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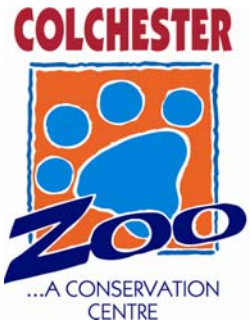

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Can Food Supplements affect Ulcerative Skin Disease in Black Rhinoceros (*Diceros bicornis*)? :An Experimental and Chromatographic Study

Jacqueline Caine

University of Manchester, Paignton Zoo Environmental Park.

jacquicaine@yahoo.co.uk

Introduction

Captive black rhinoceros (*Diceros bicornis*) suffer from a host of diseases not seen in other rhino species, many of which have a nutritional origin (see Ball *et al*. 2005; Dierenfeld 1997; Dierenfeld *et al*. 2005a; Kock and Garnier 1993; Wright and Brown 1997). One such disease is the highly prevalent and sometimes fatal 'Muco-cutaneous Ulcerative Syndrome' (MCUS), characterised by the sudden occurrence of necrotic skin lesions on the lateral body, oral mucosa and feet of affected rhinos (Lee 1997, Munson *et al*. 1998). Free-ranging black rhino do not suffer from MCUS, and as such nutritional discrepancies between wild and captive diets may explain the aetiology of the disease. Wild black rhino will preferably feed solely on 'browse'; i.e. the branches and leaves of trees and shrubs, such as the Acacia shrub, rather than grazing on grasses like the white rhino *(Ceratotherium simum)* (Grant *et al*. 2002). This 'browser' diet is difficult to recreate in the temperate UK climate due to seasonal availability of leafy browse, and the limited space, time and cost needed to grow and harvest the >20,000 kg of browse eaten by each African black rhino every year (Grant *et al*. 2002). African browse contains appreciable quantities of essential fatty acids (EFA) (Ghebremeskel *et al*. 1991), which have been shown to play a therapeutic role in dermatoses such as psoriasis and atopic dermatitis in humans and other mammals (Calder and Miles 2000). Since EFA by nature must be obtained exogenously through the diet, EFA deficient diets of captive rhino may be implicated in the onset of MCUS.

EFA act as precursors of long chain fatty acids, and can be grouped into distinct omega-3 (n-3) and omega-6 (n-6) fatty acids according to structure and function. Eicosanoids derived from n-3 EFA have anti-inflammatory and anti-thrombotic properties while n-6 EFA act in an opposite manner (Simopoulos 2002). These groups of fatty acids compete with one another in the body, so that the ratio of n-3: n-6 in the diet bears certain health implications, manifested in certain cases through dermatological disorders (Callaway *et al*. 2005).

Gas-Chromatography techniques have enabled the full EFA profile of food types to be determined and compared. Using this technique, previous studies of US captive diets revealed not only a quantitative lack of EFA, but also a bias towards n-6 EFA, when compared to African browse. Supplement trials aiming to rectify this imbalance have resulted in positive health responses of certain zoo-housed rhino and other captive exotics (Clauss *et al*. 1999; Dierenfeld 2005b). Such trials utilized linseed oil (*Linum usitatissimum*), a plant matter high in n-3 fatty acids (>50% Alpha-Linolenic acid), which may relieve MCUS through anti-inflammatory action. This is a promising avenue in MCUS studies, however conclusive evidence of the therapeutic role EFA supplements have in skin conditions is rare, and often lacks external validity.

This study aims to expand on these claims and explore the relationship between dietary EFA and the prevalence of MCUS in captive UK black rhino, based on the following hypotheses and predictions:

1. Increasing dietary EFA (specifically n-3) through a linseed oil supplement will improve skin condition, prevent MCUS onset, and have a therapeutic influence on affected animals.

2. The EFA content of food offered to captive black rhino in all UK institutions will differ significantly from that of free-ranging animals.

