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Safety and efficacy of a novel iron chelator (HBED; (*N*,*N*′-Di(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid)) in equine (*Equus caballus*) as a model for black rhinoceros (*Diceros bicornis*)

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Abstract

While iron overload disorder (IOD) and related disease states are not considered a common occurrence in domestic equids, these issues appear prevalent in black rhinoceroses under human care. In addressing IOD in black rhinos, altering dietary iron absorption and excretion may be the most globally practical approach. A main option for treatment used across other species such as humans, is chelation therapy using iron-specific synthetic compounds. As horses may serve as an appropriate digestive model for the endangered rhinoceros, we evaluated the potential use of the oral iron chelator *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*-diacetic acid (HBED) in horses for safety and efficacy prior to testing in black rhinoceros. Health and iron digestibility and dynamics were assessed in horses ($n = 6$) before, and after treatment with HBED (50 mg/kg body weight) for 8 days using a crossover design with serum, faecal and urine collection. A preliminary pharmacokinetic trial was also performed but no trace of HBED was found in serially sampled plasma through 8 h post-oral dosing. HBED increased urinary iron output in horses compared to control by 0.7% of total iron intake ($p < 0.01$), for an average of 27 mg urinary iron/day, similar to human chelation goals. Blood chemistry, blood cell counts and overall wellness were not affected by treatment. As healthy horses are able to regulate iron absorption, the lack of change in iron balance is unsurprising. Short-term HBED administration appeared to be safely tolerated by horses, therefore it was anticipated it would also be safe to administer to black rhinos for the management of iron overload.

KEYWORDS

chelation, diet, hemochromatosis, horse, iron overload, rhino

1 | INTRODUCTION

As large hindgut fermenting herbivores in order Perissodactyla, horses serve as the most appropriate domestic model of digestion for the black rhinoceros (*Diceros bicornis*; Clauss et al., 2007; Nielson et al., 2012; Sullivan, 2016). Black rhinoceroses are critically endangered with ~87 individuals managed under human care in the United States and ~240 black rhinos managed by humans worldwide (Ferrie, 2020). Black rhinos have encountered health challenges under human care, with multiple cases related to iron overload,

including reports of hemosiderosis in organs at necropsy, while their wild counterparts do not (Dennis et al., 2007; Kock et al., 1992; Molenaar et al., 2008; Paglia & Tsu, 2012; Smith et al., 1995; Sullivan et al., 2020). Iron overload can be seen in other mammalian species as well including humans, dolphins, bats, tapirs and lemurs, though not commonly in horses (Clauss & Paglia, 2012; Ganz & Nemeth, 2012; Johnson et al., 2009; Kellon & Gustafson, 2019; Klopfleisch & Olias, 2012; Lowenstine & Stasiak, 2015; Nielsen et al., 2012; Theelen et al., 2019). Mitigation of iron overload includes dietary iron reduction, phlebotomy and the use of chelators to reduce absorption and/or induce excretion (Clauss & Dierenfeld, Goff, et al., 2012; Sullivan et al., 2020).

Recently, there have been initial investigations into the interplay of potential iron loading with hyperinsulinemia in horses (Kellon & Gustafson, 2019), as well as documenting how chronically high iron content in drinking water lead to hemochromatosis and hepatopathy iron in a small herd of equids (Theelen et al., 2019). Ponies fed ferrous sulfate in excess of requirements with their diet for 8 weeks did not demonstrate clinical signs of hemochromatosis or histologic lesions in the liver, though hepatic iron as well as circulating iron parameters did increase (Pearson & Andreasen, 2001). Dietary overconsumption of iron is not generally associated with iron storage disease in horses, indicating they likely utilise regulatory mechanisms typical in most species to avoid over absorption at the gastrointestinal barrier (Nemeth et al., 2004; Pearson & Andreasen, 2001; Pearson & Hedstorm, 1994).

In addressing iron overload in black rhinos, reducing iron intake and promoting excretion may be the most globally practical approach, as frequent phlebotomies require time and training, and must be repeated due to being only temporarily palliative (Sullivan et al., 2020). Documented plant species consumed by wild black rhinos vary in iron concentration, but appear lower than what is reported for many black rhino diets under human care (Helary et al., 2012). Commercial diets have been formulated to contain the minimal amount of iron possible; however, iron concentrations continue to be higher than those recommended for iron-sensitive animals (Koutsos et al., 2016; Mylniczenko et al., 2012; Sullivan et al., 2020).

