Variation in deficient serum vitamin E levels and impact on assessment of the vitamin E status in horses

Variatie in deficiënte serum vitamine E-concentraties en de impact op het bepalen van de vitamine E-status bij paarden

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ABSTRACT

A large fluctuation in normal serum vitamin E (alpha-tocopherol) concentrations (> 2 μg/ml) has been demonstrated in normal horses. The purpose of this study was to verify the fluctuation in serum vitamin E in horses with *deficient* levels (< 1.5 μg/ml) and to assess the diagnostic value of a single serum sample to determine the vitamin E status in a deficient horse. Serum vitamin E levels were monitored over a period of 24 hours in 6 normal horses and in 22 horses with clinical signs that may be related to vitamin E deficiency. The serum vitamin E levels varied widely within each horse, and the coefficient of variation (CV) was significantly larger in the deficient horses (mean CV: 41%), compared to the horses with normal levels (mean CV: 14%). In a small proportion of the deficient horses, the vitamin E levels varied from deficient to normal within 24 hours. The sensitivity of a single sample compared to the "true value" determined by the average of the samples, was 91%. The sensitivity in case of 2 serum samples increased to 97%. These findings suggest that a single serum value does not always provide reliable information about the true vitamin E status in a deficient horse and analyzing 2 serum samples allows a more accurate estimation of the vitamin E status. The conclusion therefore is that when a serum vitamin E value > 1.5 μg/ml is found in clinically suspected horses, a second serum sample should be evaluated.

SAMENVATTING

Bij normale paarden is er een ruime fluctuatie van de normale vitamine E-concentraties (> 2 μg/ml) in het serum aangetoond. Het doel van deze studie bestond erin om de fluctuatie van serumvitamine E bij paarden met deficiënte concentraties (< 1,5 μg/ml) aan te tonen en om de diagnostische waarde van één enkel serumstaal bij het bepalen van de vitamine E-status van deficiënte paarden na te gaan. Serumvitamine E-concentraties werden bepaald gedurende 24 uur bij 6 normale paarden en bij 22 paarden met klinische tekenen die konden te wijten zijn aan een vitamine E-deficiëntie. De serumvitamine E-concentraties waren sterk verschillend tussen de dieren onderling en de variatiecoëfficiënt (CV) bleek significant groter bij de deficiënte paarden (gemiddelde CV: 41%) dan bij de normale paarden (gemiddelde CV: 14%). Bij een klein aantal deficiënte paarden varieerden de vitamine E-concentraties van deficiënt tot normaal gedurende een periode van 24 uur. De sensitiviteit van één serumstaal, vergeleken met het werkelijke vitamine E-gehalte bepaald door het gemiddelde van de stalen, was 91%. De sensitiviteit aan de hand van 2 serumstalen nam toe tot 97%. Deze bevindingen tonen aan dat één enkel serumstaal mogelijk geen betrouwbare interpretatie van de werkelijke vitamine E-status bij deficiënte paarden toelaat. Door 2 serumstalen te meten is een accuratere benadering mogelijk. Wanneer bij klinisch verdachte paarden een serumvitamine E-concentratie groter dan 1,5 μg/ml wordt gemeten, is het aangewezen om minstens een tweede serumstaal te analyseren.

INTRODUCTION

Vitamin E (alpha-tocopherol) is a fat-soluble vitamin with important anti-oxidative properties, and the deficiency of this vitamin is related to oxidative disorders. In horses, vitamin E is associated with deficiency syndromes such as equine motor neuron disease (EMND) (De la Rua-Domenèch *et al.*, 1997; Mohammed *et al.*, 2007), equine degenerative myelopathy (EDM) (Mayhew *et al.*, 1987), degenerative myopathies (Valentine *et al.*, 2002; Pearson *et al.*, 2005; Ludvikova *et al.*, 2007) and white muscle disease in foals (Löfstedt, 1997). Similar clinical signs are reported in humans, in whom prolonged vitamin E deficiency can cause ataxia, muscle weakness, diminished reflexes, peripheral neuropathy, blindness, cardiac arrhythmias and dementia (Tanyel and Mancano, 1997).