Methods

1. A randomised clinical trial of EFA supplement was conducted on black rhino housed at Paignton Zoo (1.1), which aimed to measure any resulting change in skin condition due to increased dietary n-3 fatty acids. Supplementation via Masham Micronised Feeds Linseed Lozenges commenced on a date randomly assigned by a keeper, and was given gradually over a three-day period to a final dose of 1kg per rhino per day for the remainder of the trial. Any improvement in skin condition was assessed through a standardized condition scoring survey and digital photographs (4 weeks prior, and 8 weeks post supplement addition), while behavioural changes were monitored through regular focal follows of each animal (10 days pre and post treatment). Neither animal suffered from acute ulcers at the time of the trial, although the male had shown previous signs of foot ulcers, while the female's torso skin was considered to be generally in bad condition.
2. The EFA present in diets offered to black rhino in all UK institutions was then determined, taking into account the seasonal and geographical effects of browse availability. For each animal, each dietary component offered was weighed to the nearest gram every day for a five-day consecutive period. Data were collected for three blocks of five days during the summer 2005 at Paignton Zoo Environmental Park (PZ), and, due to time constraints, for one block of five days in winter 2006 at Chester Zoo (CZ) and Port Lympne Zoo (PLZ). The mean daily intake of each food type was then calculated per rhino at each institution, so that the mean daily nutritional value of the diet could be determined for each animal. Samples of dry pellet feed, hay, and browse were taken for proximate and EFA analysis. Since browse was given on an ad hoc basis at both CZ and PLZ, this could not be quantified, however hay, pellets and produce were quantified for all Zoos.

EFA Analysis

Hay, browse and pellet feeds were sub-sampled and analysed for the presence of EFA using the extraction method by Grant *et al*. (2002). Samples were injected onto a 30m x 0.2mm capillary column (ID – BPX70 0.25UM, SGE Forte Inc.) using the Varian Cp-3800 Gas Chromatograph, and Saturn 2200 Mass Spectrometer. The temperature program followed that of Grant *et al*. (2002), using an external standard of Methyl-Caproate. This external standard meant that data could not be quantified as in the Grant *et al*. (2002) paper, and so qualitative data for each fatty acid present was determined and analysed using Chi-squared goodness of fit tests. A subsequent quantitative analysis of the samples is currently underway, the results of which will not be presented in this report. Since qualitative data may mask conclusive findings, determining the actual quantities of each EFA consumed by animals at each institution will allow for comparison with African browse, and enable any dietary EFA deficiencies to be determined. Degradation experiments will also be carried out to assess any EFA loss due to the drying and storing of samples.

Results

Supplement Trial

Behaviour

Mean daily activity budgets as determined by focal follows were not significantly affected by the addition of EFA supplement. $P>0.05$ for all state behaviours –Locomotion, Rest, Feed and 'Other'. Time spent in various forms of resting and locomotion were calculated, and remained largely unaffected by the supplement. The male did however spend less time standing (Mann Whitney $U = 9.00$, $P<0.05$) and more time lying down (Mann Whitney $U= 19.50$, $P <0.05$) after addition of the dietary EFA supplement. None of the female's locomotary behaviour was affected due to the supplement ($P>0.05$ for time spent standing, lying and walking). The frequency of each prolonged foot lift was recorded for each animal, where the male showed a significant change in foot lifts after treatment (Male: $\chi^2 = 161.47$, d.f. $=3$, $P< 0.05$); left fore foot lifts decline and right fore foot lifts increase following EFA supplementation, while both hind

feet lifts decline. Female foot lifts remained unaffected throughout the trial (Female: $\chi^2 = 3.13$, d.f. = 3, P >0.05).

Skin Condition

Inter-observer reliability for the condition scores proved to be acceptable. Skin condition did not alter significantly throughout the trial according to this scoring system, whereby the male showed consistently higher quality skin (mean score+/- SE): 7.94 +/- 0.08, than the female: 5.58 +/-0.07. Mean values and regression coefficients for the general skin condition of each animal over time are shown in Table 1 and show little change in skin score throughout the trial. R^2 values correspond to the scores for weeks 1-8 after supplementation. Neither animal showed a change in skin colour during the trial, as measured through the RGB values of digital photographs.

