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Antioxidant Status of Faeces of Captive Black Rhinoceros (*Diceros bicornis*) in Relation to Dietary Tannin Supplementation

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With 2 figures and 1 table

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Summary

In context with the frequent observations of excessive iron (Fe) storage in captive black rhinoceroses (*Diceros bicornis*), it has been suggested that both an excessive dietary Fe content and a lack of dietary Fe-chelating substances, such as tannins, is the underlying cause. Therefore, studies on the effects of tannin supplementation to captive diet are warranted. Six captive rhinoceroses were fed their normal zoo diet (N), and a similar diet supplemented with either tannic acid (T, hydrolysable tannin) or quebracho (Q, condensed tannins), and the total antioxidant capacity (TAC) was measured as mmol Trolox equivalents per kg fresh faeces. The TAC values on diets N (1.24 ± 0.39 mmol/kg fresh faeces) and T (1.34 ± 0.33 mmol/kg fresh faeces) were similar, but significantly higher on diet Q (2.32 ± 0.61 mmol/kg fresh faeces). In contrast to expectations, faecal TAC increased with increasing faecal Fe, possibly as a result of the fact that the faecal Fe content was positively correlated to the proportion of concentrate feeds in the diet, which also contain antioxidants, such as vitamin E, in addition to Fe. Increased antioxidant status caused by the use of tannin substances could have a beneficial effect on animal health, but if tannins should be incorporated in designed diets, other tannin sources, such as grape pomace should be tested.

Introduction

Captive black rhinoceroses (*Diceros bicornis*) are prone to excessive iron storage (Smith et al., 1995; Paglia and Dennis, 1999; Dierenfeld et al., 2005), which has been linked to various disease syndromes in this species. As compared to the forages these animals consume in the wild, diets in captivity are high in iron (Castell, 2005) and low in iron chelators, such as tannins (Wright, 1998). Therefore, the excessive iron storage observed could be a consequence of the high amounts of iron that is not restricted in its availability. Accordingly, there is substantial interest in the consequences of adding tannin sources to captive diets on the health of the animals (Clauss, 2003).

A major problem in studies that introduce a dietary substance with the aim of improving the long-term effects of the overall ration is the choice of an adequate parameter for the documentation of a measurable influence of that substance. Ideally, such trials should be performed on a long-term basis with a large number of animals, which can all be bled regularly, and whose overall long-time health performance can be evaluated. However, with a species like the black rhino, such conditions are very difficult to meet. On the one hand, animal numbers at one facility will hardly be enough for a statistical evaluation, but on the other hand, it will be difficult to find facilities with a similar commitment to animal training, proficiency in bleeding the animals and readiness to perform controlled feeding trials on identical diets.

Using eight animals from three different zoos, it could be shown that the inclusion of tannins to their regular diets influenced the production of salivary tannin-binding proteins (Clauss et al., 2005) but had no measureable influence on iron absorption as measured by conventional balance trials (Clauss et al., 2006b). It had been planned, in addition, to evaluate iron parameters and antioxidant status in regular blood samples from these animals, as the feeding of tannic acid had resulted in an enhanced antioxidant status in juvenile roe deer (*Capreolus capreolus*) (Clauss et al., 2003). However, as it soon became evident that regular bleeding would not be possible as planned in advance with the different zoos, we looked for an alternative parameter for measuring any potential influence on the antioxidant status. Consequently, a published protocol for the measurement of antioxidant status in human faeces (Garsetti et al., 2000) was adopted, as access to freshly defaecated faeces was not a limiting factor in the study.

In the study in humans, antioxidant capacity had been correlated with the intake of polyphenol-containing substances (coffee and red wine); therefore, we hypothesized that the antioxidant status in rhinoceros faeces should increase with tannin supplementation. Additionally, as hydroxyl radical formation in faeces was correlated with faecal iron content in studies with humans and rats (Erhardt et al., 1997; Stone et al., 2002), we expected a negative correlation between faecal iron content and antioxidant status.