Early and accurate detection of vitamin E defi-

ciency in the horse is important because a chronic deficiency can lead to EMND (Divers *et al.*, 2006; Mohammed *et al.*, 2007). In addition, non-specific clinical signs such as fatigue, poor performance and weight loss may also be encountered and are associated with EMND (Divers *et al.*, 2001). In these horses it is of particular importance that a deficiency is recognized at an early stage, before the horses develop overt clinical signs. The fact that residual symptoms have been described in stabilized cases of EMND is proofs the importance of early recognition of deficient vitamin E levels in the individual horse and the initiation of the appropriate treatment. Early recognition and treatment result in less irreversible loss of motor neurons, which may mean a better prognosis.

In horses, evaluation of the vitamin E status poses a particular problem as the serum vitamin E concentrations (even in normal horses) are often low compared to humans, in whom serum vitamin E concentrations below 5μg/ml are considered deficient (Machlin, 1991). The reported values for normal horses range from 1.0 μg/ml to 9.5 μg/ml (Maylin *et al.*, 1980; Baker *et al.*, 1986; Steiss *et al.*, 1994). In man, a more accurate assessment of the vitamin E status is achieved by relating serum vitamin E to serum lipids or serum cholesterol (Horwitt *et al.*, 1972), or by determination of vitamin E concentrations in adipose tissue (Kayden *et al.*, 1983). However, these methods do not apply to horses because of the poor correlation between serum vitamin E and serum total lipids or cholesterol (Craig *et al.*, 1989) and the large variability of vitamin E concentrations in adipose tissue in clinically normal adult horses (Steiss *et al.*, 1994). As a result, the measurement of serum vitamin E remains the most practical procedure for determining the vitamin E status in the horse. However, it has been shown that, over a 72-hour period, serum vitamin E levels can vary widely within the individual, *normal* horse (Craig *et al.*, 1989). The fluctuation of serum vitamin E levels in *deficient* horses had not yet been described previously. The objectives of this study were to determine the fluctuation in serum vitamin E in horses with deficient levels (< 1.5 μg/ml) and to verify whether this fluctuation can occasionally result in overestimation of the serum vitamin E levels as 'low' (between 1.5 μg/ml and 2 μg/ml) or 'normal' (> 2 μg/ml), when in fact the true vitamin E status in the horse is 'deficient'.

MATERIALS AND METHODS

Twenty-eight horses (13 mares, 3 stallions and 12 geldings), aged 5 to 14 years, were included in the study. The mares were not pregnant or lactating.

The sample population consisted of 22 horses (horses 7-28) that were admitted to the Department of Large Animal Internal Medicine, Ghent University, with clinical signs such as weight loss, dull hair coat, poor musculature (or even muscle atrophy) and poor performance that might be attributed, at least in part, to a vitamin E deficiency. In addition, six clinically healthy horses (horses 1-6) were included to serve as controls. Samples were collected on the day after admission, and for all horses the feeding conditions and stable management were similar during the sampling period. None of the horses received a vitamin E supplement prior to or during the study, and each was fed a ration composed of a grain-based concentrate and hay at 7:30 hours and 16:00 hours. Blood sampling started at 8:00 hours and blood was collected over a 24-hour period every 4 hours from 11 horses (horses 7-8, 11, 12, 14, 15, 17-20, 28) and every 8 hours from 17 horses (horses 1-6 and 9, 10, 13, 16, 21-27). When the blood samples were collected, care was taken to avoid contact between the blood and the rubber tube cap and the blood tubes were protected from light. The blood was immediately centrifuged, and the serum was put in a polypropylene tube and frozen at -18°C within 1 hour after blood collection. All samples were analyzed in a single day's run to eliminate between-day laboratory variances and to allow only 1 frost-defrost cycle. Serum alpha-tocopherol was quantified by high pressure liquid chromatography (HPLC) with UV detection (340nm/295nm), as described previously (Craig *et al.*, 1989).

