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Safety and efficacy of subcutaneous alpha-tocopherol in healthy adult horses

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Summary

Vitamin E is essential for neuromuscular function. The primary treatment, oral supplementation with natural ('RRR') α-tocopherol, is not effective in all horses. The objectives of this pilot study were to evaluate the safety and efficacy of a subcutaneously administered RRR-α-tocopherol preparation. Horses were randomly assigned in a cross-over design to initially receive RRR-α-tocopherol (5000 IU/450 kg of 600 IU/mL) subcutaneously (n = 3) or orally (n = 3) or were untreated sentinels (n = 2). Tissue reactions following injection in Phase I of the study necessitated adjustment of the preparation with reduction of the RRR-α-tocopherol concentration to 500 IU/mL in Phase 2. Following an 8-week washout period, horses received the reciprocal treatment route with the new preparation (5000 IU/450 kg of 500 IU/mL). Serum, CSF and muscle α-tocopherol concentrations were determined by high-performance liquid chromatography over a 14-day period during each phase. Serum and CSF α-tocopherol concentrations increased significantly postinjection only when the 500 IU/mL product was administered ($P<0.0001$). There was no significant difference in the muscle concentration of α-tocopherol following either

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Authorship

C. Donnelly, C. Finno and R. Stuart contributed to study design, sample collection and data analysis. E. Burns, S. Katzman and C. Easton-Jones assisted with sample collection. S. Cook assisted with histopathology interpretation. All authors contributed to the manuscript.

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Authors' declaration of interests

R. Stuart is the director and owner of Stuart Products which produces nutritional products.

Ethical animal research

Ethical approval by the University of California-Davis Institutional Animal Care and Use Committee (Protocol number 2009).

Manufacturers' addresses

treatment. All eight horses had marked tissue reaction to subcutaneous injection, regardless of product concentration. Whilst we have demonstrated that this route may be a useful alternative to oral supplementation, the marked tissue reaction makes use of such products limited at this time to only the most refractory of cases.

Keywords

horse; vitamin E; alpha-tocopherol

Introduction

Horses appear to be particularly susceptible to vitamin E deficiency mediated neurological and muscular degeneration (Finno and Valberg 2012). Horses at risk of deficiency, most commonly due to dry-lot management, or with diagnosed deficiency (serum α-tocopherol <2 μg/mL) are typically supplemented with RRR- α-tocopherol, the most potent enantiomer of vitamin E and the natural form found in lush pasture (Finno and Valberg 2012). However, there are anecdotal reports of horses administered appropriate doses and formulations of oral α-tocopherol failing to respond clinically, with serum concentrations not increasing as expected. Recently, malabsorption of orally administered α-tocopherol secondary to eosinophilic enteritis has been implicated in the aetiology of equine motor neuron disease (Díez de Castro et al. 2016). Currently, there are no parenteral α-tocopherol products approved for use in horses. Whilst E-Se® contains alpha-tocopherol, it is in the synthetic formulation and has been demonstrated not to affect serum alpha-tocopherol concentrations in foals (Finno et al. 2015). Administration by parenteral routes is routinely performed in food producing species with apparent efficacy (Hidiroglou and Karpinski 1991). The objective of this study was to provide preliminary safety and efficacy data for the subcutaneous administration of α-tocopherol formulated for injection. We hypothesised that subcutaneous administration of RRR-α-tocopherol would increase serum, cerebrospinal fluid and muscle concentrations in vitamin E-deficient horses.

Materials and methods

Animals

Eight adult mixed breed horses (mares n = 4; geldings n = 4), weighing 547.7 ± 47.7 kg and aged 3 to 12 years were used in this study. Based on a noninferiority power analysis for a cross-over design with an $\alpha = 0.05$, $\beta = 0.8$ and $\delta = 0.4$, a sample size of three animals would be necessary.

All horses were housed at the same facility on dry lots. All horses were fed twice daily with grass hay at ~ 2% bodyweight per day and underwent annual routine husbandry such as dental exams and core vaccinations. No animals in this study were provided with an oral α-tocopherol supplement prior to the study. The Institutional Animal Care and Use Committee at the University of California, Davis approved the study design.