Materials and Methods

The feeding trials with captive black rhinoceroses have been described in Clauss et al. (2005, 2006a,b). As −80°C freezing facilities were available only at two of the three facilities participating in the trials, the antioxidant status samples were taken only for the six animals from these two facilities (Table 1). Three different zoo rations, consisting of varying proportions of roughage, pelleted compound feeds, produce and browse were fed to each animal, resulting in a total of 18 individual feeding trials. The animal 'Mtoto' received a diet consisting mainly of concentrates and pelleted fibre sources because of a chronic, recurring oral abscess that prevented the ingestion of roughage (Hatt et al., 2004). The adaptation periods for new diets lasted for more than 2 months. All animals received basically identical diets three times in the following sequence: the diet normally fed at the respective institutions (N), a similar diet with an inclusion of 5% tannic acid (a source of hydrolysable tannins; Merck, Darmstadt, Germany; laboratory grade) in the pelleted diet compound (T), and again a similar diet with an inclusion of 5% quebracho (a source of condensed tannins; Tannin Corporation, MS, USA; non-purified, estimated condensed tannin content 75% according to Robbins et al., 1991) in the pelleted compound feed (Q). This resulted in an additional tannin source intake of 5–15 g/kg dietary dry matter.

Twelve fresh faecal samples per animal and diet period were collected immediately after defaecation over a period of 3–5 days; a subsample of approximately 2 g was mixed with 10-ml phosphate-buffered saline (PBS) using an electric mixer. The resulting mix was centrifuged at 3420 ***g*** for 10 min to obtain a supernatant free of solid cellular debris. This supernatant was frozen within approximately 2 h at −80°C.

The analysis for total antioxidant capacity was performed according to the protocol of Garsetti et al. (2000), and measured as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalents. All 12 samples per animal were pooled per experimental period. Three readings per pooled sample were performed with an average standard deviation of the mean of 2.8%. The faecal values of total antioxidant capacity (TAC) (mmol Trolox/kg fresh faeces) were obtained by multiplying the TAC of the faecal supernatant by dilution. The proportion of non-roughage feeds (pelleted compound feeds, produce, grains and bread) in the diets and the faecal iron concentrations for the dietary periods were taken from Clauss et al. (2006a,b).

The differences between the treatments were evaluated by repeated measurements using ANOVA followed by pair-wise *t*-tests. Bonferroni adjustment served to control multiple tests.

Table 1. Black rhinoceroses (*Diceros bicornis*) used in the feeding trials

Animal	Studbook no.	Sex	Age years
Emma	451	f	10
Quinto	430	m	11
Mtoto*	150	f	31
Sabi	217	f	About 30†
Parky	318	m	19
Wanda	662	f	5

*Animal had an oral abscess, and received a diet with little roughage items (Hatt et al., 2004).
†Exact age unknown as animal was caught from the wild.

Correlations between dietary and/or faecal parameters were tested using Pearson's correlation coefficient (PCC). Analyses were performed using SPSS 12 (SPSS Inc., Chicago, Illinois, USA). The significance level was set to 0.05.

Results

Repeated measurements by ANOVA indicated a difference in the faecal TAC between the feeding periods (Fig. 1; $P < 0.001$). When compared to the diet fed regularly to the animals (TAC 1.24 ± 0.39 mmol Trolox/kg fresh faeces), the tannic acid-containing diet did not alter the faecal antioxidant status (1.34 ± 0.33 mmol Trolox/kg fresh feces; N versus T: $P = 0.459$). In contrast, the faecal antioxidant status increased in all animals receiving the quebracho-containing diet (2.32 ± 0.61 mmol Trolox/kg fresh faeces; N versus Q: $P = 0.001$, T versus Q: $P = 0.006$). There was a tendency towards positive correlations with similar slopes (Fig. 2) between the faecal iron content and antioxidant status for each dietary period (N: PCC = 0.81, $P = 0.052$; T: PCC = 0.78, $P = 0.065$; Q: PCC = 0.86, $P = 0.029$). The lack of significance might be attributed to the small sample size. If, however, the outlying values for the animal 'Mtoto', which received a peculiar diet (see Methods) were excluded, these correlations disappeared or, at least, were no longer significant. In general, the faecal iron content was positively correlated to the proportion of non-roughage items in the diet (all diets combined: PCC = 0.69, $P = 0.002$). However, again, this correlation was dependent on the deviating feeding regime of 'Mtoto'. If this animal was excluded from the data, no significant correlation remained.