The identification of a cost-effective, palatable compound that sequesters iron without compromising health has been recommended (Clauss & Dierenfeld, Goff, et al., 2012). A polyanionic amine, *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*-diacetic acid (HBED) is a synthetic hexadentate ligand (mW = 388) that forms a 1:1 complex with ferric iron with high selectivity and affinity (Eplattenier et al., 1967; Hershko et al., 1984; Martell et al., 1986). HBED has previously been successfully tested for toxicity and efficacy in cell culture, rats, non-human primates, dogs and through Phase 1 clinical trials in humans (Bergeron et al., 1999b; Grady & Hershko, 1990; Grady & Jacobs, 1981; Grady et al., 1994). As HBED is poorly absorbed when administered orally in humans and non-human primates (Bergeron et al., 1999b; Grady et al., 1994), we surmised that HBED had potential to decrease iron availability in the gastrointestinal tract (GIT), as well as increase iron excretion in horses and black rhinos. We hypothesised the oral chelator HBED could be successfully used in black rhinos and horses to safely increase excretion of iron. Our objective was to evaluate the safety and efficacy of orally administered HBED in horses, prior to pursuing its use in black rhinos. We achieved this objective in horses 6 months prior to testing in three black rhinoceros with a similar design for HBED efficacy (Sullivan, 2016).

2 | MATERIALS AND METHODS

2.1 | Animals

Two experiments were conducted utilising horses owned by the University of Florida. The first experiment evaluated the potential pharmacokinetics and dosage of orally administered HBED using mature American quarter horse ($n = 3$) and thoroughbred ($n = 4$) geldings (mean body weight ± standard error [SE], 511 ± 12 kg). This experiment was performed at the Institute of Food and Agricultural Sciences (IFAS) Equine Sciences Centre in Ocala, Florida, where horses were held in individual 3.7 × 3.7 m stalls for the study duration (approximately 24 h). The second experiment, conducted approximately 1 month later, evaluated the safety and efficacy of orally administered HBED using six of the same horses (American quarter horses, $n = 3$; thoroughbreds, $n = 3$). This experiment was performed at the IFAS Horse Teaching Unit in Gainesville, Florida. When not wearing faecal collection harnesses, horses were given access to 3.7 × 12 m grass-free paddocks adjacent to their stalls for 60 min twice daily for free exercise. All study protocols and procedures were approved by the IFAS Animal Research Committee (project 008-13ANS) before the start of the experiments.

2.2 | Experiment 1: Pharmacokinetic and dosage evaluation

To assess the pharmacokinetic response of orally-administered HBED, horses received a single dose of HBED at one of two dosages: either 50 ($n = 3$) or 80 mg/kg body weight ($n = 3$). The higher dosage selected for this study is based on the dosage demonstrated safe and effective for increasing iron excretion in humans (Grady et al., 1994), whereas the lower dosage accounts for allometric scaling factors and expected differences in oral bioavailability between species, which could minimise the amount of HBED necessary for effective reduction of iron uptake in horses (Morris, 1999). The HBED (van Iperen International) was mixed with 100 g of a natural non-fat, non-flavored yogurt (Trader Joe's Company) and placed into two 60 ml syringes with the tips removed to allow for faster dosing via oral gavage. A control animal ($n = 1$) received only the yogurt vehicle. All horses were fasted overnight (approximately 12 h) before blood sampling and HBED administration. Horses had been maintained on diet of coastal bermudagrass hay and bahiagrass pasture prior to being relocated to stalls for this study. All animals were weighed the day before the experiment to allow for accurate HBED dosing.

To facilitate frequent blood sampling, all horses receiving HBED ($n = 6$) were fitted with a 16 gauge × 13 cm intravenous catheter (Abbocath-T, Hospira, Inc) in the right jugular vein under local anaesthesia using an aseptic technique. Blood sampling from the control animal was performed via jugular venipuncture. An initial blood sample was obtained from all horses at Time 0 (pre-dose), followed by HBED dosing and subsequent blood collection at 0.5, 1, 2, 3, 4, 6 and 8 h post-dosing for treatment animals and at 8 h for the control horse. Catheters were flushed with a minimum of 40 ml heparinized saline (2000 U heparin per liter of saline) after each blood sample to maintain patency of the catheter and extension tubing. Blood was immediately transferred to three 3 ml tubes containing 3.2% buffered sodium citrate (Vacutainer, Becton Dickinson Co) to permit HBED measurement. Tubes were immediately placed on ice and centrifuged at 1250×*g* for 18 min at 4°C within 1 h of collection. Plasma was harvested and stored at −80°C until it was sent via overnight mail on dry ice for analysis. Measurement of HBED in sodium citrated plasma was performed by Craft Technologies Laboratories, Inc (currently Eurofins Craft Technologies), using a procedure developed in the laboratory of Dr. Raymond Bergeron at the University of Florida. In brief, the procedure included plasma protein precipitation in acetonitrile, followed by HBED detection using reverse phase HPLC on a Luna C18 (2) column (Phenomex; Inc) using HBED and iron bound HBED as standards.

2.3 | Experiment 2: Safety and efficacy evaluation

A second study was conducted to evaluate the safety and efficacy of daily HBED administration. Geldings ($n = 6$) were randomly assigned to receive HBED (50 mg/kg body weight (BW)/day) or control (no HBED) in a crossover design study. Horses received their assigned treatment for 8 day, followed by a 4-day washout phase to reduce possible carry-over effects of HBED ingestion. After the washout phase, treatments were switched and horses began receiving the opposite treatment for an additional 8 day. This study design permitted each animal to serve as its own control.