The true vitamin E level of a horse was determined as the mean level of the combined samples taken over 24 hours and the horses were classified according to their mean vitamin E level. Serum vitamin E levels < 1.5 μg/ml were considered deficient. Levels > 2 μg/ml were considered normal and serum vitamin E levels between 1.5 and 2 μg/ml were considered low. To quantify the variation in serum vitamin E levels within each horse, the coefficient of variation (CV) was calculated for each horse.

To determine the characteristics of a single test, the sensitivity and specificity were calculated. The test was considered positive when the vitamin E level was below 1.5 μg/ml and negative when the vitamin E level was greater than 1.5 μg/ml.

To determine the predictive value of a single sample in terms of estimating the true vitamin E level of a horse, the positive predictive value (PPV) and negative predictive value (NPV) were calculated. The PPV is the probability that a positive sample is indicative of a truly deficient horse. The NPV is the probability that a negative sample is indicative of a truly non-deficient horse. With respect to the vitamin E deficiency, it is important that the diagnostic test has a high sensitivity and NPV because it is more important to detect the deficient animals correctly in order to start treatment than it is to prevent normal animals from receiving unnecessary treatment because of a false-positive diagnosis. Subsequently we evaluated the sensitivity, specificity, PPV and NPV when 2 serum samples were taken into consideration. The estimation of the sensitivity, specificity, PPV and NPV was done by means of the bootstrap method. In brief, this method makes it possible to sample randomly one result per horse and to compare this result to the true status of the horse. This procedure is repeated 1000 times, thus making it possible to estimate the parameters of interest and the

Table 1. Fluctuation in serum vitamin E concentration (µg/ml) over a 24-hour period in horses with normal, low and deficient vitamin E levels. Horses 1 – 6 are control horses and horse 7 – 28 are horses with clinical signs that might be related to a vitamin E deficiency.

group 1 (normal : mean serum vitamin E level > 2µg/ml)												
horse	1	2	3	7	8	9	10	11	mean			
vitamin E												
range	3.8 – 4.75	1.77 – 2.78	1.89 – 2.18	2.8 – 4.2	2.06 – 2.93	1.85 – 2.55	3.23 – 3.57	2.1 – 2.7				
mean (SD)	4.38 (0.51)	2.44 (0.58)	2.02 (0.15)	3.5 (0.52)	2.41 (0.4)	2.1 (0.38)	3.41 (0.17)	2.35 (0.25)				
CV	11%	23%	7%	15%	16%	18.2%	5%	11%	14%			
group 2 (low : mean serum vitamin E level between 1.5 µg/ml and 2 µg/ml)												
horse	4	5	6	12	13	14	15	16	mean			
vitamin E												
range	1.84 – 1.94	1.7 – 1.72	1.42 – 1.79	1.26 – 2.34	1.14 – 1.97	0.17 – 3.93	0.47 – 2.1	1.49 – 1.95				
mean (SD)	1.88 (0.05)	1.7 (0.01)	1.65 (0.2)	1.87 (0.5)	1.61 (0.42)	1.71 (1.7)	1.52 (0.63)	1.69 (0.24)				
CV	2.8%	0.84%	12%	27%	26%	101%	41%	13%	28%			
group 3 (deficient: mean serum vitamin E level < 1.5 µg/ml)												
horse	17	18	19	20	21	22	2	24	25	26	27	28	mean
vitamin E													
range	0.5 – 1.26	0.22 – 0.53	0.18 – 0.39	0.1 – 1.75	0.1 – 1.49	0.1 – 0.21	0.41 – 0.66	1.07 – 1.45	1.13 – 1.83	1.05 – 1.2	0.93 – 1.55	0.79 – 2.1	
mean (SD)	0.75 (0.35)	0.33 (0.11)	0.23 (0.09)	0.76 (0.73)	0.78 (0.69)	0.14 (0.06)	0.53 (0.13)	1.3 (0.2)	1.37 (0.4)	1.11 (0.08)	1.29 (0.32)	1.37 (0.55)	
CV	48%	34%	38%	96%	89%	43%	24%	15%	29%	7%	25%	40%	41%

CV : coefficient of variation / SD : standard deviation

observed variation around them. The analysis was performed by using the @RISK 4.5 (Palisade) Excel add-in.

