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Iron Overload Syndrome in the Black Rhinoceros (*Diceros bicornis*): Microscopical Lesions and Comparison with Other Rhinoceros Species

P. Olias[*], L. Mundhenk[*], M. Bothe[*], A. Ochs[†], A. D. Gruber[*] and R. Klopfleisch[*]

[*] *Department of Veterinary Pathology, Faculty of Veterinary Medicine, Freie Universität Berlin, Robert-von-Ostertag-Straße 15, 14163 Berlin and [†] Zoologischer Garten Berlin AG, Hardenbergplatz 8, 10787 Berlin, Germany*

Summary

The African black rhinoceros (*Diceros bicornis*) has adapted to a low iron diet during evolution and is thus prone to iron overload in captivity, which is associated with a number of serious disorders. A S88T polymorphism in the *HFE* gene has been suggested as a potential genetic basis of increased iron uptake in the black rhinoceros, while the Indian rhinoceros is thought to be unaffected by iron overload in captivity. In the present study, the histopathology and distribution of iron accumulations in five black rhinoceroses with iron overload syndrome were characterized and compared with three Indian rhinoceroses (*Rhinoceros unicornis*) and one African white rhinoceros (*Ceratotherium simum*). At necropsy examination, iron storage in black rhinoceroses was not associated with gross lesions. Microscopically, the most consistent and highest degree of iron load was found in the spleen, liver, small intestine and lung. There was minimal fibrosis and single cell necrosis in the liver. Endocrine organs, lymph nodes, heart and kidney were less often and less markedly affected. Unexpectedly, Indian rhinoceroses also showed iron load in the spleen and smaller amounts in organs similar to the black rhinoceros except for in the heart, while the white rhinoceros had only minor detectable iron storage in intestine, liver and lung. Sequence analysis confirmed the *HFE* S88T polymorphism in black but not in Indian rhinoceroses. The results indicate that Indian rhinoceroses may also be affected by iron storage in captivity, although in a milder form than the black rhinoceros, and therefore challenge the relevance of the S88T polymorphism in the *HFE* gene of black rhinoceroses as the underlying cause for iron overload.

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Introduction

The African black rhinoceros (*Diceros bicornis*) is evolutionarily adapted to a diet low in iron content such as leaves and twigs (Smith *et al.*, 1995). In captivity this may lead to an iron overload syndrome when a diet with inappropriately high iron content is provided (Smith *et al.*, 1995; Paglia and Radcliffe, 2000). The main lesions include widespread deposition of iron in the spleen, liver, bone, small intestine and lungs, with only mild parenchymal degeneration in affected organs and minimal, early hepatic fibrosis. Other animal species affected by iron overload conditions are mynah birds (Randell *et al.*, 1981), toucans (Spalding *et al.*, 1986), Salers cattle (O'Toole *et al.*, 2001), tapirs (Bonar *et al.*, 2006) and, as recently described, red deer (Olias *et al.*, 2011). In man, iron overload, known as primary haemochromatosis, is determined genetically and characterized by accumulation of iron in the liver, heart, intestine and other parenchymal organs (McLaren *et al.*, 1983; Andrews, 1999). Although a genetic background in animals is proposed in several studies, proof is still lacking for all forms of animal iron overload syndromes.

Two pivotal studies by Kock *et al.* (1994) and Smith *et al.* (1995) identified excessive iron storage in captive black rhinoceroses as a potential predisposing factor

Correspondence to: R. Klopfleisch (e-mail: robert.klopfleisch@fu-berlin.de).

for a number of secondary diseases, including increased susceptibility to infection, haemolytic anaemia, mucocutaneous ulcerative disorder and stress intolerance (Kock *et al.*, 1994; Smith *et al.*, 1995; Khan *et al.*, 2007; Molenaar *et al.*, 2008). In 2001, a haemochromatosis gene (*HFE*) nucleotide and protein polymorphism were identified as the potential cause for the increased sensitivity of the black rhinoceros to iron overload when compared with other rhinoceros species (Beutler *et al.*, 2001). This evolutionary genetic adaptation, leading to amino acid change S88T, is located in a highly conserved region of the HFE protein, which interacts with the transferrin receptor (Beutler *et al.*, 2001). The location of the amino acid change suggests a possible genetic mechanism for adaptation to low iron diet in black rhinoceroses, but experimental confirmation of this hypothesis is lacking (Beutler *et al.*, 2001). Importantly, S88T was not present in the analyzed Indian rhinoceros (*Rhinoceros unicornis*) or African white rhinoceros (*Ceratotherium simum*). These rhinoceros species both rely on a different grass diet in their natural habitat, which is generally of higher iron content and both species are therefore regarded as not being susceptible to excessive iron storage (Paglia *et al.*, 1986; Smith *et al.*, 1995; Beutler *et al.*, 2001).