Before starting the study, horses were transitioned from a hay-based diet to a cubed, forage-based pelleted ration (Mazuri Zulife Browser Rhino Cube 5Z1P; Mazuri) over 20 days. This is the same pelleted feed with lower iron content than other exotic pellets, used for black rhinos with iron overload disorder (IOD) at zoological institutions. For the duration of the experiment, horses were fed the pelleted diet at 1.9% body weight (dry matter [DM] basis) divided into three equal-sized meals offered at 0700, 1500 and 2300 h. A single batch of pellets was used for the entire study (Table 1). Each meal was also top-dressed with approximately 18 g of loose salt (NaCl) to meet daily sodium requirements and encourage water consumption. Pelleted diets were fed in buckets positioned approximately 1 m from the stall floor. Water was available for *ad libitum* consumption from two, 20 L buckets provided in each stall. Buckets were emptied and refilled with fresh water once daily and additional water was added during the day when the water level dropped below half a bucket.

TABLE 1 Nutrient composition of feeds used in Experiment 2 shown on a dry matter basis

Nutrient[a]	Unit	Browser pellet[b] Mean	Browser pellet[b] SEM	Pancake syrup[b]
Moisture	%	10.8	0.6	35.1
Dry matter	%	89.3	0.5	64.9
Crude protein	%	15.6	1.1	0.1
Acid detergent fibre	%	31.7	1.9	0.1
Neutral detergent fibre	%	45.1	2.3	1.0
Lignin	%	6.0	0.0	0.1
Starch	%	5.8	0.7	0.3
Water soluble carbohydrate	%	9.1	0.4	87.9
Ethanol soluble carbohydrate	%	7.4	0.9	95.6
Crude fat	%	4.6	0.5	0.9
Ash	%	8.7	0.1	0.3
Calcium	%	0.9	0.0	0.0
Phosphorus	%	0.4	0.0	0.0
Magnesium	%	0.3	0.0	0.0
Potassium	%	1.5	0.1	0.0
Sodium	%	0.8	0.0	0.2
Iron	mg/kg	284.5	31.5	1.0
Zinc	mg/kg	150.0	22.0	0.0
Manganese	mg/kg	146.5	16.5	0.0
Molybdenum	mg/kg	2.1	0.1	0.0
Sulfur	%	0.6	0.0	0.0
Selenium	mg/kg	0.9	0.1	0.0
Cobalt	mg/kg	1.1	0.1	0.1
Gross energy	kcal/g	4.5	0.2	3.6

Abbreviation: SEM, standard error of the mean.

[a]With the exception of % dry matter and % moisture, all values are on a 100% DM basis.

[b]Mazuri Rhino Browser Cube 5Z1P (Mazuri); mean ± SE of $n = 2$ samples; Walmart Generic Great Value Brand pancake syrup; $n = 1$ sample.

Initial testing of the raw HBED product, from the same source as Experiment 1, indicated a purity of 85%. To reduce potential contaminants, HBED was further purified before administering to horses. Briefly, 150 g HBED was added to a 4 L Erlenmeyer flask, filled with 2010 ml of 18 MΩ double distilled water and heated to boiling on a hot plate until solubilized (indicated by colour change from clear to a brown-red). The solution was hot filtered through Whatman #10 filter paper under vacuum, allowed to sit overnight at 4°C to recrystallise, and subsequently freeze dried until a consistent weight was achieved. Evaluation of the purified HBED revealed a purity greater than 95%, which was deemed safe for oral

administration in Experiment 2. To dose horses, one-third of the daily dose of HBED was top-dressed on each meal of pelleted feed. To mask the bitterness of HBED, it was mixed with approximately 30 g of store brand pancake syrup free of iron (Table 1; Walmart Great Value) immediately before feeding. When on the control treatment, horses received only the syrup vehicle. Body weight of horses was measured using a livestock floor scale on Days 1 and 8 of each period, and feeding rate and HBED dosing adjusted accordingly for each period.

Feed intake was measured daily and 100% consumption of HBED or control was verified at each feeding. Feed samples were collected daily and composited within each period for analysis. Orts were collected and analysed for nutrient composition if greater than 50 g remained; however, there were few incidences of orts for each animal. Orts were subtracted from the daily feed offered to calculate intake.

At the beginning (Day 1, before dosing) and end (Day 8) of each period, blood was collected via jugular venipuncture into evacuated tubes containing either 3.2% buffered sodium citrate, potassium EDTA or no anticoagulant. Tubes were immediately placed on ice until further processing. Tubes containing sodium citrate were centrifuged at 2000×*g* at 4°C for 18 min within 1 h of collection, and the plasma was subsequently stored at −80°C until analysed. Tubes containing no anticoagulant were held at room temperature to allow clot formation and then centrifuged at 2000×*g* at 4°C for 18 min. A portion of the serum was stored at −80°C until further analysis of iron biomarkers (see below). The remaining serum and blood tubes containing potassium EDTA were submitted to the University of Florida Veterinary Diagnostic Laboratory within 2 h of collection for analysis of equine serum chemistry panel and complete blood cell count (CBC).

