

The absorption of natural vitamin C in horses and anti-oxidative capacity: a randomised, controlled study on trotters during a three-month intervention period

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Abstract

The primary aim of the present study was to assess whether low dose vitamin C supplemented in its natural form, as a dried powder from selected subspecies of rosehip (*Rosa canina*), would result in a detectable increase in the concentration of blood vitamin C in horses supplemented daily for three months. In a secondary part of the study, the anti-oxidant capacity of the present treatment was also tested. Sixteen horses were randomly allocated to two groups and supplemented with either 25 g rosehip powder daily (equal to 125 mg natural vitamin C), or with 50 gram rosehip powder daily (equal to 250 mg vitamin C). Serum vitamin C was analysed both prior to supplementation and then again 14, 28 and 84 days following supplementation. Anti-oxidative capacity was assessed as the release of oxidative anions from polymorph-nucleated leucocytes prior to supplementation and after 14 and 84 days, respectively. Whilst there was a trend towards an increase in serum vitamin C concentration following 84 days of supplementation with 125 mg of natural vitamin C, this was not statistically significant. However, a significant increase in serum vitamin C concentration was observed following 14 days of supplementation with 250 mg vitamin C ($P<0.02$). The magnitude of this increase was greater following 28 and 84 days of supplementation ($P<0.02$ and $P<0.02$), respectively. Additionally, serum vitamin C concentration was significantly higher following supplementation with 250 versus 125 mg of vitamin C daily ($P<0.03$). The higher dose of natural vitamin C also resulted in a significant reduction in the release of oxidative anions ($P<0.001$). These results suggest that natural vitamin C from rosehip is absorbed sufficiently to raise serum vitamin C concentration and to reduce the liberation of oxidative anions in horses supplemented with less than 500 mg daily.

Keywords: vitamin C, rosehip, horses, antioxidants, dietary supplements

1. Introduction

Exercise and the associated increase in oxygen consumption and increased flux through the aerobic energy generating pathways are linked with an increased production of free radicals. When the production of free radicals overwhelms the body's antioxidant defences, oxidative stress can occur with subsequent deleterious effect on cellular structures. In horses, oxidative stress has been shown to occur during exercise (Mills *et al.*, 1996) and has also been implicated

in the aetiology of a number of equine diseases including recurrent airway obstruction (Kirschvink *et al.*, 2002), exercise induced pulmonary haemorrhage (Derksen, 1997), joint disease (Dimock *et al.*, 2000) and muscle damage (Perkins *et al.*, 1998). Oxidative stress is more likely to occur when dietary antioxidant provision is sub optimal, as reviewed by (Deaton and Marlin, 2005). Ascorbic acid is a major component of the antioxidant defences in lung lining fluid and vitamin C status has been identified as a factor in the pathogenesis of chronic inflammatory airway

diseases in horses (Kirschvink *et al.*, 2002a). Furthermore, a significant antioxidant function for vitamin C in synovial fluid has also been described in horses (Murray *et al.*, 2009). However, unlike humans, but like most other vertebrates, the horse under normal circumstances has no dietary requirement for ascorbic acid, as it is synthesised in the liver ultimately from glucose. Vitamin C status is reported to be reduced, however, following prolonged exercise (Marlin *et al.*, 2002). In a study on 2 and 3 year old trotters (n=81) a mean blood vitamin C level of 32.5 and 36.4 $\mu\text{mol/l}$, respectively (range 9.1 to 51.1 $\mu\text{mol/l}$) was demonstrated (Rasbeck and Kofoed-Johnsen, 1987). In the same study, it was also documented that blood vitamin C was up to 25% lower in the autumn than in the spring in horses in active race training, most likely as the result of cumulative training and racing during the season. The lowest vitamin C level in blood in this aforementioned study was found in horses with infection (Rasbeck and Kofoed-Johnsen, 1987). Dietary supplementation with vitamin C has, therefore, been suggested to be a significant factor in maintaining ascorbic acid status during times of 'stress', or where disease is present (Deaton and Marlin, 2005).

Specifically it has been suggested that supplementation with vitamin C could moderate oxidative stress and thereby ameliorate muscle damage (Hargreaves *et al.*, 2002) inflammatory airway disease (Kirschvink *et al.*, 2002b; White *et al.*, 2001) in horses and inflammatory joint disease in humans (Peregoy and Wilder, 2011). The role of vitamin C in inflammatory disease in horses is therefore reasonably well established. Vitamin C status is also found to be reduced with infection (Jaeschke, 1984; Jaeschke and Heller, 1978) and previous studies have shown an improved antibody response to vaccine in aged horses following short-term bolus administration of vitamin C (Ralston, 2001).

