CASE STUDY: WINOS FOR RHINOS: FEEDING GRAPE POMACE TO BLACK RHINOCEROS (*DICEROS BICORNIS*) AS A METHOD FOR MITIGATING IRON STORAGE DISEASE

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Abstract

Iron storage disease has been detrimental to the captive browsing rhinoceros population. One of the proposed methods for reducing available iron in the gut of these animals is to increase iron chelators into the captive diet that are normally present in the wild diet. This study used the wine making by-product grape pomace, which is naturally high in tannins, as a browse item fed to two black rhinos over the course of two separate trials. The first trial, with the male rhino Ruka, showed promising results with increased fecal iron excretion and lowered serum iron levels when he was fed the grape pomace diet over a 10 day period compared to being fed bamboo leaves and stems as the browse item. However, serum indicators of iron storage status, ferritin, were increased. A second trial was conducted using both Ruka and his female cohort, Zuri. With the same design, serum iron levels did not respond as in the previous trial, but serum ferritin levels were again greater when the animals were fed grape pomace as the browse item. These results would indicate that the increased tannins in the diet did not improve iron storage status for these animals.

Introduction

Iron storage disease has been plaguing browsing rhinoceros species for decades (Candra et al., 2012; Olias et al., 2012). The species' inability to regulate the uptake of iron can be evidenced by the increased serum iron and transferrin saturation levels. More precise diagnosis of iron levels requires more invasive organ biopsies to determine tissue level iron. Because of the risk of such procedures, actual confirmation of iron storage disease in rhinos is generally not confirmed until post-mortem exams; however, these excess iron stores has been implicated in several clinical disorders observed in the species , such as hemolytic anemia, mucocutaneous ulcerative disorder, congenital leucoencephalomalacia, and decreased immune system response (Olias et al., 2012; Paglia & Tsu, 2012). Although work is still needed to help determine the exact genetic factors that relate to iron uptake in browsing rhinos, what is accepted is the regulatory mechanism that prevents excessive iron absorption most mammals, including grazing rhino species, has been lost over time in browsing rhinos species. This adaptation has evolved out of necessity for these animals that live in areas with low soil iron and subsist on plants high in iron chelators, such as tannins (Lavin, 2012). Although this adaptation serves the species well in the wild, captive managed rhinos are fed diets that are high in iron and low in chelators. The result is that most (75%) captive managed black rhinos will eventually succumb to the effects of this disease (Paglia & Tsu, 2012).

Recent work has suggested that captive diets could be improved for rhinos by adding back the chelators found in their natural diets through addition of either diet items naturally high in the compounds, oils and extracts rich in the compounds, or artificially produced chelators (Huntley, 2016, Lavin, 2012; Sullivan, 2016). Dietary chelators, such as naturally occurring tannins, bind strongly to iron in the digestive track. This bound iron would then pass through the rest of the tract unabsorbed, effectively decreasing dietary iron as a method to mitigate the accumulation of iron in the rhinos. Red grape seeds and skins contain high levels of catechin, epicatechin, and gallic acid, which are three phytochemicals that will strongly bind to iron when given the opportunity (Yilmaz & Toledo, 2004). Through contacts within the Oregon Zoo Horticulture Department, a sizable amount of grape pomace was obtained from a regional winery. This pomace is the solid remains of the grapes after they have been pressed for their juice for making wine. This pomace contains the skins, pulp, seeds, and stems of the fruit. It was the intent of this study to evaluate the addition of this tannin-rich diet item to the diet of the Oregon Zoo black rhinos to determine its viability in mitigating dietary iron absorption.

Materials & Methods

Grape Pomace & Bamboo

The grape pomace used for this study was the by-product of the production of a rosé wine where the red grape skins and stems were pressed for their juice but not fermented with the wine as would be done with a red wine. The pomace was obtained from the Mt. Hood Winery and brought back to the Oregon Zoo where it was bagged into 2-gallon size freezer bags and stored at -20°C. Bamboo was collected on zoo grounds fresh daily, and stems and leaves were stripped from the stalk and bagged stored in a walk-in cooler kept at 2°C.

Feeding Trial A

During March of 2016, a feeding trial was conducted that included 10 days of our male rhino, Ruka, on a control diet (CON) with his normal diet and bamboo browse consisting of only leaves and stems, a 10 day wash-out period of his regular diet and no browse, and then a 10 day experimental diet with his normal diet and grape pomace (POM, Table 2). Both the POM and CON diets were designed to adjust the bamboo or grape pomace amounts to keep a consistent dry matter intake during the 10 day trial. During the trial, all feed was weighed in and all orts were weighed out to determine intake. The morning diet consisted of half of the hay diet. Browse was fed in the evening feeding with all of the grain and half of the hay portions of the diet. A blood sample was taken on each of the last three days of the CON and POM diet periods. During the last three days, total fecal collection was done to determine weight, and a subsample of feces was taken each day and composited over the three days. All fecal samples were stored in a -20°C freezer for later analyses.