On the last 5 day of each 8-day period, each horse was fitted with a harness (Stablemaid PTY, LTD) enabling separate collection of all voided faeces and urine. All horses had been previously acclimated to the collection harnesses as part of other studies. During this collection phase, all wood shaving bedding was removed from stalls, but rubber mats (2.5 cm in thickness) were retained to maintain comfort of the horses on the concrete-floored stalls. Horses remained in their stalls during the collection phase, with the exception of 15 min twice daily where horses were given access to individual 3.7 × 10 m outdoor, grass-free pens attached to each stall for self-exercise. Horses were supervised during exercise to prevent dirt intake.

Harnesses were emptied of their contents at 8 h intervals at 0700, 1500 and 2300 h, which aligned with mealtimes. At each collection interval, total faeces excreted were weighed and the total volume of urine was measured. A 10% representative subsample was retained for each and stored at 4°C. Urine was mixed thoroughly prior to subsampling. The urine subsample was acidified with 20 µl of 12 M HCl per 1 ml urine prior to storage. At the conclusion of each 24 h interval, subsamples obtained at the three collection times were thoroughly mixed and composited into samples representing a single day within the 5-day collection phase and stored at −20°C. For example, the Day 6 composite began with subsamples obtained at 1500 h on the first day of collection and ended with the subsamples obtained at 0700 h on the second day of collection. This resulted in five faecal and five urine samples per horse per period. Nutrient analysis was performed on each 24 h composited sample, and data were averaged resulting in one mean for each nutrient or variable of interest per horse per diet for statistical analysis.

Feed and faecal samples were analysed at Dairy One Laboratories for proximate composition and minerals, gross energy, neutral detergent and acid detergent fibre and acid insoluble ash using Association of Official Analytical Chemists-recognised wet chemistry procedures Dairy One Analytical Laboratory (2016). Digestibility of DM, crude protein (CP), neutral detergent fibre (NDF) and iron were determined by subtracting nutrient excretion in faeces from nutrient intake, and dividing this by nutrient intake. Balance of iron was calculated on a DM basis per day, as grams of iron intake—grams of iron output in the faeces—grams of iron output in the urine, standardised by body weight. Water collected from multiple hydrants used to provide drinking water to horses was analysed at Dairy One Laboratories for mineral content, nitrates and coliforms using recognised United States Environmental Protection Agency authorised procedures.

Serum was analysed for iron biomarkers. Serum ferritin was analysed at Kansas State Veterinary Diagnostic Laboratory by sandwich ELISA utilising a polyclonal rabbit anti-horse ferritin antibody (Smith et al., 1995). Total non-haem iron and total iron binding capacity (TIBC) analyses were performed on serum by using a colorimetric ferrozine procedure (M. D. Knutson et al., 2000). Transferrin saturation percentage was calculated by dividing serum non-haem iron by TIBC. Urinary iron was analysed at Cornell University Veterinary Diagnostic Laboratory. Plasma derived from sodium citrate tubes was analysed for HBED at Craft Technologies Laboratory, Inc, as described above for Experiment 1. Urine samples were also analysed at Craft Technologies for the presence or absence of HBED and HBED with bound iron using ultraviolet visible spectroscopy and a standard of HBED saturated with iron in double distilled water.

2.4 | Statistical analyses

Based on HBED supplementation and iron balance data in five humans (50 ± 17% increase in iron excretion; Grady et al., 1994) and a one-sample, one-tailed *t*-test using an alpha level of 0.05, $n = 3$ animals per species would result in >90% power to detect a statistical effect of treatment should one exist (Systat 13). However, as this was a test of safety prior to use in an endangered species, the sample size of six horses increases the power to close to 100%. All data were analysed using a mixed model ANOVA suitable for a cross-over design (proc mixed function; SAS version 9.4; SAS Institute). Blood cell distribution, blood serum chemistry and serum iron biomarker data were analysed with time (Days 1 vs. 8) as a repeated measure and covariate structures of heterogenous compound symmetry or

compound symmetry based on Akaike Information Criteria (AIC) and finite-sample corrected AIC for best model fit. Treatment, period, time and all interactions were included in the model as fixed effects and horse nested within day was used as random effect. The PDIFF option of the least significant difference test was used to separate means when the model was significant ($\alpha \leq 0.05$). Digestibility, intake and output data were also analysed with a mixed model ANOVA with treatment and period as fixed effects and horse as a random variable. Breed was also assessed in the full model for digestibility; however, after no significant differences were detected, breed was removed from the model. One horse presented with signs of colic on Day 6 of period 2 while on the HBED treatment. Treatment and sample collection were discontinued for this horse for the remainder of the period. However, data collected from this horse through Day 5 of period 2 were retained in the dataset. A Kenward Rogers adjustment for the degrees of freedom was used for all statistical analyses. Data are presented as least squared means with SE except where otherwise noted. Differences were considered significant at $p < 0.05$ or as tendencies at $0.05 < p \leq 0.10$, and noted if physiologically relevant.