HFE encodes for a protein that complexes with the transferrin receptor (Feder *et al.*, 1996). Defective *HFE* expression results in increased iron absorption via enterocytes of the small intestine and subsequent iron storage by several parenchymal cell types (Powell *et al.*, 1970; Waheed *et al.*, 1999). In addition, recent studies have established that the HFE protein, together with the transferrin receptor-2 protein, affect the expression levels of hepcidin, a protein which is involved in the control of iron release into the bloodstream (Camaschella *et al.*, 2000; Pigeon *et al.*, 2001).

The aims of the present study were (1) to compare the histopathological changes of iron overload in black rhinoceroses with tissue histology of three Indian rhinoceroses and one white rhinoceros and (2) to analyze and compare the *HFE* gene sequences of the black rhinoceros and Indian rhinoceros.

Materials and Methods

Animals and Clinical Data

All rhinoceroses from the Berlin Zoological Garden submitted for necropsy examination to the Department of Veterinary Pathology at the Freie Universität Berlin during the past 30 years were included in this study. These included five African black rhinoceroses (*D. bicornis*; animals 1–5), three Indian rhinoceroses (*R. unicornis*; animals 6–8) and one African white rhinoceros

(*C. simum*; animal 9) (Table 1). Seven animals were >10 years of age (animals 1 and 3–9) and one animal was 2 years old (animal 2). The causes of death or humane destruction varied considerably. Two animals were humanely destroyed after trauma and two animals were humanely destroyed due to severe uraemia. Enterotoxaemia, cachexia, haemolysis and intestinal colic were the cause of death of one animal each. The clinical signs and the cause of death were not recorded for animal 9 (Table 1). Only tissue samples of lymph node, lung and skin were available for animal 6.

Histopathology and Turnbull Blue Staining

Tissue samples were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (2–4 μm) were stained by haematoxylin and eosin (HE) and the Turnbull blue method.

DNA Extraction

DNA was extracted from formalin-fixed and paraffin wax-embedded tissue samples (Klopfleisch *et al.*, 2011; Weiss *et al.*, 2011). Briefly, 20–40 shavings (each 2 μm) were taken from each wax block and collected into a microcentrifuge tube. Samples were dewaxed with xylene and resuspended in 300 μl cell lysis solution (Gentra® Puregene® Tissue Kit; Qiagen, Hilden, Germany). The samples were heat pretreated at 65°C for 45 min followed by 98°C for 15 min. This was followed by proteinase K digestion for 48 h, 3 h or no digestion and RNase A digestion for 15 min. Protein precipitation solution (Gentra® Puregene® Tissue Kit; Qiagen) was used to eliminate protein contamination and DNA was precipitated by addition of 1 volume of 99% isopropanol. DNA pellets were washed twice in 70% ethanol, air dried and resuspended in 25 μl DNA hydratation solution (Gentra® Puregene® Tissue Kit; Qiagen). DNA quality and concentration was determined photometrically (Nanodrop, Peqlab, Erlangen, Germany) and

Table 1
Animals included in the study

Animal	Species	Age (years)/Sex	Body condition	Cause of death
1	Black rhinoceros	16/M	Poor	Uraemia
2	Black rhinoceros	2/F	Poor	Enterotoxaemia
3	Black rhinoceros	Adult/M	Poor	Cachexia
4	Black rhinoceros	Adult/F	NR	Haemolysis
5	Black rhinoceros	38/F	Good	Trauma
6	Indian rhinoceros	Adult/F	Good	Trauma
7	Indian rhinoceros	30/F	Poor	Uraemia
8	Indian rhinoceros	30/M	NR	Colic
9	White rhinoceros	32/F	Decreased	NR

M, male; F, female; NR, not recorded.

adjusted to 100 ng/µl by adding 5 mM Tris buffer, pH 7.4.