Absorption of vitamin C is reported to occur in the horse by passive diffusion in the ileum (Lewis, 1995) and not by sodium dependent active transport as for humans (Rumsey *et al.*, 1999). Absorption of vitamin C in the horse appears to be affected by both the level and form in which the vitamin C is supplied. Although the plasma level of vitamin C was elevated by feeding 20 g of ascorbic acid daily for 25 days, it was not affected by a single 20 g administration (Snow and Frigg, 1987, 1989, 1990). Ascorbyl palmitate, a lipid soluble derivative of ascorbic acid, is more bioavailable than either ascorbic acid or ascorbyl stearate, although there is significant reported variation between individuals (Snow and Frigg, 1989, 1990). Calcium ascorbyl 2 monophosphate also failed to increase plasma vitamin C following 2 weeks of daily supplementation (20 mg/kg bodyweight).

In contrast, it has recently been reported that the administration of a single dose of natural vitamin C (1 g) as a standardised powder from certain selected subspecies of rosehip significantly increased vitamin C concentration

in blood 2 hours following administration (Winther *et al.*, 2010).

The present study aimed to investigate whether long-term administration of a relatively low level of natural vitamin C (from 125 to 250 mg daily) would result in any detectable increase in serum vitamin C in horses and further to elucidate if such changes have any impact on oxidative stress, when estimated as the release of oxidative anions from polymorph-nucleated leucocytes (PMN's). As the demand for dietary vitamin C in horses in trotting training is expected to be most pronounced in the autumn towards the end of the racing season, as previously reported (Rasbeck and Kofoed-Johnsen, 1987), the horses in this study were supplemented between August and the end of October.

2. Materials and methods:

Sixteen horses (Standardbred trotters), 9 mares and 7 geldings, mean age 3.4 years (range 2-6 years) were all maintained on the same standardised long-term dietary management and training programme. All of the horses used in this study were from the same stable, were in an identical training programme and undertook regular competition. The training programme would normally run as follows:

Monday: relaxing and 30 minutes running at low speed;
 Tuesday: intervals;
 Wednesday: relaxing;
 Thursday: intervals;
 Friday: relaxing and 30 minutes running at low speed;
 Saturday: relaxing;
 Sunday: competition.

The training and competition programme had been unchanged during the previous 6 months, before entering this trial and continued unchanged during the 3-month treatment period. This is important as exercise itself can influence oxidative stress (Mills *et al.*, 1996). All horses included in the trial were assessed at the same time of year (August to October) where vitamin C levels are reported to be lower (Rasbeck and Kofoed-Johnson, 1987). This was done in order to limit any potential effect of seasonal variation on vitamin C status. In addition, all horses were free from known respiratory diseases, previous injury or behavioural issues – all of which have been previously reported to depress vitamin C status.

Horses were randomly divided into two groups of 8 horses and were daily supplemented with either 25 g or 50 g of rosehip powder (LitoVet, Hyben-Vital, Langeland, Denmark). The amount of rosehip powder fed was equivalent to 125 mg or 250 mg of vitamin C, respectively. In order to ensure that the study was carried out on

a blind basis, the group of horses given 25 g of rosehip was also supplemented with a placebo powder of similar colour, odour and taste fed at a ratio of 1 to 1. Each horse in the study was therefore given an identical weight of powdered supplement daily. The supplement containers were randomly labelled from 1 to 16 and randomisation took place in blocks of 4 yielding two groups of eight horses, one group receiving 125 mg and the other receiving 250 mg of natural vitamin C.

The dose of 250 mg and 125 mg were chosen for this study, because natural vitamin C is absorbed within 2 hours by horses supplemented with the same ingredient at a level of 1 g daily, as shown by the authors in a previous study (Winther *et al.*, 2010). In addition, supplementation as low as 125 mg is absorbed in humans within a few hours (K. Winther, personal communication).

The rosehip powder was top-dressed onto the standardised daily diet for a period of three months from August to October. The two groups were balanced for age, but not for sex, as there is no reported effect of gender difference on vitamin C status in horses. There were 6 mares and 2 geldings in the high-dose group and 6 geldings and 2 mares in the low-dose group. The study was completed by all 16 horses with none being withdrawn.

