhinoceros diets across three of the same geographical locations showed significant differences between sites with variable differences between seasons.[8] These sites covered three of the same areas that were included in this study (Karoo, Eastern Cape, and Madikwe-Marakele-Pilansburg). The highest iron content was found in the Karoo (Tswalu Kalahari Reserve, seasonal means 100.2–175.0 ppm) followed by the Eastern Cape (Great Fish River Reserve, seasonal means 76.4–86.1 ppm) and lowest in Madikwe-Marakele-Pilansburg area (Waterburg Plateau Park, seasonal means 49.5–61.2 ppm). The range

of iron dietary content was 49.5 ± 10.9 ppm to 175.0 ± 31.5 ppm. However, the highest level is still significantly lower than dietary iron levels in previously published captive black rhinoceros diets (374 ± 224 ppm).[2] Current dietary concentration recommendations for black rhinoceros are 50–100 mg Fe/kg diet (dry-matter basis).[3] Although iron bioavailability may have a greater effect on iron status than diet composition, it is difficult to measure. There are numerous factors that can impact iron absorption, including the presence of phytates and polyphenols in plant-based diets.[11] These compounds may reduce iron bioavailability when rhinoceros are fed wild-type diets.[3] Therefore, iron content may be more important in captive diets than in wild-type diets.

In contrast to previous published reports, this study did not find evidence of increased iron stores in boma-confined black rhinoceros.[14] However, the number of samples from boma-confined individuals was small. Mean serum iron levels for boma-confined black rhinoceros (40.6 ± 13.8 μmol/L) were similar to those observed in other free-ranging black rhinoceros populations (27.9 μmol/L, 95% CI 0.7–55.1).[15,18] The lack of significance may be a product of time in the boma as well as low sample numbers. Further investigation of the effects of diet and duration of boma confinement should be conducted.

No correlation was observed between GGT and serum iron or ferritin levels. GGT is a liver-specific enzyme that has been shown to be a good indicator of hemochromatosis in horses.[22] However, because none of the rhinoceros in this study had evidence of pathological levels of iron markers, it was not unexpected that these values were not statistically linked. Therefore, it is unclear whether GGT levels in free-ranging black rhinoceros are an indicator of iron overload disorder. GGT levels (range 7–30 U/L) were similar to those reported in other studies (range 3–21 U/L) and may vary with different laboratories used for analyses.[16,20]

Although the majority of values for individual ferritin were <500 ng/ml, there were three free-ranging black rhinoceros that had ferritin values >1,000 ng/ml. These included a 4.5-yr-old female from Madikwe Game Reserve (1,761 ng/ml), an adult female from the Eastern Cape (1,274 ng/ml), and an 8.2-yr-old male from Mokala National Park (Northern Cape-Kimberly) (1,330 ng/ml). None of these animals had any evidence of clinical disease. Ferritin is also an acute phase protein, and increased levels have been associated with inflammation in humans.[10,12] Potential causes of inflammation in the black rhinoceros in this study include changes in nutrition, subclinical injuries/disease, or responses to increased parasite loads. Levels in these individuals were still considered low compared to those in captive black rhinoceros that exhibit clinical manifestations associated with increased iron loads.[21]

This is the first study to examine a large population of free-ranging black rhinoceros with the use of the rhinoceros-validated ferritin assay to provide comparative values for captive animals. No significant differences were found when results were compared for different age categories or gender. Although a few geographic locations had statistically significant differences in ferritin, the clinical significance was considered to be minor. This may be important in investigating the role of dietary iron in wild rhinoceros. An important result that needs additional research was the lack of change in ferritin levels during boma confinement. Additional research into management factors may provide clues for prevention of IOD in captive black rhinoceros. Results suggest that environmental factors such as diet play an important role in prevention of IOD.

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