Semi-nested Polymerase Chain Reaction Amplification and Sequence Analysis

Specific primer sites for exon 2 of published *HFE* genes of black and Indian rhinoceros were identified (GenBank accession numbers AY007542 and AY007544). Three consensus polymerase chain reaction (PCR) primers were designed using the GeneFisher 2.0 software (Giegerich *et al.*, 1996). The three new primers were denoted as HFE152for (5′-TTTGAAGCCTTGGGCTA TGT-3′), HFE201 rev (5′-CTTGCTGTGGTTGTGG TTGT-3′) and HFE152rev (5′-ATGTGATCCCAC CCTTTCAG-3′). A semi-nested PCR was performed. In brief, all amplification reactions were performed in 50 µl reaction mixtures containing 200 pM of each primer, 10 µl of 5× Green Flexi reaction buffer, dNTP mix (0.2 mM each), 1.5 mM $MgCl_2$ and 1.25 U GoTaq DNA polymerase (Promega, Madison, Wisconsin, USA). The thermal profile used was 2 min at 94°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 58°C and 1 min at 72°C, with a time increment of 2 sec, and finally 10 min at 72°C. Amplicons were resolved on a 2% agarose gel stained with ethidium bromide and photographed under ultraviolet light. Processing and negative controls were included in each PCR run. PCR amplicons were purified using the NucleoSpin Extract II system (Macherey–Nagel, Düren, Germany) and sequenced using primers HFE152for and HFE152rev. The sequences were compared by MEGA 5.05 and BLAST software (Altschul *et al.*, 1990; Tamura *et al.*, 2007).

Results

Necropsy Examination

Reported gross findings varied depending on the cause of death (Table 1). Histological diagnosis of iron overload was not associated with any consistent gross finding in the affected animals. Furthermore, gross lesions reported for Salers cattle with proposed haemochromatosis, including abnormal brown–red staining of the intestine, were not found in any of the animals.

Microscopical Findings in African Black Rhinoceroses

Severe iron overload was detected in all five black rhinoceroses (Table 2). All five animals had haemosiderosis of small intestine, liver and spleen (Figs. 1–5). Turnbull blue staining revealed the highest iron contents in small intestine, liver, spleen and lung, which showed iron overload in four of five cases (Figs. 2–6; Table 2). Iron storage in the small intestine was characterized by moderate to marked amounts of brown–black, coarse haemosiderin, mainly in the deep submucosa (Figs. 1 and 2) and to a lesser extent in the lamina propria of the tips of the villi (Fig. 3). The submucosa and the muscularis propria contained minimal deposits of haemosiderin, mainly in perivascular macrophages. The iron accumulation was occasionally associated with mild atrophy of the intestinal villi. The other segments of the gastrointestinal tract, large bowel and stomach also contained multifocal small aggregates of haemosiderin-laden, mainly perivascular macrophages. The liver of all five animals contained large amounts of iron within Kupffer cells and, to a lesser extent, within hepatocytes (Fig. 5). Hepatic iron accumulation was associated with mild fibrosis in three of five animals and mild, acute, hepatocellular necrosis in all five animals. The spleen of all five animals contained large numbers of histiocytic cells and extracellular iron, but no further histopathological changes (Fig. 5).

Four of the five animals had iron storage in the lung, brain, lymph nodes, adrenal and thyroid gland. The lung of four animals contained moderate to

Table 2
Level of iron storage in affected organs

Number	Small intestine	Liver	Pancreas	Lymph node	Spleen	Bone marrow	Heart	Lung	Kidney	Skin	Adrenal/thyroid
1	+ + +	+ + +	+ +	+	+ + +	+	NT	+ + +	+	−	+ +
2	+	+ +	NT	+	+ +	NT	NT	−	−	NT	+
3	+ + +	+ + +	+ +	+	+ +	NT	NT	+ +	−	−	−
4	+ + +	+ +	−	−	+ + +	NT	+	+ + +	+	−	+
5	+ + +	+ +	−	+	+ +	+ + +	+	+ + +	−	−	+ +
6	NT	NT	NT	+	NT	NT	NT	+ +	NT	−	NT
7	−	+	−	−	+ + +	NT	−	+ +	+	NT	+
8	+ +	+ +	−	+	+ + +	NT	−	+	−	−	+
9	+	+	NT	NT	NT	NT	NT	+	NT	NT	−