Study design

Initially, a balanced cross-over design was employed with horses randomly assigned to receive the same α-tocopherol preparation once by subcutaneous injection (n = 3) or orally (n = 3). Horses were administered a total dose of 5000 IU (i.e. 10 IU/kg) α-tocopherol by either route. Following an 8-week washout period, animals received the reciprocal treatment. The washout period was determined from previously published data (Brown et al. 2017). All subcutaneous injections were performed after aseptic preparation of the skin over the left pectoral area. The α-tocopherol product was sterilely filtered via a 170- to 250-μm blood component filter (Y-type blood solution set)[1] prior to administration. Due to marked tissue reactions with the original 600 IU/ml formulation (customised preparation, Phase 1)[2], a 500 IU/ml preparation (Vital-E)[2] was used for Phase 2 in an attempt to mitigate the swelling associated with the injection. However, the total dose (5000 IU per horse) remained unchanged for both phases. Two animals (one mare, one gelding; aged 7 and 11 years, respectively) served as environmental sentinels for the entire study period, with only serum samples collected from these animals. The University of California Animal Use and Ethics Committee approved all procedures.

Cerebrospinal fluid (CSF) was collected on day 0 of the experiment. A jugular catheter was placed in all horses. Horses were premedicated with xylazine (1.1 mg/kg bwt i.v.), and general anaesthesia was induced with ketamine hydrochloride (2.2 mg/kg bwt i.v. and midazolam (0.05 mg/kg bwt i.v.). Horses were placed in right or left lateral recumbency and CSF fluid collected in sterile fashion by atlanto-occipital (AO) centesis using an 8.9-cm 18 gauge spinal needle. CSF was collected again in a similar fashion on day 7 post α-tocopherol administration. AO centesis was performed under general anaesthesia in preference to standing collection techniques in order to obtain a higher volume sample with less risk of blood contamination. Samples were collected into plain light-protected plastic vials and kept on ice. Samples were centrifuged at 4°C within 3 h of collection, and the supernatant stored at −80°C until analysis. The same sampling protocol was followed for the reciprocal treatment in phase 2 of the experiment.

Serum was collected immediately before anaesthesia on day 0 into plain light-protected vacutainer tubes. Further serum samples were collected 24, 48, 96 h, 7 and 14 days following α-tocopherol administration. All samples were centrifuged within 3 h of collection and stored at −80°C until analysis. The same sampling protocol was followed for the reciprocal treatment in phase 2 of the experiment.

Muscle was sampled from the gluteus medius whilst the horses were anaesthetised on days 0 and 7. An additional sample was collected on day 14 understanding sedation (xylazine 0.4 mg/kg bwt). Muscle samples were aseptically collected using a Bergström biopsy needle as previously described (Snow and Guy 1976). The sample site was alternated for each sample. All samples were flash-frozen in liquid nitrogen immediately at collection and stored in plastic, light-protected vials at −80°C until analysis. The same sampling protocol was followed for the reciprocal treatment in phase 2 of the experiment.

[1]Baxter, Deerfield, Illinois, USA.
[2]Stuart Products, Bedford, Texas, USA.

Serum, CSF and muscle α-tocopherol concentrations were measured at the Iowa State Veterinary Laboratory. Additionally, both injectable products were subjected to independent α-tocopherol quantification. All samples and both injectable products were analysed by HPLC as previously described (Finno et al. 2015).

Data analysis

Data were analysed by commercial software (GraphPad Prism 7.4)[3]. Due to small sample size, baseline samples for serum, CSF and muscle were evaluated by Kruskal–Wallis test for each treatment phase. There was no significant difference between baseline measurements; therefore, the washout period was considered appropriate. Serum, CSF and muscle tissue α-tocopherol concentrations were then evaluated by two-way repeated measures ANOVA, with time and experimental group as fixed factors and horse as the random effect. Post hoc testing was performed using Sidak's multiple comparison test. Significance was set at $P<.05$.