Feeding Trial B

During March of 2017, a second feeding trial was conducted that included 10 days of both our male rhino, Ruka, and female rhino, Zuri, on a CON and POM diet with the same design as Trial A, outlined above (Table 2). The animals were fed all diets separately, but they were allowed access to each other in the yard when no diet was available. The only change between the protocols was to remove the fecal collection from the second trial.

Diet Analysis
All feed samples were collected, packed on dry ice, and sent to Dairy One Forage Laboratory (730 Warren Rd., Ithaca, NY 14850 USA) for proximate analyses. Feed samples and fecal samples were collected and sent on dry ice to the lab of Dr. Harley Naumann at the University of Missouri (110 Waters Hall, Columbia, MO 65211) for analysis of proanthocyanidins and protein precipitable phenolics. Proanthocyanidins were analyzed via acid butanol method (Hagerman, 2011) as modified by Johnson (2014). Protein precipitable phenolics were analyzed by the method outlined by Hagerman & Butler (1978) as modified by Johnson (2014). These samples were then sent to the University of Missouri Agricultural Experimental Station Chemical Laboratories (700 Hitt Street, Columbia, MO 65211) for analysis of Fe, Zn, and Cu using AOAC Official Method 985.01(A, B, D).

Blood Analyses
Blood samples were always taken in the morning before the morning feed, can change over the course of a day, and fasting levels are preferred (Sauberlich, 1999). Blood was collected into serum separator tubs, centrifuged to separate serum, and serum was decanted into cryotubes and stored at -80°C before shipping. All blood samples were shipped to Kansas State Veterinary Diagnostic Laboratory (1800 Denison Ave., Manhattan, KS 66506) for analysis of serum iron, ceruloplasmin, ferritin, haptoglobin, and total iron binding capacity (TIBC). Transferrin saturation percentage was calculated as the serum iron/TIBC*100.

Results

Phenolic Analysis
Grape pomace was the only diet item to contain measurable values of proanthocyanidins and protein precipitable phenolics (Table 1). As all diet items were similar within each trial, except the browse component, only the mineral levels of the bamboo and grape pomace are shown in Table 1. Although Fe and Zn were greater in the bamboo than the grape pomace, both browse items contained physiologically low levels of all three minerals.

Fecal Output
Fecal output and mineral concentration was only recorded during Trial A of this study. During Trial A, intake, fecal output, and Fe, Cu, and Zn intake had comparable values between both diets (Table 3). Cu and Zn output were also comparable between the two diets tested. However,

measured Fe output was higher than Fe intake for both diets, and Fe output was also numerically greater in the POM diet than in the CON diet.

Blood Analysis

As the *n* for this study was only 1 for Trial A and 2 for Trial B, no real statistical conclusions can be made from the data. What is observable from the data is that during both Trial A & B, serum iron did not seem to differ at a physiologically significant level, although it was slightly higher for Zuri during Trail B. Also, ceruloplasmin and TIBC remained consistent for both trials and treatments on both rhinos. Haptoglobin did not appear to differ between the CON and POM diets, but it was greater in Trail B compared to Trial A. Transferrin saturation percentage was lower in POM in Trial A, unchanged in Ruka in Trial B, and greater in POM with Zuri on Trial B. Ferritin values varied between Trial A & B, but was consistently seen was that ferritin levels were greater in the POM diets when compared to the controls within animal within trial.

Discussion

When this idea was initially introduced, a great deal of research went into the viability of this item as a feed source. Through some study into the field of oenology, it was determined that only the pomace from a certain wine would work for our situation. In the making of full bodied red wines, such as a Cabernet Sauvignon or Burgundy, the grapes and skins and stems are pressed and then fermented with the wine. The resulting grape pomace, therefore, contains a high alcohol and lower sugar content than would be suitable for feeding to rhinoceros. White wines do not ferment the skins and stems with the wine, but they use only white grapes. This resulting pomace would be too low in tannins for the purposes of our study. However, when a lighter bodied red wine is made, such as a Pinot Noir or Rosé, the amount of time the grape skins come in contact with the juice is limited to a short period, typically 4 hr to 2 days. This does not allow for enough time for the sugars to be converted to alcohol in large amounts, resulting in a grape pomace that is high in tannins and sugars and low in alcohol. Nutritionally, grape pomace is a great browse feed that is 42% DM, 12% CP, 28% ADF, and only 80.5 ppm Fe. The added practical benefits to the use of grape pomace were that it is a waste product for the producer that they will gladly give away for free, it can be easily bagged into useable amounts with very little effort, and it can be stored in a freezer and thawed readily to be fed out to the collection animals. Nutritionally, the grape pomace is very similar to the control browse we chose, bamboo. Bamboo leaves and stems are 52% DM, 13% CP, 29% ADF, and only 165.5ppm Fe. Further, the grape pomace had a greater palatability, which led to increased consumption of overall diet by our female rhino, Zuri, who is normally a very reluctant eater.