Observer	Male R^2	Male Mean Score (+/- SE)	Female R^2	Female Mean Score (+/- SE)
Keeper 1	0.17	8.08 (+/-0.08)	0.02	5.75(+/-0.12)
Keeper 2	0.46	7.91(+/-0.17)	0.04	5.62(+/-0.14)
Researcher	0.01	7.83(+/-0.15)	0.05	5.38(+/-0.12)

Table 1. Mean score and regression coefficients (R^2) for skin condition scores given by each observer throughout the trial (8 weeks post treatment). NB. Mean score of 10 indicates excellent skin condition.

Dietary Intake Study

Intake studies show that food offered to black rhino in UK institutions contains (mean daily percentage +/- SE) 48.2 +/- 8.65% Produce, 21.91 +/- 4.02 % Pellet and 27.51 +/- 5.09% Hay. Only PZ and CZ gave an additional EFA supplement during the intake trial period, accounting for 2.31% and 4.89% of the diet respectively -see Figure 1. Browse consumption varied among institution according to seasonal availability: PZ offered an additional (mean daily weight (g) +/- SE) 4427.70 +/- 362.14 g of browse during the summer months when several species of browse were freely available. CZ and PLZ offered a limited range of browse on an ad hoc basis during the winter months, offering as much as was available at time of feeding. Winter availability of browse was limited to white poplar (*Populus alba*), willow (*Salix* spp.) and hawthorn (*Crataegus monogyna*) for CZ and willow (*Salix* spp.), apple (*Malus communis)*, sweet chestnut (*Castanea sativa)* and silver birch *(Betula pendula)* for PLZ.

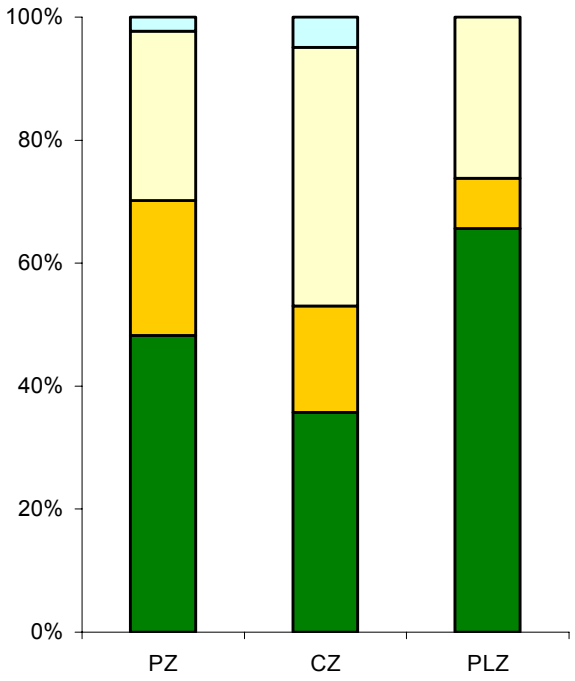

Figure 1. Mean daily percentage intake of each food group per rhino at each UK institution. Produce, Pellet, Hay, EFA supplement.

Proximate analysis

The mean daily intake of proximate nutrients did differ according to institution. One- way ANOVA test show: Total dietary fibre, F = 4.619, d.f. = 2, P<0.05; Crude fat, F = 9.438, d.f. = 2, P<0.05; Crude Protein, F = 4.459, d.f. =2, P< 0.05; Ash, F = 33.646, d.f. = 2, P<0.05. A

Bonferroni post hoc test showed the variability between each institution was dependent on the particular nutrient, with no consistent pattern according to institution.

EFA content- *Produce*

Zootrition software revealed that the produce items contain substantial amounts of Linolenic acid (n-3 EFA) and Linoleic acid (n-6 EFA). Since produce comprised nearly half of the diet offered, this was investigated further and the mean daily intake of EFA, contributed by produce was calculated-see Figure 2. A one-way ANOVA test showed a significant difference in each EFA according to institution: Linolenic acid (n-3) $F = 109.856$, d.f. = 2, $P<0.05$; Linoleic acid (n-6) $F = 4.506$, d.f. = 2, $P<0.05$.