Results

Serum α-tocopherol

For oral α-tocopherol administration, no time or treatment phase interaction was detected. Therefore, all oral α-tocopherol supplementation data were subsequently combined. A significant time treatment interaction ($P<0.0001$) was detected for the first versus the second subcutaneous injection, and therefore, these data were analysed separately. The majority of variation was derived from treatment (44%). Baseline serum concentrations of α-tocopherol were not significantly different between treatment groups or between phases (Fig 1). All animals were considered vitamin E deficient at the beginning of the trial (mean ± s.d.; 1.2 ± 0.07 μg/mL; reference range 2-4 μ/mL) (Finno and Valberg 2012). Serum concentrations were significantly increased at 24 h (18.1 ± 12.05 $P\leq0.0001$), 48 h (16.53 ± 2.04 $P\leq0.0001$) and 72 h (11.6 ± 3.08 $P<0.05$), postadministration only for the 500 IU/mL subcutaneous injection in Phase 2. By days 7 and 14, subcutaneous administration in Phase 2 was not significantly different compared to oral, Phase 1 injection or baseline concentrations.

CSF α-tocopherol

CSF α-tocopherol baseline concentrations were not significantly different between treatment groups. Similar to serum, there was a significant time treatment interaction (<0.0001), with treatments analysed separately. The majority of variation arising from time period (43.2%). All horses were considered vitamin E deficient in the CSF at the beginning of the trial (4.3 ± 1.6 ng/mL; reference range ≥10 ng/mL) (Finno et al. 2015), injection with the 500 IU/mL preparation resulted in significantly higher CSF α-tocopherol at day 7 (15.27 ± 0.95 ng/mL, $P\leq0.0001$) compared to the 600 IU/mL injection (6.7 ± 0.99 ng/mL), oral administration (6.66 ± 4.23 ng/mL) and baseline (Fig 2).

[3]GraphPad, San Diego, California, USA.

Muscle α-tocopherol

Muscle concentrations were highly variable between animals (range; 0.1-4.3 μg/g). Whilst there was a modest trend towards increased concentrations at 14 days post-subcutaneous administration in Phase 2, this was not significant.

Tissue reaction

All animals developed marked swelling at the injection site, regardless of concentration of the α-tocopherol formulation. For seven of eight animals, this tissue reaction was self-limiting. One horse had persistent swelling and eventual drainage requiring surgical intervention. Histology of the area was consistent with a sterile granuloma (Fig 3).

Independent assessment of product α-tocopherol concentrations

Each of the products was independently verified using HPLC (Finno et al. 2015). The product in Phase I contained 336 mg/mL (equivalent to 672 IU/mL), with the product in Phase 2 containing 290 mg (equivalent to 580 IU/mL).

Discussion

Currently, there are no labelled parenteral vitamin E preparations suitable for use in treatment or prevention of horses with existing or recurrent deficiencies in vitamin E. Additionally, with reports of malabsorptive conditions leading vitamin E deficiency, a need exists for a safe and efficacious parental means of supplementing horses (Finno and Valberg 2012).

Despite the importance of vitamin E as a potent antioxidant, the exact mechanisms of its biokinetics in horses are poorly understood and extrapolated from studies in other species (Finno et al. 2011, 2015). The primary route of absorption of vitamin E is alimentary and is closely associated with fat absorption requiring appropriate pancreatic, biliary and small intestinal function (Desmarchelier and Borel 2018). A specific mechanism for malabsorption in horses has not been evaluated. Speculatively, it is likely that perturbation of small intestinal function plays a role, and however, this is yet to be substantiated. Horses require a constant dietary supply to maintain appropriate serum concentrations of vitamin E, with serum levels falling quickly in the absence of supply (Stuart et al. 2010; Brown et al. 2017). Horses in the current experiment demonstrate this, with all animals considered deficient at the beginning of the trial, presumably subsequent to dry-lot management (Stuart et al. 2010). Despite this deficiency, no horses demonstrated any clinical signs of neuromuscular disease throughout this trial. Further, horses in this experiment appeared to have normal intestinal absorption of α-tocopherol, with an expected approximate doubling of serum concentrations 24 h after oral administration (Lodge et al. 2004; Stuart et al. 2010). Whilst this increase was not statistically significant, it was a biologically appropriate response and indicates both a normal ability to absorb and also to distribute α-tocopherol (Finno and Valberg 2012).