Initial results from Trial A showed promising results with increased iron excretion and possibly decreasing serum iron when Ruka was fed the POM diet. Also, the decreased serum iron levels and stable TIBC levels indicated decreased transferrin saturation percentage when Ruka was fed POM. However, fecal results were showing that there were endogenous iron sources in the fecals that were unable to be accounted for and were making fecal iron excretion greater than fecal intake. What was seen was an increase in ferritin on the POM diet. As ferritin is the serum indicator for iron stores, this lead us to question if this method would lead to a viable high tannin feed source. Previous work has shown that there can be individual variation regarding serum parameters and effectiveness of chelators (Sullivan *et al.*, 2015), so a second trial was proposed to determine if the results could be repeated in the same rhino and also in his female cohort.

Trial B included both of our Black rhinos, Ruka and Zuri. Both animals are young rhinos, 6 yrs and 9 yrs old, respectively. From 2015 until 2017, their normal ferritin levels range between 480-2200 ng/mL, as measured monthly, with their average values being 887 ng/mL for Zuri and 1413 ng/mL for Ruka. Two dietary changes occurred between the first and second trail. First, the rhinos were both switched off of the higher iron/protein alfalfa hay in favor timothy had and more orchard grass hay. Second, produce was increased in the diet to accommodate shifting and training. However, since Ruka is still growing, his overall intake was increased, which lead to an increased overall iron intake. Fecal collection was done as part of Trial B due to the labor required and the confounding factor of the endogenous iron seen in the first trial. Serum parameters in Ruka showed similar numbers in both Trials, with the exception of haptoglobin being increased and ferritin values decreasing in both the CON and POM diets. What did appear to remain consistent through all three trials was that when fed the POM diet, ferritin levels were between 20-70% greater compared to the CON diet.

Ferritin can be affected by many factors including time of day, age, sex, fasting or fed state, or inflammation (Sauberlich, 1999). Ceruloplasmin and haptoglobin were measured in the serum as indicators of inflammation. As values remained consistent within each rhino within each trial, there was no indication that inflammation was causing the increase in serum ferritin. Further, these animals were kept on the POM diet for another 14 days after Trial B was concluded without any breaks. The ferritin value obtained from Zuri on day 23 of being the POM diet was 1126 ng/mL with the other values remaining consistent to what was seen during the trial, which indicated a sustained increased ferritin iron load compared to her normal diet.

Conclusion

What was concluded from this study, was the initial promising results seen when feeding grape pomace browse as a natural chelator of iron in the diet did not overcome the negative results seen in the serum ferritin levels. The elevated serum ferritin levels measured during each portion of the trials where the animals were fed grape pomace as a browse item indicate that the use of the grape pomace is increasing the iron load on the animals, or, it is at least not improving the iron status beyond just feeding a low iron diet.

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Table 1. Mineral and phenolic concentration of bamboo leaves & stems and grape pomace fed as browse to black rhinoceros (*Diceros bicornis*) to evaluate the effects on iron storage disease.

Parameter	Unit	Bamboo Leaves & Stems	Grape Pomace
DM	%	52	42
Fe	ppm DM	165.5	80.5
Zn	ppm DM	86.0	14.3
Cu	ppm DM	10.2	13.6
Proanthocyanidins	g/kg DM	UD[1]	45.9
Protein Precipitable Phenolics	g/kg DM	UD[1]	22.2

[1]Undetectable levels

Table 2. Nutritional analyses of diets fed to two black rhinoceros (*Diceros bicornis*) to evaluate the effect of grape pomace on iron storage disease.