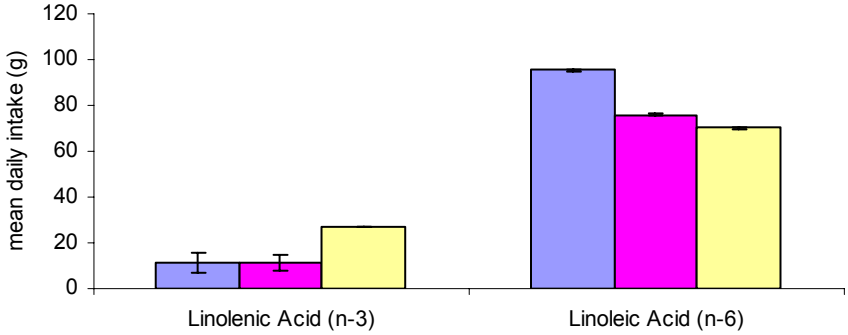

Figure 2. Mean daily intake of Linoleic and Linolenic essential fatty acids available from produce only. Data shown for Paignton Zoo, Chester Zoo and Port Lympne Zoo.

EFA content - *GC-MS analysis*

Gas chromatographs show several n-3 and n-6 EFA, and their presence/absence was recorded. Total fatty acid profiles of browse varied according to species and institution, as did pellet feeds and hay types- although unreliable data from PZ hay types calls for repeated experiments. The two EFA of particular interest to this study, Alpha-Linolenic Acid (18:3n-3) and Alpha-Linoleic Acid (18:2n-6), were investigated further by comparing their presence/absence according to each institution. There was a skewed distribution of n-3 and n-6 EFA in the total diets of each institution, as shown in Figure 3, whereby diets repeatedly contained more n-6 EFA.

Chi-squared goodness of fit tests revealed there was no significant difference in the presence of either EFA in *total* dietary components, between institutions: For (18:3n3) $\chi^2 = 0.33$, d.f. =2, $P > 0.05$; (18:2n6) $\chi^2 = 0.14$, d.f. =2, $P > 0.05$. Furthermore, the browse components *alone* were compared between institution in the same way, revealing similar non-significant results: (18:3n3) $\chi^2 = 0.36$, d.f. =2, $P > 0.05$; (18:2n6) $\chi^2 = 0.13$, d.f. =2, $P > 0.05$.

Although data from PZ was unreliable for hay analysis, CZ and PLZ showed that lucerne had similar fatty acid profiles, both containing n-6 but not n-3 EFA. Dry pellet feeds showed similar results across all institutions, whereby most contained n-6 EFA, and all but the Linseed supplement and PLZ pellet did not contain n-3 EFA.

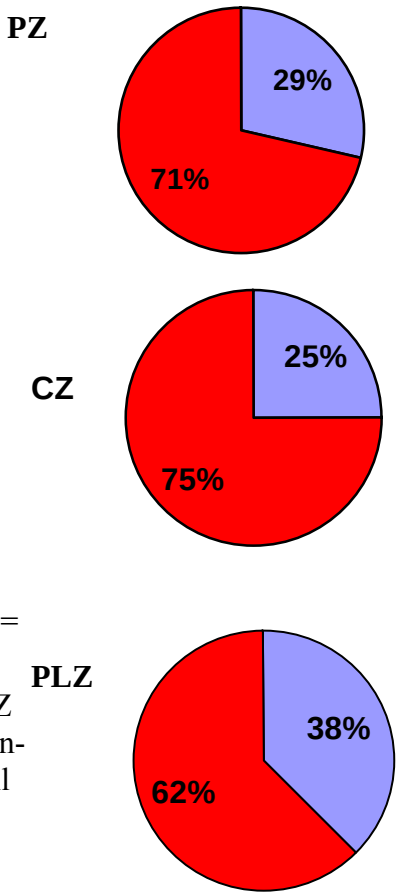

Figure 3. Percentage presence of 18:3n3, 18: 2n6 in total diets of PZ: Paignton Zoo, CZ: Chester Zoo and PLZ: Port Lympne Zoo.