RESULTS

Group 1 consisted of 8 horses with adequate mean serum vitamin E levels, group 2 of 8 horses with low mean serum vitamin E levels and group 3 of 12 horses with deficient mean serum vitamin E levels (Table 1).

In 3 control horses normal mean serum vitamin E levels were found while the other 3 showed low mean levels.

In each group, the single serum samples presented a distribution over normal, to low and deficient values (Figure 1). In group 1, no deficient serum concentrations were measured but low serum vitamin E levels were found, at 1 sampling in 2 horses and at 2 samplings in 1 horse. In group 2 a normal value was found in 2 horses at 2 samplings and in 1 horse at 1 sampling, and a deficient value was found at 2 samplings in 3 horses and at 1 sampling in 3 horses. In group 3, 3 out of the 12 horses each had 1 low serum concentration, and 1 horse out of the 12 had one normal value in the 24-hour period.
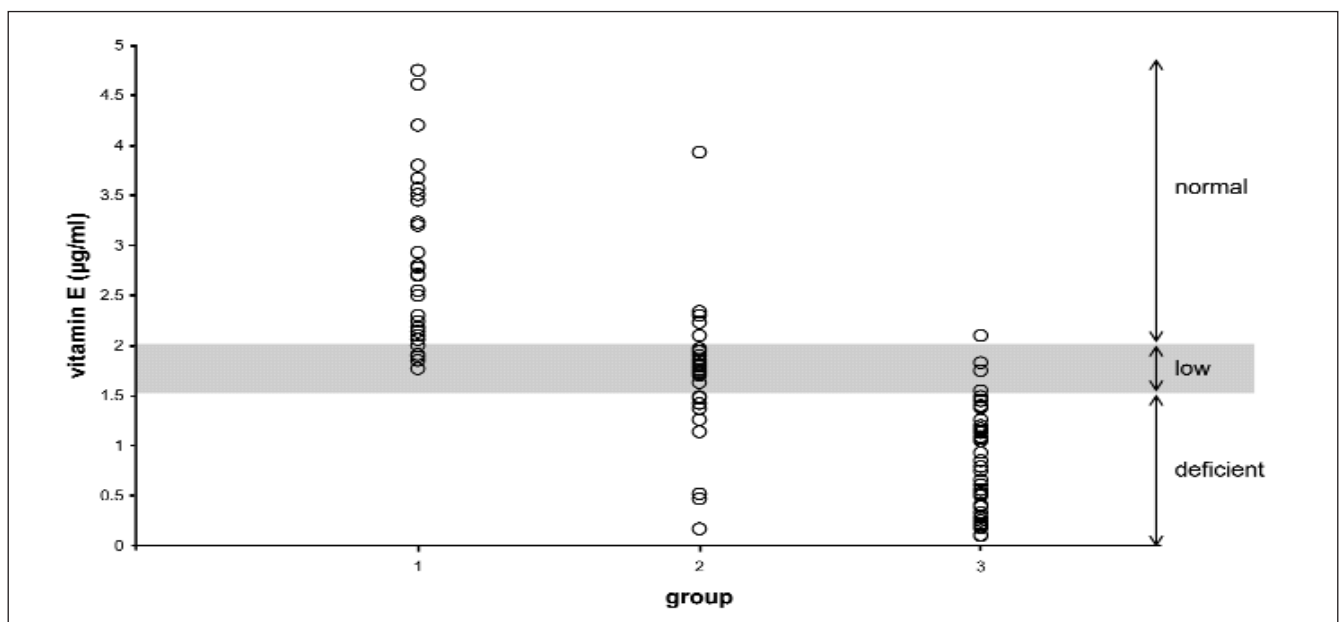

Figure 1. Single serum vitamin E values of horses with normal (group 1), low (group 2) and deficient (group 3) mean levels of serum vitamin E. In group 1, the single serum samples presented a distribution over normal to low values; in groups 2 and 3, the single serum samples presented a distribution over normal to low to deficient values.