3 | RESULTS

3.1 | Experiment 1: Pharmacokinetic and dosage evaluation

Horses appeared to tolerate the single dose of HBED and exhibited no outward clinical signs or adverse reactions. HBED was undetectable in all plasma samples, regardless of dosage and sampling time, in contrast to both a HBED control, and a HBED sample saturated with ferric iron.

3.2 | Experiment 2: Safety and efficacy evaluation

Short-term, daily supplementation of HBED had no effect on blood chemistry variables (Table 2). With the exception of a trend ($p = 0.06$) for eosinophils to be higher when horses received HBED, which may indicate potential mild allergy, chelation treatment also had no effect on CBC (Table 3). Across treatments, most haematological variables also did not change between Days 1 and 8. The exceptions included albumin, phosphorus, bicarbonate, cell haemoglobin concentration mean and corpuscular haemoglobin in intact red blood cells, which were higher at the beginning of each period compared to the end ($p < 0.05$), and calcium, magnesium, creatinine and chloride which were lower on Day 1 compared to Day 8 ($p < 0.05$; Table 3). However, for all of these variables, values remained within the normal reference range for this species. Similar to Experiment 1, HBED was not detected in the plasma of horses at any time point.

Feed intake was consistent for all horses with minimal orts. There was not a difference in BW found between treatments (Table 4). There were no differences in iron intake or faecal iron output between treatments, though faecal iron concentration did show some variability (Table 4). In contrast, daily iron excretion in urine was greater ($p < 0.01$) when horses received the HBED treatment than control (Table 4). The control treatment resulted in 2.4 mg iron excreted via the urine per day, while this amounted to 27 mg iron per day with HBED, which is a difference of 0.08% versus 0.83% of total iron intake. For scale, both HBED and control net excreted between 102 and 159 mg of iron in faeces (iron consumed mg–iron excreted in faeces mg; calculated using iron concentration of feed, faeces and animal body weights). Additionally, iron-bound HBED and what appeared to be HBED derivatives were detected (though not quantified) in urine when horses received the HBED treatment (data not shown). Iron digestibility ($p = 0.98$) and iron balance ($p = 0.55$) were not different between treatments (Table 4). HBED supplementation had no effect on DM, CP or NDF digestibility (Table 5). Treatment with HBED had no effect on serum iron biomarkers (Table 6).

4 | DISCUSSION

Chelation treatment may be a viable option for reducing iron uptake and/or increasing iron excretion in black rhinos with IOD based on these initial studies in horses. Using horses as a model for black rhinos, we found that short-term, daily HBED administration increased iron excretion without any apparent adverse impacts to health. Within the order Perissodactyla, the closest related domesticated species to rhinos would be the horse (*Equus caballus*), who share similar digestive anatomy, site of primary fermentation and size (Stevens & Hume, 2004). Based on the performance of HBED in horses in the current study, as well as the findings in other species, investigation of this chelator in black rhinos as a means to manage iron overload was warranted. Chelation with HBED was performed after this study was completed with three black rhinos (Sullivan, 2016) and is currently being prepared for publication.

Faeces are the dominant route of iron excretion in mammals (Emerit et al., 2001). This was demonstrated in the current study. HBED did not further increase iron excretion in the faeces, but did increase urinary iron excretion (0.08% vs. 0.83% of iron intake), although not enough to impact iron retention (~127 mg with HBED and ~157 mg on control) or create an overall net excretion. In contrast to all other minerals, iron cannot be actively regulated to be excreted from the body. While recent studies have documented biliary excretion of iron in genetically iron loaded knockout mice models (Mercadante et al., 2019; Prajapati et al., 2021), that has not been demonstrated as an active mechanism of iron regulation in other species, especially non-iron loaded species such as the horse. The level of iron in the body is primarily regulated via absorption in the small intestine at the brush-border membrane of duodenal enterocytes. Whole-body iron homoeostasis is regulated by the interplay between the site of iron absorption and the site of iron recycling from the macrophages of the spleen, liver and bone marrow (M. Knutson & Wessling-Resnick, 2003). Hexadentate chelators of ferric iron like HBED do not redistribute iron in the body (Hider

TABLE 2 Blood chemistry when horses received the control or HBED treatment for 8 days