Discussion

EFA Supplement Trial

Results show that the addition of an EFA supplement did not significantly affect skin appearance and coloration of either rhino, or the behaviour of the female. State behaviours of the male also remained unaffected, however his locomotion and frequency of foot lifts did vary after receiving the supplement. Locomotion bouts and foot lifting were analysed due to the chronic foot problems previously suffered by this male, and therefore a change in these behaviours in particular is to be expected. After supplementation the male spent an increased amount of time lying down and less time standing. This is counter-intuitive to expectations of therapeutic effects of EFA supplementation, since improved skin condition due to the supplement should encourage more weight bearing on the affected skin (i.e. the feet). Any benefit or detrimental effects of the EFA supplement on the male's feet would also be reflected in the locomotary actions, and although standing and laying behaviour was affected, time spent walking remained unchanged. It is reasonable to assume that any major effects of the supplement on foot lesions would inhibit walking primarily, since this involves periodic weight bearing, and an abrasive action on the footpads. As this was not the case, behavioural observations reveal that the therapeutic effects of an EFA supplement trial via 'Linseed Lozenges' remain inconclusive.

Neither Skin assessment through condition scores or photographs revealed any improvement in skin condition in the subject animals during this trial, which indicates the aetiology of MCUS lies elsewhere. However the experimental limitations of this study may mask more conclusive findings; namely the short period of the trial (Nordstrom *et al*. (1995) suggest that 3 months of linseed oil supplementation may not be sufficient to change the n-3: n-6 balance in humans) and the relatively small amount of n-3 EFA contained in the Linseed lozenge (3.74% Alpha Linolenic Acid).

Dietary Intake Study

Intake data revealed produce to be the main component of diets offered in UK institutions followed by hay and lucerne. There was however significant variability in diet according to institution, as reflected by the proximate analysis of feedstuffs. The type of produce in the diet also varied according to institution. Browse, the primary dietary component for free ranging rhino, was unable to be quantified for most zoos, and was given on an ad hoc basis, such that as much as was available was given per feed. The seasonal availability of browse was apparent both in variety and quality of vegetation; warmer months allowed for a richer variety of leafy trees such as willow (*Salix* spp.) and hazel (*Corylus* spp.), which lacked greenery during the winter. Browse availability also varied according to each institution, and depended primarily on the space available for growing edible trees and shrubs.

EFA Profile

The presence of n-3 or n-6 EFA in browse did not significantly vary according to each institution. Since browse species did differ according to institution, this suggests that the amount of browse offered, rather than the particular browse species, is the foremost factor concerning EFA intake. Quantitative analysis of each EFA is needed to further support this claim. There is a slight bias towards the presence of n-6 in relation to n-3 EFA in the browse offered at each institution. This bias is more apparent in the hay and pellet feeds, and is reflected in the entire diet including produce. This trend is also seen in US institutions but is reversed in African browse (Grant *et al*. 2002; Ghebremeskel *et al*. 1991), which supports the notion that captive diets offer a skewed n-3: n-6 diet not present in wild browse. Lack of quantitative evidence for this study masks any conclusive evidence of n-3 deficiency; however supportive quantitative data from produce, the primary dietary component of UK captive rhino, suggests this is a reasonable assumption. The more balanced n-3: n-6 ratio seen in browse more than any other food type reinforces the view that browse is the most appropriate food for this species. However since browse availability is still a limiting factor, re-addressing the n-3: n-6 imbalance via supplementation remains the best approach.

EFA Biochemistry

The role of EFA in skin structure and function is twofold; firstly, due to their structural incorporation into membrane phospholipids, and secondly as the shortest precursors of eicosanoids; a group of intracellular messengers controlling several biological processes including inflammatory reactions (Kurowska *et al*. 2003). N-3 and n-6 derived long chain fatty acids compete and displace each another in cell membranes, as well as causing an appropriate shift in the inflammatory response (Simopoulos 2002). Thus the n- 3 ALA present in linseed supplement acts in an anti-inflammatory, anti-thrombotic manner, and potentially alleviates the inflammatory state linked to MCUS prevalence (Ball *et al*. 2005). The skewed ratio of n-3: n-6 shown in UK captive diets therefore bears significance, since n-6 may have a detrimental affect on MCUS prevalence.