−, No iron detected; +, small amounts of iron detected; + +, moderate amounts of iron detected; + + +, large amounts of iron detected; NT, not tested.
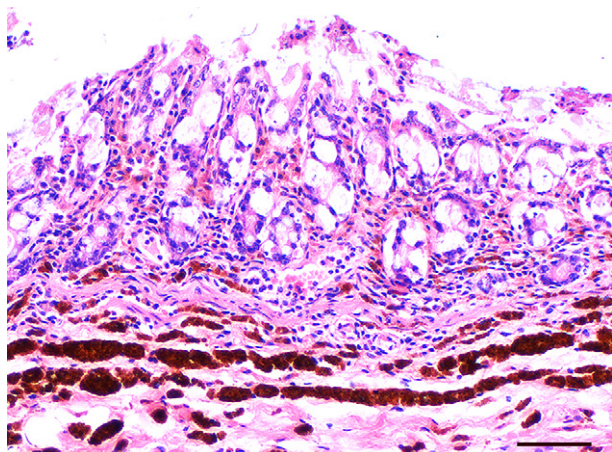
Fig. 1. Small intestine, black rhinoceros. There is severe iron storage in the lamina propria and submucosa. HE. Bar, 150 µm.

Fig. 3. Small intestine, black rhinoceros. Multifocal areas of iron storage restricted to the tips of the villi. Turnbull blue stain. Bar, 150 µm.

marked amounts of iron in the interalveolar septa and the peribronchial connective tissue (Fig. 6). There were few intra-alveolar iron-laden macrophages. Pulmonary haemosiderosis was, however, not associated with any degenerative or fibrotic lesion. In the brain there were multifocal, mainly perivascular haemosiderin-laden macrophages (Figs. 7 and 8). Lymph nodes contained small to moderate amounts of haemosiderin within macrophages. Adrenal gland and thyroids contained few, randomly dispersed haemosiderin-laden macrophages.

Two of the five animals had iron overload in the pancreas and kidney. Iron was detected extracellularly and within macrophages in the renal interstitium and the glomeruli. Pancreatic tissue samples were only available from three of the five animals with iron storage disease. In two of the three, a few randomly dispersed haemosiderin-laden macro-

phages were present in the perivascular or parenchymal interstitium.

Bone marrow and myocardial tissue samples were only available for two animals. The bone marrow samples contained mild and severe iron accumulation, respectively. Iron was mostly located in macrophages, but to a lesser extent was also extracellular (Table 2). Both myocardial tissue samples contained few, mainly perivascular haemosiderophages. Very rarely, occasional scattered cardiomyocytes contained intracytoplasmic iron as identified by Turnbull blue staining (Fig. 9).

Microscopical Findings in Indian and African White Rhinoceroses

All three Indian rhinoceroses had iron deposits in diverse organs (Table 2). Hepatic iron was present as

Fig. 2. Small intestine, black rhinoceros. Turnbull blue staining confirms the massive accumulation of iron in the submucosa of the small intestine. Turnbull blue stain. Bar, 150 µm.
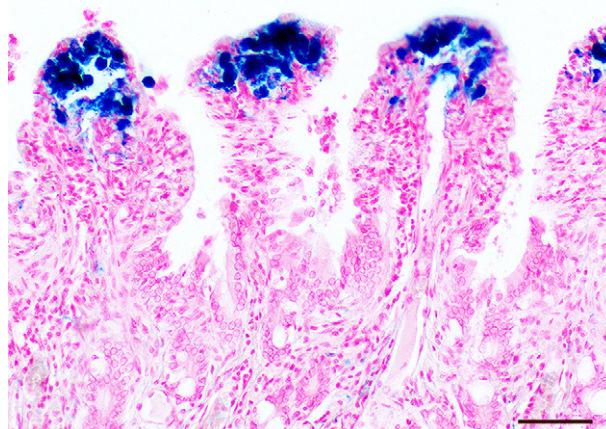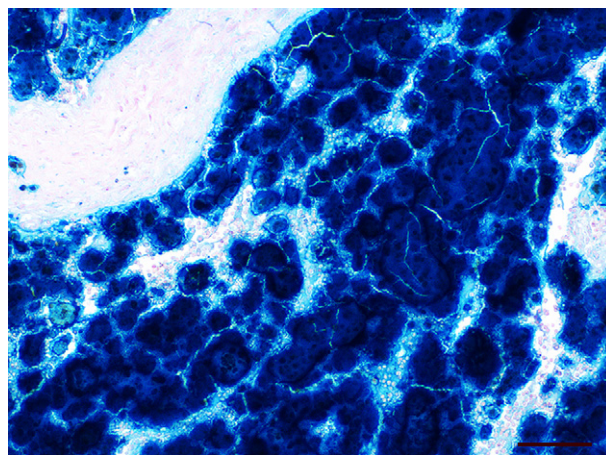