Serum vitamin E concentrations within horses varied widely, with a CV of up to 101% in one horse. The within-animal CV was significantly larger (P<0.005, independent samples t-test) in horses with deficient levels of vitamin E (mean CV: 41%) compared to animals with normal levels of vitamin E (mean CV: 14%). The mean CV of the group with low mean levels of vitamin E was 28%.

Group 3 consisted solely of horses which presented clinical signs. In group 2, five out of 8 horses presented clinical signs (horses 12 - 16). These five horses accounted also for the largest variation in this group (CV: 13% - 101%), and in 3 of them, normal, low and deficient vitamin E levels were all found in each horse at different testing moments. The control horses that fell in group 2 presented a CV (CV: 0.84% - 12%) comparable to the CV found in the horses of group 1 (CV: 5% - 18.2%).

There was no evident cycle or circadian rhythm in the serum vitamin E level.

The estimated sensitivity of a single vitamin E determination was 91% (95% confidence interval 66-100%), indicating that on average in 91% of the cases a single test of a truly deficient horse will in fact indicate that the horse is deficient. The specificity was 82% (95% confidence interval 50-100%), indicating that on average in 82% of the cases a single test result of a truly non-deficient horse will in fact indicate that the horse is non-deficient. From a clinical perspective it is important to know the prognostic value of a single serum sample to assess the true vitamin E status of a horse. This is reflected by the positive and negative predictive value of a serum sample. In this study, the PPV was 85% (95% confidence interval 62-100%), indicating that if a single serum sample is positive (<1.5µg/ml), there is an 85% chance that the horse is truly deficient, whereas in 15% of the cases the horse is not deficient. The NPV was 90% (95% confidence interval 67-100%), indicating that if a single test is negative (>1.5µg/ml), there is a 90% chance that the horse is truly not deficient, whereas in 10% of the cases the horse is actually deficient. When 2 samples were evaluated, the sensitivity increased to 97% (95% confidence interval), the specificity to 91% (95% confidence interval), the NPV to 98% (95% confidence interval) and the PPV to 90% (95% confidence interval).

DISCUSSION

The results in this study yield further evidence that a large fluctuation in serum concentration of vitamin E exists within horses. Previous studies did not take into account the fluctuation in deficient animals. Accurate assessment of the vitamin E status is important, not only to diagnose specific diseases such as EDM, EMND and muscular pathologies in horses with overt clinical signs, but also to detect deficient levels in subclinical cases. Several studies attribute a major causative role to vitamin E deficiency in the multifactorial

etiology of EMND and demonstrate an increased risk of EMND with deficient vitamin E levels (De La Rua-Domènech *et al.*, 1997; Divers *et al.*, 2006; McGorum *et al.*, 2006; Mohammed *et al.*, 2007). It has also been proven that clinical signs of EMND develop after a prolonged time of deficiency (Divers *et al.*, 2001; Mohammed *et al.*, 2007) and do not manifest until more than 30% of the motor neurons are involved in the degenerative process (Weber *et al.*, 1998), making the existence of subclinical cases very likely (Divers *et al.*, 2001). The purpose of the present study was to extrapolate the results obtained to a clinically relevant population. Because low or deficient vitamin E levels are not always related to clinical disease and can be found in normal horses, we decided to compose the sample population based on clinical signs rather than on the vitamin E level. In this study it was not possible to determine the exact contribution of the vitamin E deficiency to the clinical signs presented by the horses and we need to be cautious about attributing a decisive causative role to vitamin E deficiency. However, fatigue, poor performance, unusual gaits and failure to gain weight are the most common complaints with the chronic form of EMND (Divers *et al.*, 2001), and the role of vitamin E in poor performance has been recognized (Dewes, 1981). The horses in this study were considered representative of a clinically relevant population because they represent a group at risk of developing EMND or other vitamin E related pathologies and they might benefit from supplementation with vitamin E.