		Ruka Trial A[1]		Ruka Trial B[2]		Zuri Trial B[2]	
Diet Item	**Unit**	**Control**	**Pomace**	**Control**	**Pomace**	**Control**	**Pomace**
Apple	g/d	220		1760		1760	
Carrot	g/d	-		500		500	
Emcelle Tocopherol	g/d	13		13		13	
Mazuri ZNN Herbivore Diet 5M64	g/d	2000		2400		4200	
Mazur Browser Rhino Cube 5Z1P	g/d	1000		1200		1200	
Alfalfa Hay	g/d	5000		-		-	
Orchard Grass Hay	g/d	5000		6000		8000	
Timothy Hay	g/d	-		6000		6000	
Bamboo Leaves & Stems	g/d	1800		1800		2200	
Grape Pomace	g/d		2400		2380		2910
<u>Parameter</u>							
DM	%	81.4	80.9	78.0	75.7	79.6	77.3
CP[3]	%	15.4	14.2	11.6	11.5	12.2	12.0
Crude Fat[3]	%	2.7	2.5	2.7	2.7	2.8	2.8
NDF[3]	%	47.0	49.5	57.2	55.0	55.8	53.7
ADF[3]	%	31.3	32.2	32.8	32.7	32.0	31.8
Ash[3]	%	7.6	7.8	6.8	6.7	7.0	6.8
DM Intake	kg/d	12.64		15.34		19.00	
Iron	mg/d	1749	1798	1883	1932	2373	2433
Copper	mg/d	246	240	239	233	318	310
Zinc	mg/d	742	721	877	855	1196	1170
Protein Precipitable Phenolics[4]	g/d	0	20.9	0	20.8	0	25.4

[1]Trail A was conducted March 2016

[2]Trial B was conducted March 2017

[3]DM Basis

Table 3. Mineral intake and output over a 3-d period[1] of a black rhinoceros (*Diceros bicornis*) fed for 10 days on a diet which included either bamboo browse or grape pomace browse.

Parameter	Unit	Ruka Trial A	
		Control Diet[2]	Grape Pomace Diet[3]
Intake	kg DM/3 d	38.1	38.2
Fecal Output	kg DM/3 d	25.8	24.0
Protein Precipitable Phenolics	g/3 d	0.0	62.9
Fe Intake	mg/3 d	13368	12422
Fe Output	mg/3 d	14835	17904
Cu Intake	mg/3 d	506	551
Cu Output	mg/3 d	387	415
ZN Intake	mg/3 d	2523	1672
ZN Output	mg/3 d	1780	1752

[1]Each diet was fed for 10 days with a 10-day wash-out period in between each feeding. All parameters were measured over the last 3 days of each 10 day feeding period.

[2]Diet consisted of 33% Alfalfa hay, 33% Orchard Grass hay, 13.4% Mazuri ZNN Herbivore Diet 5M64, 7% Mazuri Browser Rhino Cube 5Z1P, 1.5% Apple, 0.1% Emcelle Tocopherol, and 12% Grape Pomace

[3]Diet consisted of 32% Alfalfa hay, 32% Orchard Grass hay, 13% Mazuri ZNN Herbivore Diet 5M64, 6% Mazuri Browser Rhino Cube 5Z1P, 1.5% Apple, 0.1% Emcelle Tocopherol, and 15.4% Grape Pomace

Table 4. Serum parameters of two black rhinoceros (*Diceros bicornis*) fed for 10 days on a diet which included either bamboo browse or grape pomace browse.

Serum Parameter[3]	Unit	Ruka Trial A[1]		Ruka Trial B[2]		Zuri Trial B[2]	
		Control	Pomace	Control	Pomace	Control	Pomace
Iron	µg/dL	192.67	163.00	176.33	182.00	171.67	245.33
Ceruloplasmin	mg/dL	113.33	87.00	89.60	81.83	87.37	91.10
Ferritin	ng/mL	1457.33	2035.33	849.67	1450.67	830.33	1028.33
Haptoglobin	mg/dL	118.00	108.67	702.00	607.67	567.00	583.00
TIBC[4]	µg/dL	320.00	316.33	339.00	353.67	406.67	383.33
Transferrin Saturation[5]	%	60.00	52.00	52.02	51.52	42.32	63.99

[1]Trail A was conducted March 2016, and daily diets included Alfalfa hay, Orchard Grass hay, Mazuri ZNN Herbivore Diet 5M64, Mazuri Browser Rhino Cube 5Z1P, Apple, Emcelle Tocopherol, and either Bamboo leaves and stems (Control) or Grape Pomace (Pomace). Iron content of both diets was 140 ppm.

[2]Trial B was conducted March 2017 and daily diets included Timothy hay, Orchard Grass hay, Mazuri ZNN Herbivore Diet 5M64, Mazuri Browser Rhino Cube 5Z1P, Apple, Emcelle Tocopherol, and either Bamboo leaves and stems (Control) or Grape Pomace (Pomace). Iron content of both diets was 125 ppm.

[3] Each diet as fed for 10 days with a 10-day wash-out period between the Control and Pomace diets. All serum parameters are the average of 3 blood samples taken on the last three days of each 10-day portion of the trials.

[4]Total iron binding capacity

[5]Transferrin Saturation = Iron/TIBC*100