Fig. 4. Spleen, black rhinoceros. All the black rhinoceroses had moderate to marked accumulation of iron in the spleen. Turnbull blue stain. Bar, 150 µm.
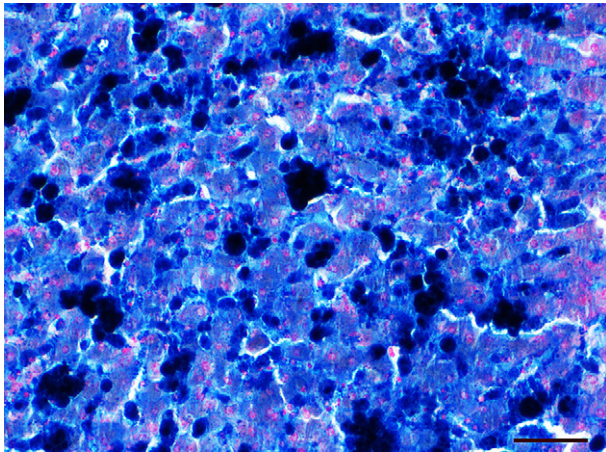
Fig. 5. Liver, black rhinoceros. Livers of all animals contained moderate to high amounts of extracellular iron and iron within macrophages and hepatocytes. Turnbull blue stain. Bar, 150 μm.

Fig. 7. Brain, black rhinoceros. The brain of animal 5 contained few, multifocal, mainly perivascular haemosiderophages. HE. Bar, 150 μm.

moderate amounts of iron in Kupffer cells, but not in hepatocytes (Fig. 9). Both spleens had severe, multifocal to coalescing aggregates of haemosiderin-laden macrophages with extracellular iron accumulation (Fig. 10). All three lungs were affected by mild to moderate iron storage (Fig. 11). Lymph nodes and adrenal glands of two animals had significant iron deposition. The pancreas and kidney of animal 7 contained few haemosiderin-laden macrophages and mild to moderate iron accumulation was identified in the tips of the small intestinal villi of animal 8 (Fig. 12). Iron storage was not associated with microscopical lesions.

The African white rhinoceros (animal 9) had minimal amounts of haemosiderin within macrophages in the small intestine, liver and lung. Iron storage was not associated with microscopical lesions.

Sequence Analysis of the HFE *Gene*

Amplifiable genomic DNA was extracted from the wax blocks of tissue from animals 1–3, 5, 7 and 8. The partial *HFE* sequence of all four black rhinoceroses confirmed the presence of the S88T amino acid change reported previously. The S88T polymorphism was not detected in either of the two Indian rhinoceroses. The *HFE* sequence of the Indian and black rhinoceroses also differed consistently in two positions of the sequenced segments of the *HFE* gene (Fig. 13). However, these two changes do not change the amino acid sequence of the protein.

Discussion

The increased iron deposits in captive black rhinoceroses are thought to be the result of an evolutionary
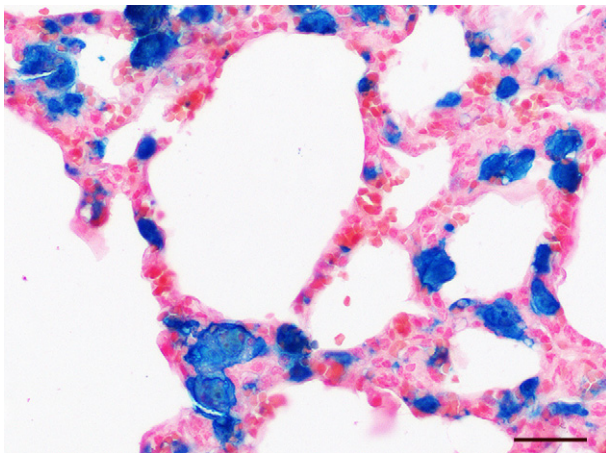