Variable	Units	Reference range[a]	Control	HBED	SEM	*p* value		
						Trt	Time	Time x Trt
ALP	U/L	69–228	181.6	186.3	21.6	0.88	0.30	1.00
AST	U/L	148–322	305.2	309.4	22.5	0.90	0.36	0.58
Total bilirubin	mg/dl	0.3–1.9	1.2	1.4	0.3	0.64	0.91	0.21
Total protein[b]	g/dl	6.1–8.4	6.9	6.9	0.1	0.96	0.02	0.53
Albumin[b]	g/dl	2.7–4.5	3.5	3.5	0.1	0.96	<0.01	0.73
Globulin	g/dl	2.4–4.9	3.4	3.5	0.1	0.67	0.94	0.82
A/G ratio		0.6–1.4	1	1	0.1	0.87	0.34	0.34
Calcium[c]	mg/dl	10.7–13.3	12.1	12	0.2	0.54	0.01	0.46
Phosphorous[b]	mg/dl	2.3–4.7	3.3	3.5	0.2	0.51	<0.01	0.62
Creatinine[c]	mg/dl	1.1–2.0	1.5	1.5	0.1	0.94	0.02	0.81
BUN	mg/dl	9.0–22.0	16.2	15.5	1.3	0.72	0.41	1.00
Glucose	mg/dl	62–128	100.9	101.5	3.5	0.83	0.38	0.65
Triglyceride	mg/dl	4–44	19.6	22.2	3.6	0.63	0.12	0.94
Magnesium[c]	mg/dl	1.2–1.9	2	1.8	0.1	0.12	0.01	0.71
GGT	U/L	17–50	23.8	25.6	1.6	0.45	0.08	<0.01
CPK	U/L	117–555	306.8	460.5	125.1	0.41	0.50	0.29
Sodium	mEq/L	136–144	136.4	136.8	0.3	0.43	0.06	0.73
Potassium	mEq/L	2.2–5.3	4.2	4.1	0.1	0.42	0.41	0.74
Chloride[c]	mEq/L	96–105	100	100.3	0.3	0.43	<0.01	1.00
Bicarbonate[b]	mEq/L	22–30	29.3	28.9	0.3	0.33	<0.01	0.85
Anion gap	mEq/L	10.0–20.0	11.3	11.6	0.5	0.67	0.10	0.67

Note: Data represent the mean of Days 1 and 8 samples, as the effect of time was not significant except where noted.

Abbreviations: A/G ratio, albumin/globulin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CPK, creatine phosphokinase; GGT, Gamma-glutamyl transferase; HBED, *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*-diacetic acid; SEM, standard error of the mean.

[a]University of Florida Veterinary Diagnostic Laboratory, Gainesville, FL, USA.

[b]Day 1 > Day 8; $p < 0.05$. All values remained within the normal reference range for horses.

[c]Day 1 < Day 8; $p < 0.05$. All values remained within the normal reference range for horses.

et al., 1999). In humans, both urine and faecal excretion of iron increased with oral HBED administration; however, the 50% increase in total daily iron output was attributed primarily to increased iron in the faeces (Bergeron et al., 1998a; Grady et al., 1994). The source of excreted iron was not determined in their study. Deferoxamine (DFO), a hexadentate ligand similar to HBED, has been shown to have the potential to enterally bind unabsorbed iron in the GIT (Bergeron et al., 2002c). However, iron in the faeces potentially could have originated from labile iron pools in the liver, rather than from dietary iron due to the chelator. Intraperitoneal (i.p.) injection of HBED (100 mg/kg body weight) into iron hyper-transfused rats increased net iron excretion in faeces threefold, and in urine twofold compared to DFO equivalents (Grady et al., 1994). In the same study, radioactive labelled iron used to track which iron pools were affected by injected HBED showed that faecal iron originated from liver storage pools, and iron excreted in the urine originated from the processing of transfused red blood cells by the reticuloendothelial system (Grady et al., 1994). By comparison, HBED administered orally to rats retained only 25% of its documented iron excretion activity compared to i.p. administration, at 384 µg/kg/day net iron excreted with 87% in the faeces and 13% in the urine (Grady & Hersko, 1990). The current study demonstrates a net iron retention on HBED and control (with 96% iron excretion in faeces and 4% in urine) rather than a net excretion (Table 4) however, the horses were not iron overloaded. Despite the apparent absorption of HBED in the horses tested as evident from the increased urinary excretion of iron, the extent of absorption is unclear, as the current study was not designed to identify the origin of iron excretion, and because HBED itself was not detected in plasma after oral application. Due to a lack of difference in faecal iron output between treatments, it is possible that all of the HBED treatment increase in urinary iron excretion was solely due to absorbed HBED. While an increase of excreted urinary

TABLE 3 Complete blood cell count when horses received the control or HBED treatment for 8 days