The LitoVet rosehip supplement is a powder, which consists of the entire rosehip fruit from selected subspecies of rosehip (*Rosa canina*). This means that all the seeds and the entire shell of the rosehip fruit are included. Immediately after harvesting, the fruits were frozen and kept frozen throughout a patented drying process, where the temperature remains below 40 °C and runs for several days. This drying methodology ensures that heat labile active ingredients are preserved. This rosehip powder is standardised for vitamin C content and for the content of a further active ingredient, an anti-inflammatory agent and member of the galactolipid family (GOPO), which is present in higher amounts in very few plant species (Larsen *et al.*, 2003).

Blood samples for the analysis of vitamin C and a marker of oxidative stress were taken in the morning at 7:00, immediately prior to feeding by venipuncture of the jugular vein using two 5 ml vacutainers. Blood samples were taken prior to supplementation and again following 14, 28 and 84 days of supplementation with rosehip. Blood samples were brought to the laboratory in less than one hour to avoid breakdown of active ingredients. Vitamin C was analysed using an established photometric methodology (Hausman Lench and Lewis, 1961). The release of oxidative anions from PMNs was assessed at the start, after 14 days and at the end of the study after 3 months using chemiluminescence (Khazazmi and Winther, 1999).

All data are presented as mean with lower and upper 95% confidence interval given in brackets. The non-parametric Wilcoxon test was used for statistical comparison within horse groups, whereas the non-parametric Mann-Whitney test was used for statistical comparison between horse groups. The comparison of data between the two groups over time was performed using analysis of variance for repeated measures (ANOVA). The ANOVA repeated analysis was based on the difference from day 0 to day 14, day 28 and day 84, respectively, stated in the text as delta values. Statistical significance was declared at $P < 0.05$.

3. Results

Mean pre-supplementation concentration of serum vitamin C in the 25 g group was 20.0 (16.4-23.7) $\mu\text{mol/l}$, which was not significantly different from that observed in the group supplemented with 50 g of rosehip: 18.5 (13.9-23.0) $\mu\text{mol/l}$. Mean concentration of vitamin C in serum following 14, 28 and 84 days of supplementation with 25 g of rosehip is presented in Figure 1A. Whilst there was a small increase in serum vitamin C concentration over the period of supplementation in the group fed 25 g of rosehip, this was not significantly different from the baseline pre supplementation concentration. In contrast, mean serum vitamin C was significantly increased compared to the pre-supplementation level in the group fed 50 g of rosehip after 14 days of supplementation (21.4 (16.7-26.0) vs. 18.5 (13.9-23.0), $P < 0.02$) and was also significantly different from that observed in the group fed 25 g of rosehip after the same time point ($P < 0.03$) (Figure 1B). There was a further significant increase in mean serum vitamin C, in the 50 g supplemented group after 28 and 84 days, respectively ($P < 0.02$) reaching a concentration 30% above the initial pre-supplementation concentration (Figure 1B).

Whilst the increase in vitamin C concentration in the group of horses fed 25 g rosehip (125 mg of vitamin C) was not significant compared to the pre-supplementation level, it had increased sufficiently by 84 days of supplementation to ensure that there was no longer a significant difference in serum vitamin C concentration between horse groups.

The relative increase in serum vitamin C concentration above the pre-supplementation level was calculated for both groups (Δ vitamin C). Mean Δ vitamin C was -1.2 (-4.6-2.2) $\mu\text{mol/l}$ following 14 days of supplementation with 25 g of rosehip and 2.9 (1.5-4.3) $\mu\text{mol/l}$ following 50 g of rosehip supplementation, respectively ($P < 0.02$). Comparison of the two groups over the period of supplementation (ANOVA) revealed a significant difference in Δ vitamin C ($P < 0.03$).

Chemiluminescence analysis revealed that there was no significant difference in the initial liberation of oxidative anions between the 25 g and 50 g supplementation with rosehip: 4,498 mV vs. 4,304 mV ($P < 0.96$), respectively