Fig. 6. Lung, black rhinoceros. Multifocally, large amounts of iron are present in the interalveolar and peribronchiolar interstitium. Turnbull blue stain. Bar, 150 μm.
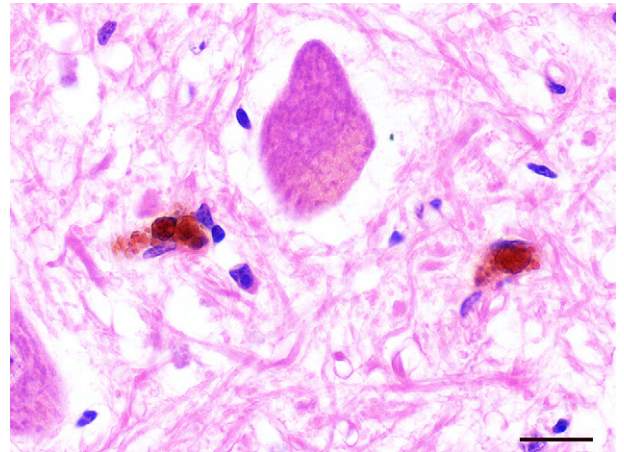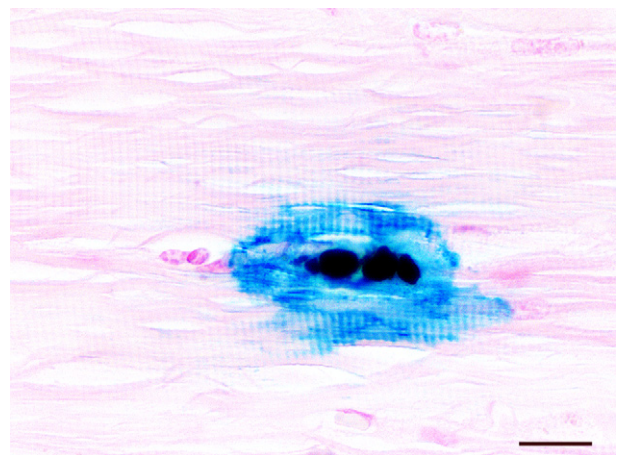

Fig. 8. Heart, black rhinoceros. Both available myocardial tissue samples of black rhinoceros had mild, mainly intrahistiocytic iron storage, which was rarely present in myocardiocytes. Turnbull blue stain. Bar, 150 μm.
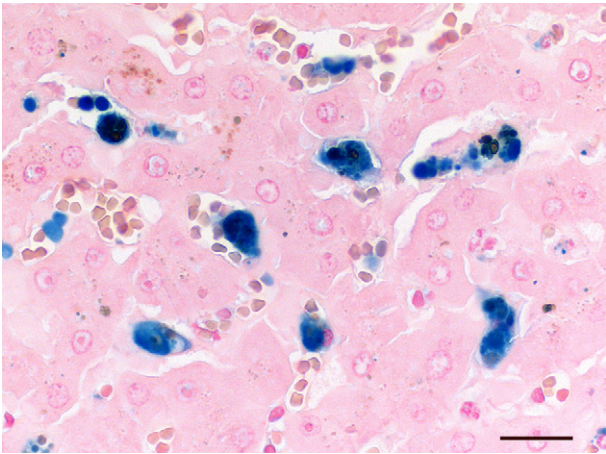
Fig. 9. Liver, Indian rhinoceros. Livers of two animals had small to moderate amounts of intrahistiocytic iron. Turnbull blue stain. Bar, 150 µm.
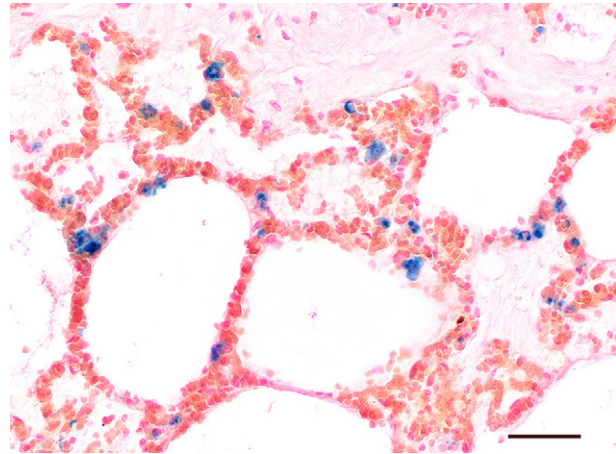