The CV in serum concentrations in horses with normal levels found in this study corresponded to those found in normal horses in a previous study (mean CV: 12%) (Craig *et al.*, 1989). In our study it was not only apparent that the CV in the deficient group was significantly larger compared to the CV in the normal group, but also that the amount of variation differed according to the level of serum vitamin E because a larger CV was also seen in the group of horses with low mean levels of serum vitamin E. Also, the largest CV was encountered in the horses with clinical signs when compared to the control horses that fell in this group. Despite the low number of horses, this finding might indicate that a distinction can be made between normal horses with normal to low levels and horses with clinical signs with low to deficient levels, suggesting an altered vitamin E metabolism in the latter group. The higher variation obtained in the horses of the latter group might reflect a modified uptake, processing, distribution or utilization of the vitamin E. There is no storage of vitamin E in the serum and the vitamin E levels in the serum appear to reflect the daily intake of the vitamin (Ronéus *et al.*, 1986), with an increase and decrease according to the dietary vitamin E level. Moreover, a circadian rhythm of fat soluble vitamins is reported in horses with the highest levels of serum vitamin E measured at 16:00 hours (Piccione G. *et al.*, 2004). Together with the pathophysiological mechanisms underlying low vitamin E levels in horses, this may contribute to the variation in the serum levels.

However, in this study and in the study described by Craig (1989), the serum vitamin E levels revealed no circadian pattern, nor did they present a postprandial variation. Besides individual factors, the vitamin E serum concentration can also be influenced by several methodological factors, and the influences of light, contact with the rubber tube cap, hemolysis, duration of freezing, and repeated frost and defrost cycles have all been proven (Craig *et al.*, 1992). Particular precautions were taken to eliminate these influences on the serum vitamin E concentration in the present study.

In normal horses, various factors that cause a physiological variation in serum vitamin E concentration have already been identified: seasonal variation with lower levels in the winter (Mäenpää *et al.*, 1988a; Blakley and Bell , 1994), type of feed (Mäenpää *et al.*, 1988b; Blakley and Bell, 1994), age, the fact that foals have a lower vitamin E status than adults (Mäenpää *et al.*, 1988a; Craig *et al.*, 1989; Blakley and Bell, 1994), level of exercise (Watanabe *et al.*, 1982) and breed, with Thoroughbreds having lower concentrations than other breeds (Steiss *et al.*, 1994). However, these factors might not influence the serum concentration in a 24-hour period.

In this study we applied the level of 1.5 µg/ml to ascribe deficiency. Although a wide range of normal values for horses is reported and the serum vitamin E concentration can be as low as 1.0 µg/ml in normal horses (Steiss *et al.*, 1994), in several reports clinical disease was clearly associated with levels below 1.5 µg/ml (Blythe *et al.*, 1991; De la Rua-Domènech *et al.*, 1997; Divers *et al.*, 2006), and normal horses in general have vitamin E levels above 2 µg/ml (Steiss *et al.*, 1994). Therefore, for most researchers, levels > 2 µg/ml are considered normal, levels between 1.5 µg/ml and 2 µg/ml are considered low, and levels below 1.5 µg/ml are considered deficient. This implies that there will be overlap between normal horses with low to deficient levels and clinically affected horses with low to normal levels. The horses in our study demonstrated a similar distribution. On the other hand, in this study all the horses with deficient mean levels of vitamin E presented one or more clinical signs. This observation corresponds to the reported relationship between deficient levels of vitamin E and clinical signs of EMND (De la Rua-Domènech *et al.*, 1997; Divers *et al.*, 2001).

The relevance of the findings in this study is that low and normal levels of serum vitamin E can be found in deficient horses. This implies that if a diagnosis were made based solely on a single serum sample, then in some cases the vitamin E status would have been overestimated and no treatment would have been instituted. Statistical analysis yielded fair results and the confidence intervals were large, but this is believed to be due to the large coefficient of variation found within the individual animals and the small size of the study group. The results in this study indicate that evaluation of two serum samples enables a more accurate estimation of the true vitamin E status of a horse. Therefore it is recommended that when low or

normal vitamin E levels are found in a horse with clinical signs, at least 2 serum samples should be analyzed before definitive conclusions are drawn.

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