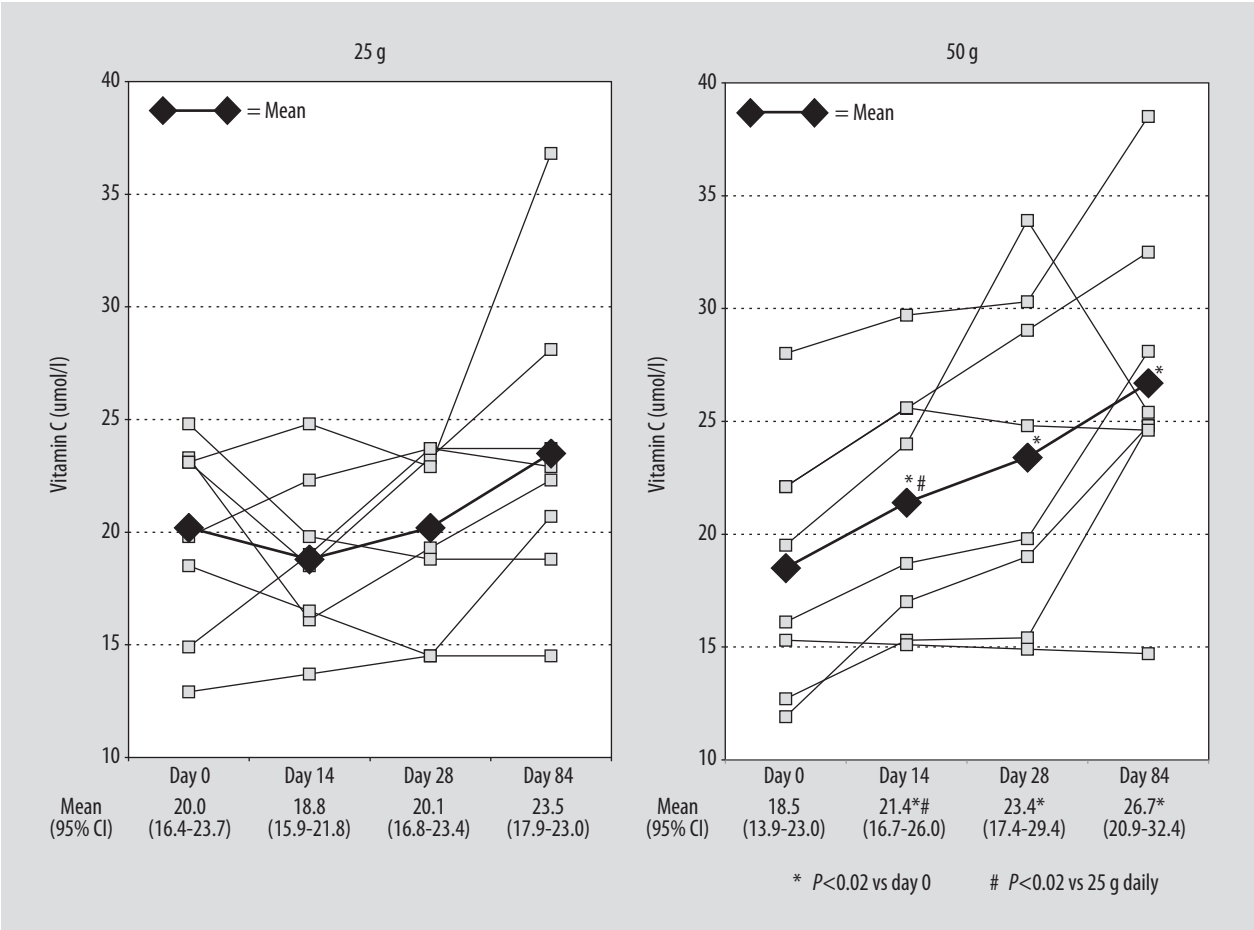


Figure 1. Serum vitamin C levels in each of the horses daily given (A) 25 g and (B) 50 g of standardised rosehip supplement. Mean values are given for each of the two groups of horses at the 4 different time points. The lower and upper 95% confidence intervals are given in brackets.

(Table 1). There was also no significant change in the release of oxidative anions during the course of the study in the group supplemented with 25 g of rosehip. In contrast, a significant decline in the liberation of oxidative anions was observed in the 50 g group after 14 days (2,028 mV, $P < 0.01$) and after 84 days of supplementation (906 mV, $P < 0.01$), respectively (Table 1). In addition, the decline in the liberation of oxidative anions observed after 14 days of supplementation for the 50 g group was significantly different to the corresponding value for the 25 g group when either the absolute values ($P < 0.03$) or Δ values (0.003) were compared (Table 1). As with the vitamin C data, comparison of the two supplementation groups after 84 days of supplementation, revealed no significant difference in the liberation of oxidative anions.