In addition to increased EFA intake, factors that limit its uptake and 'bioavailability' once in the body are also important. Once ingested, all EFA must undergo lipolysis via gastric lipases, and solubilization in the upper small intestine through bile and pancreatic secretions (Minich *et al*. 1997). Following solubilization, EFA are then taken up across the apical membrane of enterocytes; although this mechanism is unknown (Minich *et al*. 1997), Punchard *et al*. (2000) argue a facilitated, specific absorptive mechanism involving a transport protein. This theory complies with the known sensitivity of enterocyte membranes to the degree of intra-luminary fatty acid saturation (Levey *et al*. 1992). Once in the enterocyte, fatty acids are re-esterified and exported via the lymphatics into the circulation (Minich *et al*. 1997) and then to target tissues such as the liver; the primary site for the desaturation and elongation reactions that form long chain polyunsaturated fatty acids (Bezard *et al*. 1994).

In view of this process, inappropriate absorption of the EFA present in supplements and browse will limit its bioavailability to the subject animal and may result in EFA deficiency regardless of EFA intake. Changes in dietary fat, carbohydrates and protein levels have been shown to affect the intestinal uptake of EFA in rat models (Thomson *et al*. 1989), while furthermore EFA deficiency itself can perpetuate malabsorption by inhibiting solubilization by bile, and intracellular fat absorption (Minich *et al*. 1997). Therefore any deficiencies already experienced by the subject animals may hinder the response to EFA supplementation, or increased EFA intake through elevated browse consumption. Furthermore, sensitive ulcers causing pain (as foot lifting in affected black rhino suggest), indicates correct nerve function but impaired vascular circulation (F. Bowling, pers comm. 2006). This hinders white blood cells from reaching the affected area, leading to insufficient clearance of toxins and eventual breakdown of the skin (Dr. W. Edney, pers comm. 2006). As such any increase in dietary EFA may not reach the appropriate target tissues once in the circulation, again hindering any therapeutic response of supplements.

Implications for MCUS treatment

This study did reveal dietary discrepancies between captive UK and published data from free ranging diets of black rhino, suggesting a possible over-consumption of n-6 EFA, and lack of n-3 EFA. However it is unlikely that MCUS is the result of a simple EFA deficiency, and since other dietary considerations need to be made with such supplementation, it is not surprising that immediate effects of elevating dietary EFA through a linseed supplement were not observed in this trial.

Further work is required to support the deficiency claims made in this report, not least to quantify the EFA present, but also through blood sampling and adipose tissue analysis of captive animals to monitor the intake and metabolism of the nutrients once ingested. In this way, MCUS symptoms can be further correlated with concentrations of plasma EFA, in order to identify a possible causal relationship. Comparative immune responses across all rhino species have shown the lymphocyte action of black rhino to be less vigorous than other species, indicating a possible pre-disposition to diseases such as MCUS (Vance *et al*. 2004). Dierenfeld *et al*. (2005b) also show that over 50% of captive US black rhino have undetectable levels of ALA from adipose

tissue analysis; revealing a long term dietary deficiency. Evidence of such long-term deficiencies may prove difficult to reverse by supplements alone, given the previously discussed factors affecting malabsorption. However appropriate nutrition from birth may act to prevent the onset of MCUS and other related diseases later in life. Both of these studies highlight the need for close monitoring of disease prevalence in this captive species, while it seems clear that nutrition is integral to maintaining viable captive populations that will act as a safety net against species extinction.

Conclusion

This study highlights nutritional discrepancies in the EFA profile of captive black rhino in the UK, compared to published data for wild animals. A bias towards the presence of n-6 in relation to n-3 EFA was found in each food group fed to captive animals, and was reflected quantitatively in produce, the primary component of the diet. Further data is needed to quantify EFA presence in all food groups, specifically browse, which revealed a more balanced n-3: n-6 ratio and may prove vital in correcting this perceived 'imbalance'. Additional investigations are also required to correlate EFA presence with the prevalence and intensity of MCUS in captive animals, since the therapeutic effects of an n-3 EFA supplement trial remained inconclusive due to experimental limitations. The role of EFA in the prevalence of MCUS symptoms remains unclear, however results indicate comparative experiments of wild and captive nutrition may play an integral role in maintaining viable captive populations of black rhino in the UK.

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