	Units	Reference range[a]	Control	HBED	SEM	*p* value		
						Trt	Time	Time x Trt
White blood cells	K/μl	5.6–11.6	7.0	7.0	0.5	0.95	0.36	0.64
Red blood cells (RBC)	M/μl	6.6–11.0	7.6	7.9	0.4	0.68	0.09	0.52
Haemoglobin	g/dl	11.0–16.0	12.9	13.3	0.7	0.71	0.10	0.67
Haematocrit	%	30.0–44.0	35.5	37.3	2.0	0.53	0.08	0.92
Packed cell volume	%	30.0–44.0	36.5	37.8	2.0	0.66	0.93	0.42
Mean corpuscular volume	fl	38.0–51.0	47.3	47.3	0.7	0.94	0.25	0.30
Mean corpuscular haemoglobin	pg	13.0–19.0	16.9	16.9	0.3	0.96	0.57	0.39
Mean corpuscular haemoglobin concentration	g/dl	35.0–39.0	35.7	35.7	0.2	0.87	0.38	0.62
Cell haemoglobin concentration Mean[b]	g/dl	34.9–37.6	35.5	35.6	0.4	0.88	0.03	0.70
corpuscular haemoglobin in RBC[b]	pg	13.7–17.9	16.9	16.9	0.3	0.89	<0.01	0.30
Red cell distribution width	%	16.0–21.0	18.6	18.7	0.2	0.85	1.00	0.39
Haemoglobin distribution width	g/dl	1.5–2.1	2.3	2.3	0.04	0.69	0.39	0.91
Platelet count	K/μl	100–308	148.5	138.5	8.3	0.42	0.77	0.48
Mean platelet volume	fl	5.9–9.9	7.0	6.9	0.4	0.85	0.09	0.40
Protein isoelectric point[b]	g/dl	6.0–8.0	7.2	7.1	0.1	0.85	0.03	0.94
Fibrinogen	mg/dl	100–400	291.7	211.3	37.8	0.17	0.26	0.82
Neutrophil	K/μl	2.6–6.7	4.2	3.8	0.4	0.43	0.20	0.02
Lymphocyte	K/μl	1.1–5.7	2.3	2.2	0.3	0.78	0.20	0.63
Monocyte	K/μl	0–0.7	0.2	1.2	0.5	0.14	0.21	0.19
Eosinophil	K/μl	0–0.6	0.2	0.7	0.1	0.06	0.15	0.09
Basophil	K/μl	0–0.2	0.1	0.1	0.05	0.33	0.07	0.62

Note: Data represent the mean of Day 1 and 8 samples, as the effect of time was not significant except where noted.

Abbreviation: HBED, *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*-diacetic acid; SEM, standard error of the mean.

[a]University of Florida Veterinary Diagnostic Laboratory, Gainesville, FL, USA.

[b]Day 1 > Day 8; $p < 0.05$. All values remained within the normal reference range for horses.

iron from 0.08% to 0.83% of iron intake may seem minor, the resulting difference creates 27 mg/day of iron output from urine. Chelation goals for humans on IV DFO are 20–50 mg of iron (600–1500 mg per month) excreted through combined urine and faeces daily (Mobarra et al., 2016). While these may be considered conservative goals for the body size of the horse or black rhinoceros, considering the retention levels of iron seen (126–159 mg iron/kg BW), perhaps these are reasonable chelation goals, even without a net excretion in animals not particularly loaded with iron. The current study documented proof positive that HBED can pull iron to the urine in a healthy hind-gut fermenter without iron dysregulation issues.

The higher excretion of (putatively HBED-bound) iron in urine indicates HBED was taken up from the GIT in the horse. However, HBED was not detected in the plasma in either Experiment 1 or 2. Based on research in humans, we expected little absorption of HBED to occur in horses. Orally active hexadentate ligands tend to have low non-facilitated absorption in the GIT due to molecular weights that are generally larger than 500 (Hider et al., 1999; Maxton et al., 1986). HBED has been documented to have a low (5%) oral bioavailability based on in vitro cell permeability (Sugano et al., 2002). The lack of HBED detected in the plasma of horses, which would confirm absorption, may be due to several reasons. First, it is possible that the pharmacokinetic experiment did not accurately capture the window of absorption, although this is unlikely. Sampling frequency was based on human and non-human primate models of HBED supplementation, as well as expected foregut digesta passage kinetics

	Units	Control	HBED	SEM	*p* value
Average body weight	kg	511	511	8.6	0.96
Iron intake	mg/kg BW	6.0	6.4	0.3	0.36
Faecal iron concentration	mg/kg DM	599.6	609.4	34.5	0.75
Urine iron concentration	µg/dl	22.1	200.4	13.8	<0.01
Urine volume	L	13.5	14.4	0.8	0.45
Faecal iron output	mg/kg BW	5.8	6.1	0.4	0.11
Urine iron output	mg/kg BW	0.005	0.053	0.01	0.09
Digestibility of iron	%	5.3	3.4	6.9	0.98
Iron retention	mg/kg BW	0.31	0.25	0.41	0.55

Abbreviation: BW, body weight; DM, dry matter; HBED, *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*-diacetic acid; SEM, standard error of the mean.

TABLE 4 Iron intake and excretion when horses received the control or HBED treatment for 8 days (amounts on a daily basis)

TABLE 5 Digestibility of dry matter (DM), crude protein (CP) and neutral detergent fibre (NDF) when horses received the control or HBED treatment for 8 days (amounts on a daily basis)

	Units	Control	HBED	SEM	*p* Value
DM					
Intake	g DM/kg BW	19.7	19.9	0.2	0.75
Faecal output	g DM/kg BW	9.6	10.0	0.3	0.15
Digestibility	%	51.6	49.5	0.8	0.30
CP					
Intake	g DM/kg BW	3.1	3.1	0.1	0.81
Faecal output	g DM/kg BW	1.0	1.0	0.1	0.55
Digestibility	%	69.3	67.3	1.4	0.47
NDF					
Intake	g DM/kg BW	9.4	9.4	0.1	0.76
Faecal output	g DM/kg BW	6.1	6.4	0.2	0.37
Digestibility	%	34.7	32.1	2.5	0.32

Abbreviation: BW, body weight; HBED, *N*,*N*-bis(2-hydroxybenzyl) ethylenediamine-*N*,*N*-diacetic acid; SEM, standard error of the mean.