Fig. 11. Lung, Indian rhinoceros. Multifocally, moderate amounts of intrahistiocytic iron are detected in the interalveolar and the peribronchiolar interstitium. Turnbull blue stain. Bar, 300 µm.

adaption to low iron diets in their natural habitat and relatively high iron concentrations in the diets in captivity (Kock *et al*., 1994; Smith *et al*., 1995; Beutler *et al*., 2001). Iron overload syndrome in the black rhinoceros is therefore thought not to be caused by a pathological mutation of any of the genes involved in iron metabolism, but by inadequate, iron-rich diets in captivity similar to iron overload in certain avian species like mynah birds and toucans (Smith *et al*., 1995; Beutler *et al*., 2001; Mete *et al*., 2005). This clearly differentiates iron storage disease in rhinoceroses from inherited human haemochromatosis, which is caused by an inadequate uptake of normal dietary iron (Pietrangelo, 2003).

The ultimate problem in both diseases is the accumulation of iron in several organs, which eventually disrupts their physiological functions and can increase the susceptibility to infection (Beutler *et al*., 2001; Pietrangelo, 2003; Khan *et al*., 2007; Molenaar *et al*., 2008). The phenotype and the organ tropism of iron accumulation of human haemochromatosis depend on which of the iron metabolism genes is mutated (Pietrangelo, 2003). Patients with mutations in the hepcidin gene (*HAMP*) or the hemojuvelin gene (*HJV*) are affected early in life by a dramatic clinical presentation, which is mainly caused by changes in the myocardium and endocrine glands. In contrast, patients with mutations in the transferrin receptor-2 gene (*TfR2*), the ferroportin gene (*FPN*) or the haemochromatosis gene (*HFE*) have a milder, later onset phenotype with the liver as the main organ affected
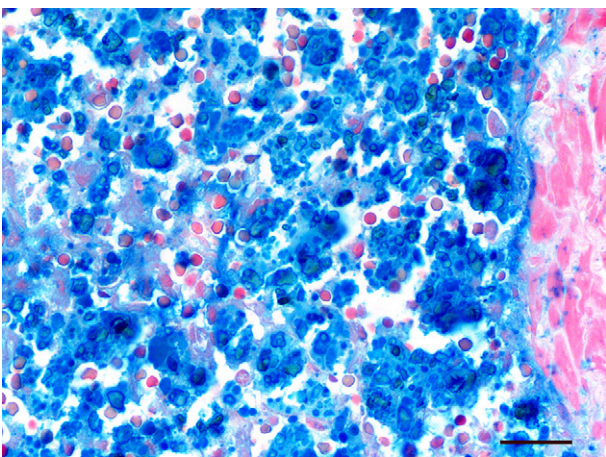

Fig. 10. Spleen, Indian rhinoceros. Two of the three Indian black rhinoceroses had serve accumulation of extracellular and intrahistiocytic iron in the spleen. Turnbull blue stain. Bar, 300 µm.
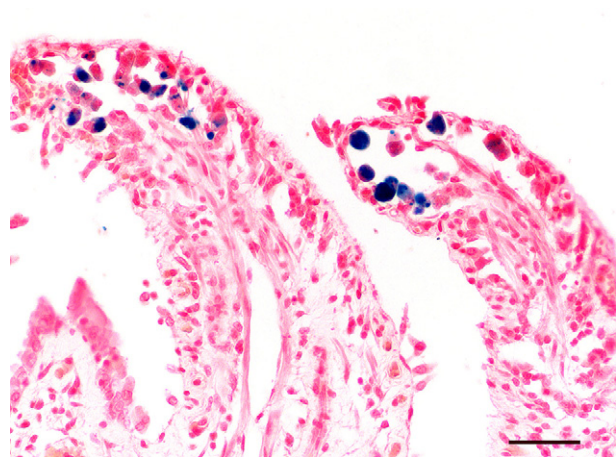

Fig. 12. Small intestine, Indian rhinoceros. Multifocal, intrahistiocytic and extracellular iron storage is restricted to the tips of the villi, but is not present in the basal lamina propria. Turnbull blue stain. Bar, 150 µm.