4. Discussion

The pre-supplementation concentration of vitamin C in plasma was similar to that previously reported in normal non-diseased horses (Rasbech and Kofoed-Johnsen, 1987). The present data reports that 250 mg of natural

vitamin C supplemented daily, in the form of 50 g of a rosehip supplement, resulted in a significant increase in serum vitamin C level of horses, after 14 days. When supplementation was maintained for approximately 84 days, the magnitude of the increase in serum vitamin C was doubled to reach a level in serum of 26.7 $\mu\text{mol/l}$, which was approximately 30% above the initial pre-supplementation level. In contrast, a lower level of supplementation with 25 g rosehip per day (equivalent to 125 mg of natural vitamin C), did not result in the same significant increase in serum vitamin C concentration, although some increase was observed after approximately 84 days of supplementation.

The preliminary measurement of oxidative stress in this study, estimated by measuring the liberation of oxidative anions from PMNs showed a similar pattern. Supplementation for 14 days with 250 mg of vitamin C from rosehip resulted in a significant decline in oxidative stress. This change was not observed in the group receiving 125 mg vitamin C from rosehip. After 84 days of supplementation, however, oxidative stress tended to decline in this group also, although statistical significance was not reached. We

Table 1. The release of oxidative anions from polymorph-nucleated leucocytes in horses given daily rosehip supplementation, estimated as chemiluminescence (mV). Data are given as mean values, with lower and upper 95% confidence intervals between brackets.

	Day 0	Day 14	D-value (day 0-14)	P-value ¹	Day 84	D-value (day 0-84)	P-value ¹
25 g/day (n=8)	4,498 (3,213-5,784)	4,340 (3,520-5,161)	109 (-1,366-1,584)	<0.30	3,351 (2,620-4,282)	940 (-451-2,331)	<0.19
50 g/day (n=8)	4,304 (3,319-5,289)	2,276 (964-3,589)	2,028 (1,517-2,539)	<0.01	3,397 (3,064-3,731)	906 (-43-1,856)	<0.01
P-value ^{2,3}	<0.96	0.03	0.003		0.71	0.50	

¹ The P-values are given in relation to pre-treatment levels within the same group.

² The P-values are given in relation to intergroup variation.

³ ANOVA comparison of the two groups over time yielded a P-value of $P < 0.01$.

have shown in the laboratory that GOPO, the galactolipid present in rosehip powder, can reduce the liberation of oxidative anions in isolation (Winther, unpublished data). The anti-oxidative capacity reported in this study could therefore be due to at least two different constituents: vitamin C and GOPO.

Based on the present data the authors suggest that 50 g of rosehip fed daily, which is equivalent to 250 mg of natural vitamin C, is needed to raise serum vitamin C concentration in horses and to modify oxidative stress. This data should, however, be interpreted with some caution as the ability to raise serum vitamin C concentration appeared to be related to pre-supplementation vitamin C status. Serum vitamin C concentration was in fact increased significantly in response to 84 days of supplementation with 25 g of rosehip in a subgroup of horses with the lowest pre-supplementation concentration of serum vitamin C (14.9-23.1 $\mu\text{mol/l}$) ($P < 0.031$). This was not apparent following 14 or 28 days of supplementation with 25 g of rosehip. It is therefore possible that serum vitamin C may have increased significantly in all horses in this group had the 25 g of rosehip been administered for more than 84 days.

It cannot be stated that a non-supplemented group would have significantly different vitamin C levels or PMN liberation. Anyway, a control group, untreated or treated with placebo, could have been added to the design of this study to elucidate a possible impact from the training programme, or any potential seasonal variation or environmental effects on the values measured for oxidative stress or for vitamin C. However, all horses underwent an identical training programme and seasonal variation was minimised by carrying out the supplementation study in the autumn, towards the end of the racing season, where vitamin C levels have been reported to plateau. Vitamin C status, in terms of analysis in synovial fluid, is unaffected by age; however, despite this the two horse groups were matched for age (Murray *et al.*, 2009). The mean value of

vitamin C on day 0 in the included mares and geldings was almost identical ($P < 0.833$) suggesting no sex difference as regards vitamin C levels in horses.

The decline in chemotaxis of PMNs observed in human clinical studies has been previously reported to be subject to a large carry-over effect which has also been observed in an earlier study in horses showing that the decline is maintained for up to four weeks after cessation of supplementation (Winther, unpublished results). For future study, it would be of interest to test for the oxidative burst reaction and vitamin C status following the cessation of supplementation in horses continuing the same training programme. It was beyond the scope of this study to evaluate further measures of oxidative stress. However, in future studies the investigation of the effect of supplementation on further biochemical parameters such as malondialdehyde, uric acid, creatine kinase, superoxide dismutase as well as uric and the cellular level of vitamin C could be undertaken. Also, the analysis of urinary vitamin C may have added to the results and conclusions drawn in this study, as urinary vitamin C (when expressed in relation to creatinine) has been shown to be correlated to dietary vitamin C in other animals when plasma vitamin C exceeds the renal threshold (Padilla *et al.*, 2007).