Discussion

There were several limitations to this study. The fact that the deviating diet of one animal had an important influence on the results regarding the correlation of faecal iron to both antioxidant status and the proportion of concentrates ingested, emphasizes that such studies should ideally be performed with a larger number of animals, and especially with identical diets for all animals. Additionally, animals should receive different diets in crossover designs, in order to eliminate any potential

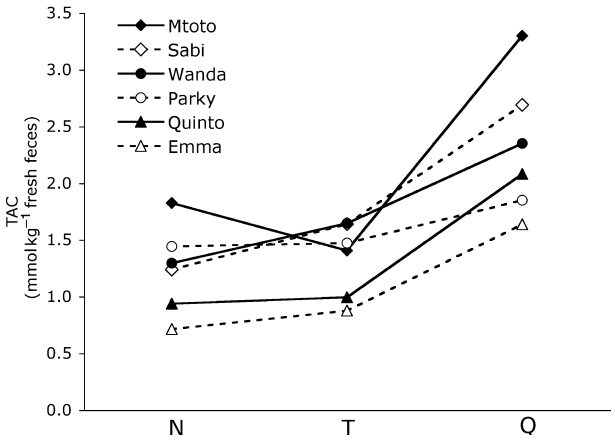

Fig. 1. Total antioxidant capacity (TAC, mmol/kg fresh faeces) in six black rhinoceroses (*Diceros bicornis*) on the normal zoo diet (N), with an addition of tannic acid (T) and quebracho (Q).

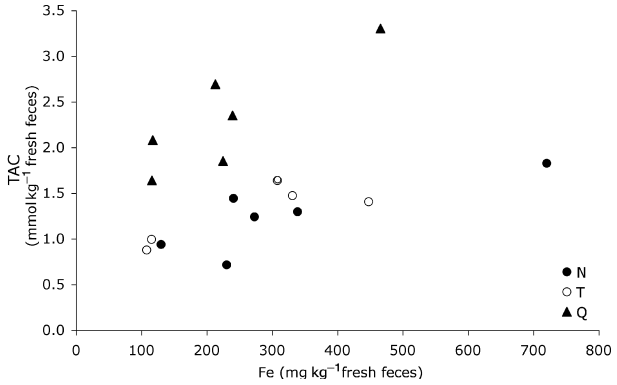

Fig. 2. Total antioxidant capacity (TAC, mmol/kg fresh faeces) in six black rhinoceroses (*Diceros bicornis*) on the normal zoo diet (N), with an addition of tannic acid (T) and quebracho (Q) in relation to faecal iron (Fe, mg/kg fresh faeces) content.

sequence effects. Working in (different) zoological gardens, such approaches might be difficult to achieve, but one should nevertheless try to increase the experimental standards as compared with this study.

In this study, quebracho, but not tannic acid supplementation led to a significant increase in faecal antioxidant status. A possible reason for the absence of an effect of tannic acid could be that, in contrast to the condensed tannins in quebracho, hydrolysable tannins are more easily degraded and/or absorbed in the gastrointestinal tract (Hagerman et al., 1992). The animals used in this study increased the production of tannin-binding salivary proteins in response to the tannin supplementation (Clauss et al., 2005). However, the results given here suggest that these salivary proteins did not prevent the effect of increasing antioxidant capacity in the quebracho period. Hagerman et al. (1992) stated that even if different tannin sources have similar effects *in vitro*, such as protein binding or iron chelation, the biological relevance of this finding must be tested in *in vivo* assays, as differences in resistance to neutralization or degradation by the animal organism will result in different effects *in vivo*. Consequently, these authors demonstrated a significant difference between tannic acid and quebracho tannins on protein digestion in ruminants, with tannic acid showing little effect. The present study reaches a similar conclusion, because the *in vitro* effects of tannins on antioxidant status are achieved *in vivo* by quebracho, but not by tannic acid.