TABLE 6 Serum iron biomarkers when horses received the control or HBED treatment for 8 days

	Units	Control	HBED	SEM	*p* Value
Serum iron[a]	µg/dl	149.5	158.8	12.1	0.60
Total iron binding capacity	µg/dl	458.4	466.2	12.9	0.68
Ferritin	ng/ml	224.5	223.3	120.8	0.99
Transferrin saturation	%	32.4	34.0	2.0	0.59

Note: Data represent the mean of samples obtained on Days 1 and 8, as the effect of time was not significant except where noted.

Abbreviation: HBED, *N*,*N*-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*-diacetic acid; SEM, standard error of the mean.

[a]Day 1 > Day 8; $p < 0.05$.

in horses (Miyaji et al., 2008). Second, it is possible the plasma contained a HBED metabolite, which was not identified with the current analysis. First pass metabolism through the liver could have resulted in the formation of a HBED derivative that was excreted by the kidneys. Analysis of urine did detect what appeared to be HBED metabolites and HBED bound to iron. Although detected, HBED was not quantified in the urine, nor was faeces analysed for HBED in the horses, so it is possible HBED was at lower levels than a HBED metabolite. The plasma may also have had levels of HBED below the detectable limit of the assay; especially considering the horse's blood volume, it might be easier to detect HBED if concentrated in urine.

While the mechanisms of HBED's chelation action in the horses remains unclear, urinary iron excretion increased by 0.7% and health did not appear to be negatively impacted by short-term HBED administration. Blood chemistry and complete CBC remained relatively static during the study period and were not altered by HBED treatment. Only eosinophils were slightly above reference ranges when horses received HBED, indicating a potential reaction; however, other white cell populations and indicators did not indicate an allergic or negative reaction. There were no changes in iron biomarkers over the 8-day period as would be expected. Non-haem iron, total iron-binding capacity and serum ferritin remained within normal ranges for horses (Smith et al., 1984). This indicates horses were able to compensate for any iron losses and maintain iron homoeostasis, as would be expected for a healthy mammal. The horses were not iron overloaded prior to the study, nor did they have genetic issues with iron dysregulation. While short term oral dosing of HBED affected excretion of iron in humans (7.9 mg/day iron excretion with 60% in faeces and 40% in urine), humans showed steady levels of all other serum measures, including ferritin (Grady et al., 1994). Apparent digestibilities of DM, CP, NDF and iron also not differ between treatments in a relevant way, indicating a lack of impact on dietary macronutrient utilisation.

Potential hazards of chelation include unintended targets such as other minerals and damage to the liver or kidneys by the process of

chelation and excretion (Crisponi & Remelli, 2008; Liu & Hider, 2002). Based on blood chemistry, there did not appear to be negative effects of short-term HBED administration on kidney or liver function of horses in the current study. The metal centre of iron complexes has the potential to participate in redox chemistry, including catalysing the formation of hydroxyl radicals through the Haber–Weiss and Fenton reactions Burkitt and Gilbert (1990). HBED has been shown as protective rather than stimulatory of several reactive oxidant species compared to other chelation drugs (Dean & Nicholson, 1994; Samuni et al., 2001). This includes inhibiting peroxidation of mitochondrial membrane lipids and oxidation of ascorbate, as well as functioning as a hydrogen donating antioxidant (Dean & Nicholson, 1994; Samuni et al., 2001). While the horse is not the ideal model for black rhinos considering their ability to keep iron well-regulated naturally under human care, they are the most appropriate digestive model for testing how HBED would potentially function in a monogastric colonic fermenting mega-herbivore. Our study demonstrated horses remained healthy, HBED treatment resulted in a 0.7% increase in urinary iron excretion compared to iron DM intake. Collectively, these findings indicate HBED is likely to be a safe oral chelator to evaluate in the critically endangered black rhino. A corresponding evaluation in three black rhinos was performed after the current study with positive results in two out of the three animals (Sullivan, 2016).

In order to improve the welfare and health of black rhinos, IOD must be prevented and properly managed. Black rhinos exhibit total iron body loads that increase with time in captivity, laboratory and histopathologic evidence of cellular injury and necrosis, and clinical signs similar to human IOD (Paglia, 2017; Paglia & Tsu, 2012; Sullivan et al., 2020). Based on the minor increase of urine iron output observed in horses in the current study, HBED administration was safe and has the potential to allow iron to be pulled out through the urinary route, though this did not create meaningful impact in horses. We still anticipated HBED administration would increase urinary iron excretion in iron-loaded similar species such as black rhinos, at least at a conservative level, which could warrant longer term use for health impacts. This would have the potential to promote iron load reduction in the black rhino for long term health through oral supplementation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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