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                                   *        20          100        Ⓣ        *
Black Rhinoceros  : GGATGACGAGCTTTTCGTGGC-//-GTGGCTGCAACTGACTCAGAGC : 112
Indian Rhinoceros : GGATGATGAGCTTTTCGTGGC-//-GTGGCTGCAGCTGACTCAGAGC : 112
                                                                   Ⓢ
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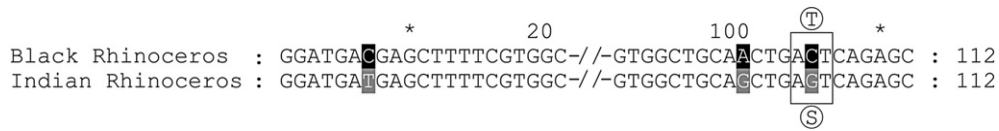
Fig. 13. Sequence alignment of partial exon 2 of *HFE* genes from the black rhinoceros (*Diceros bicornis*) and Indian rhinoceros (*Rhinoceros unicornis*). Amino acid change S88T with potential functional significance in the black rhinoceros is boxed. Two additional consistent differences in the nucleotide sequences are also indicated with no amino acid change.

(Pietrangelo, 2003). Both phenotypes are the extreme variants of a continuum of the clinical and morphological phenotype. The black rhinoceroses described in this study have a phenotype somewhere between both extremes, but closer to the late onset, mild phenotype. Age, clinical presentation and liver involvement coincide with mild, late onset iron storage in human patients and may be caused by adaptations in *HFE*, *FPN* and *TfR2* to low iron diets. The involvement of the endocrine organs and the massive iron storage in most other parenchyma indicates that a role of *HAMP* and *HJV* in rhinoceros iron storage cannot be excluded.

Sequence analysis of *HFE* confirmed the presence of S88T in the black rhinoceroses included in this study, while the two Indian rhinoceroses analyzed did not have this amino acid change. The two additionally identified genetic differences in *HFE* of black and Indian rhinoceroses do not lead to changes in the predicted amino acid sequence and are therefore most probably irrelevant for the function of the HFE protein. None of the black rhinoceroses with iron storage disease in this study had a cause of death that could be directly associated with iron storage, which is in accordance with previous reports (Kock *et al.*, 1994; Paglia *et al.*, 2001). For example, the uraemia in two of the animals was not associated with massive amounts of iron in the kidney. Iron storage may contribute to a reduced resistance to other diseases, but is not consistently associated with a specific clinical problem such as hepatic or renal failure. Furthermore, there was only mild hepatic fibrosis in the rhinoceroses, in contrast to the more severe lesions in people, Salers cattle or red deer with iron storage disease (O'Toole *et al.*, 2001; Pietrangelo, 2003; Olias *et al.*, 2011).

No consistent gross lesion was observed in the rhinoceroses that would allow a diagnosis of iron storage disease to be made without histopathological examination of tissue samples. This is in contrast to Salers cattle, which consistently have firm, dark brown livers and lymph nodes and brown-coloured small intestines, but is similar to red deer with iron storage disease (O'Toole *et al.*, 2001; Olias *et al.*, 2011). Another unique feature of the black rhinoceroses in this study and in other recent reports is the marked involvement of the lung (Paglia and Radcliffe, 2000). The alveolar septa and peribronchiolar interstitium contained significant amounts of iron, except in the 2-year-old animal, which had iron in the intestine, liver, spleen and endocrine glands. The lung therefore seems to be an organ that is affected late in the disease. The clinical relevance of the pulmonary iron storage is unclear, since parenchymal damage of the lung was mild to absent and respiratory signs were not reported for any of the five animals.

Surprisingly, all three Indian rhinoceroses had detectable, but lesser iron storage in organs similar to the black rhinoceros, except for the heart. These results indicate that this species may also have mild iron storage in captivity and therefore challenges the relevance of the S88T polymorphism in the *HFE* gene. Nevertheless, the analysis of a larger cohort of Indian rhinoceroses from a different zoo and, thus, environment is needed to confirm these findings.

Acknowledgments

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