An important factor for the antioxidant status of faeces is the production of superoxide by gut bacteria, which can be converted to hydroxy radicals under the catalytic effect of iron (Erhardt et al., 1997). Therefore, both limiting dietary iron and supplying potent iron chelators should lead to an increase in faecal antioxidant status. As the faecal antioxidant status is believed to be linked to certain types of colon cancer in humans, the chelation of iron in the digesta has been suggested as a prophylactic measure against this disease (Babbs, 1992). In our black rhinoceroses, the positive effect of the condensed tannins from quebracho – potential iron chelators – on faecal antioxidant status is in accord with these assumptions; similarly, the antioxidant status of human faeces was positively correlated to the consumption of polyphenolic-containing beverages (Garsetti et al., 2000). In contrast, the finding that faecal antioxidant status was positively correlated to the faecal iron content (if the animal 'Mtoto' was included in the dataset) was unexpected. As an explanation, we suggest that diets higher in pelleted compound feed contain not only more iron but also more supplemented vitamins, such as vitamin E. Vitamin E supplementation to captive black rhinoceroses has received particular attention over the last decades (Clauss et al., 2002). Increased dietary vitamin E levels will positively influence the faecal antioxidant status (Stone et al., 2002). In this dataset, including 'Mtoto', the faecal iron content was positively correlated to the proportion of concentrates in the ingested diet, supporting this explanation. Unfortunately, vitamin E levels of the diet items used were not analysed.

It can only be speculated whether the increased faecal antioxidant status observed could have a positive influence on the health of captive black rhinoceroses. Hagerman et al. (1998) suspected that even those tannins that are resistant to degradation by intestinal enzymes and symbiotic microbes could protect other biomolecules from possible oxidative damage during digestion, thus sparing other antioxidants, and contributing to the enhancement of the antioxidant status of the whole organism. Such a 'sparing effect' of plant phenolics on vitamin E was demonstrated *in vitro* (Facino et al., 1998; Virgili et al., 1998). In contrast, the presence of excessive amounts of dietary iron reduces the antioxidant status of the gut epithelium, when the dietary vitamin E levels are controlled (Stone et al., 2002). The binding of iron seems to be a major cause for the antioxidant effect of phenolic compounds, and would be one of the main objectives in diet design for captive black rhinoceroses. The binding of iron and the concomitant inhibition of hydroxyl radical formation (depression of antioxidant status) and of iron absorption, by tannins, has been demonstrated both *in vitro* and *in vivo* in humans (Gillooly et al., 1983; Brune et al., 1989; Lopes et al., 1999; Gaffney et al., 2004).

Should the inclusion of tannins be an objective in the development of new diets for black rhinoceroses, then sources other than those used in this study would have to be used for financial reasons (Gaffney et al., 2004). In the case of quebracho, there are also ecological problems, as this substance is gained in a destructive manner from the quebracho tree (*Aspidosperma quebracho*). Red grape extracts and juice have a high affinity for iron *in vitro* (Boato et al., 2002; Gaffney et al., 2004). The iron absorption is reduced by red wine *in vivo* (Bezwoda et al., 1985; Cook et al., 1995). Red grapes and red wine have very high antioxidant capacities (Pellegrini et al., 2003), and red wine or red grape juice show antioxidative effects *in vivo* in humans (Fuhrman et al., 1995; Stein et al., 1999). Together with moderate iron contents of approximately 30 mg/kg dry matter in grapes (Souci et al., 1989), these properties recommend red grape products (such as pomace from the wine industry) as a substance to be tested in zoo animal feeds. A number of studies have shown that polyphenols occur in relevant amounts in red grape pomace (Chidambara Murthy et al., 2002; Gonzàlez-Paramàs et al., 2004; Kammerer et al., 2004).

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