Vitamin C is known to enter the plasma and extracellular fluid relatively quickly from the gastrointestinal tract. From this extracellular phase, vitamin C is then taken up by a variety of different cell types including leucocytes. It has been suggested that a two-compartment system exists with respect to vitamin C and that serum vitamin C will only increase once certain cells have become saturated (Deaton *et al.*, 2003). This may explain why an increase in serum vitamin C in those horses fed the lower amount of rosehip (i.e. 25 g) was only achieved after 84 days compared to the 14 days in the horses supplemented with 50 g of rosehip.

It should finally be considered that other antioxidants, which are constituents of the present rosehip powder, such as vitamin E, beta-carotene and the newly isolated galactolipid GOPO, which also is a strong anti-oxidant, presumably contributed to the observed reduction in the release of oxidative anions from neutrophil leucocytes. This may explain why there was no correlation between the level of serum vitamin C and neutrophilic release of anions – as other anti-oxidants may have played an important role.

The present results are very interesting in the context of earlier studies using synthetic vitamin C. A single administration of 20,000 mg synthetic vitamin C (ascorbic acid) given to horses did not result in any detectable increase in blood (Deaton *et al.*, 2003; Loscher *et al.*, 1984). However, it has been reported that 20,000 mg of synthetic vitamin C added daily to the drinking water of 6 horses, over a period of 25 days, did result in some improvement in the concentration of vitamin C in blood, although the variation between individuals was pronounced (Snow *et al.*, 1987). The increase in vitamin C observed in this study in relation to 250 mg ascorbic acid per horse per day (~2 mg/kg body weight is comparable to that previously reported in ponies with synthetic ascorbic acid (Loscher *et al.*, 1984; Snow *et al.*, 1987). In the latter study, however, the level of supplementation with either calcium ascorbyl-2-monophosphate, or ascorbyl palmitate was significantly higher (20 mg/kg body weight). In addition, ascorbyl palmitate has been reported to offer greater bioavailability in horses compared to ascorbic acid (Snow and Frig, 1989).

This study therefore suggests that a comparable increase in serum vitamin C concentration can be achieved with this natural form of vitamin C from rosehip using a fraction of the amount of synthetic sources previously used. The bioavailability of micronutrients such as vitamin E in horses has been previously reported to increase when presented in a natural form (Pagan *et al.*, 2005). It should therefore also be borne in mind, that the seeds from rosehip contain natural vitamin E, as vitamin E itself might have some impact on the reported anti-oxidative capacity.

The authors have shown in an earlier study (Winther *et al.*, 2010) that a single administration of 1000 mg of natural vitamin C, when fed as 210 g of rosehip powder resulted in a significant increase in the serum concentration of vitamin C in horses after just 2 hours ($P < 0.05$). The present data indicate that 250 mg of natural vitamin C, when supplemented on a long-term basis improves serum vitamin C status. It is interesting to note that such a low level of supplementation with natural vitamin C, given as a powder consisting of the entire dried fruit of a subtype of rosehip, yields an increase in serum vitamin C which resembled the concentration obtained following supplementation with synthetic vitamin C as ascorbic acid at a level 80 times higher (Loscher *et al.*, 1984; Snow *et al.*, 1987) and that

this low level of natural vitamin C supplementation can influence the liberation of oxidative anions.

It was not the aim of the present study to explain the basic mechanisms of this phenomenon, but the authors suggest that within the matrix of the rosehip powder there may be some 'carriers' or co-factors, which facilitate the uptake of vitamin C from the digestive tract. The relevance of this study lies in the role of vitamin C in maintaining respiratory function and joint integrity. It has been previously established the vitamin C content of lung lining fluid is influenced by the vitamin C content of the diet (Deaton *et al.*, 2003). Furthermore, the importance of pulmonary vitamin C status in horses with recurrent airway obstruction has also been previously reported (Deaton *et al.*, 2004; Kirschvink *et al.*, 2002b). The provision of natural vitamin C in its natural form in conjunction with other known anti-inflammatory mediators (GOPO) in this specific sub-species of rosehip has reputed beneficial effects for cartilage and for osteoarthritis in man (Christensen *et al.*, 2008; Schwager *et al.*, 2011) and preliminary data suggests a beneficial effect in working horses (Winther *et al.*, 2010).

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