Vitamin E is fat-soluble and as such may also be absorbed by nonenteric routes. Following subcutaneous, intramuscular or intraperitoneal injection in other species, α-tocopherol is primarily taken up by lymphatics, before rapidly equilibrating between plasma and the cell

membrane of erythrocytes (Lodge et al. 2004). Distribution to tissues rarely results in accumulation of vitamin E to toxic levels, reflecting a highly regulated and active process (Lodge et al. 2004). This process may also take place in the absence of major transport proteins found in the liver (Irías-Mata et al. 2018). As such, the tissue-specific absorption remains tightly regulated and is able to utilise nonalimentary α-tocopherol.

Injectable formulations of α-tocopherol have been investigated in a number of domestic species (Knight and Roberts 1985; Hidiroglou and Karpinski 1987,1988). Administration by this route appears to effectively and efficiently increase serum α-tocopherol concentrations. In contrast, parenteral administration in horses has not been well studied and there are currently no approved products for use in this species. As mentioned previously, products containing synthetic vitamin E and selenium (such as E-Se®) are commonly administered parenterally to neonatal foals, and however, these products provide insufficient amounts and minimally bio-potent vitamin E to be used to treat deficiency mediated diseases (Finno et al. 2015).

Following subcutaneous administration with the 500 IU/mL preparation, supra-physiological α-tocopherol concentrations in the serum and CSF were attained. Very low levels of α-tocopherol administered parenterally are required to maintain erythrocyte stability in deficient horses (Stowe 1968). Administration by this route circumvents both alimentary losses as well as liver-mediated regulation and may account for the large difference in serum and CSF concentrations. Subcutaneous administration demonstrates an opportunity to rapidly increase nervous tissue concentrations, the main target of treatment for vitamin E-mediated neurological diseases. Oral administration of suitable formulations may take up to 14 days to increase CSF concentrations, and therefore, parenteral preparations may be more suitable for early treatment of vitamin E deficient neurological disease (Hidiroglou and Karpinski 1991).

Administration of the 600 IU/mL preparation did not result in the same robust increase in α-tocopherol serum and CSF concentrations. The primary difference between the preparations was concentration, with equipotent doses administered. Local inflammation has previously been recognised as a source of variation for absorbance of nonaqueous preparations administered subcutaneously to horses (Alvinerie et al. 1998). Given the variability in individual local inflammatory responses and the small number of animals available in this study, the disparity of absorbance between products may represent the spectrum in local reactions of individual horses. That is to say that horses with more marked tissue reaction (like those in the Phase 1) would have reduced absorbance as reflected by lower serum and CSF concentrations.

Muscle α-tocopherol concentrations were not significantly different pre- and post-supplementation. Muscle concentrations of α-tocopherol are affected by the amount of lipid within the muscle (Ronéus et al. 1986). Adipose tissue is a main storage site for α-tocopherol, and therefore, muscle with increased amounts inter-fascicle fat will have increased concentrations of α-tocopherol. The amount of adipose tissue was not standardised in the current experiment, with whole tissue submitted for evaluation.

Additionally, as these animals were deficient prior to the study, there was likely preferential distribution to adipose tissue over muscle (Lodge et al. 2004).

The tissue reaction observed in the current experiment was marked and affected all animals. The rapidity by which reactions occurred may indicate large scale degranulation of tissue-resident mast cells, leading to marked oedema in the region of injection (Krystel-Whittemore et al. 2016; Jørgensen et al. 2018). Whilst the tissue reaction was self-limiting in 7 of 8 horses and required no further intervention, the degree of tissue reaction makes use of this product in horses limited only to situations where the benefit of treatment outweighs the development of complications.

In conclusion, parenteral administration of α-tocopherol via the subcutaneous route effectively increases serum and CSF α-tocopherol concentrations. Preparations that circumvent alimentary absorption provide a novel area of investigation for more efficacious and long-term preparations of α-tocopherol. However, the current trial demonstrated that the tested product is not safe for use in horses due to local tissue reaction and as such cannot be recommended for clinical use at this time.

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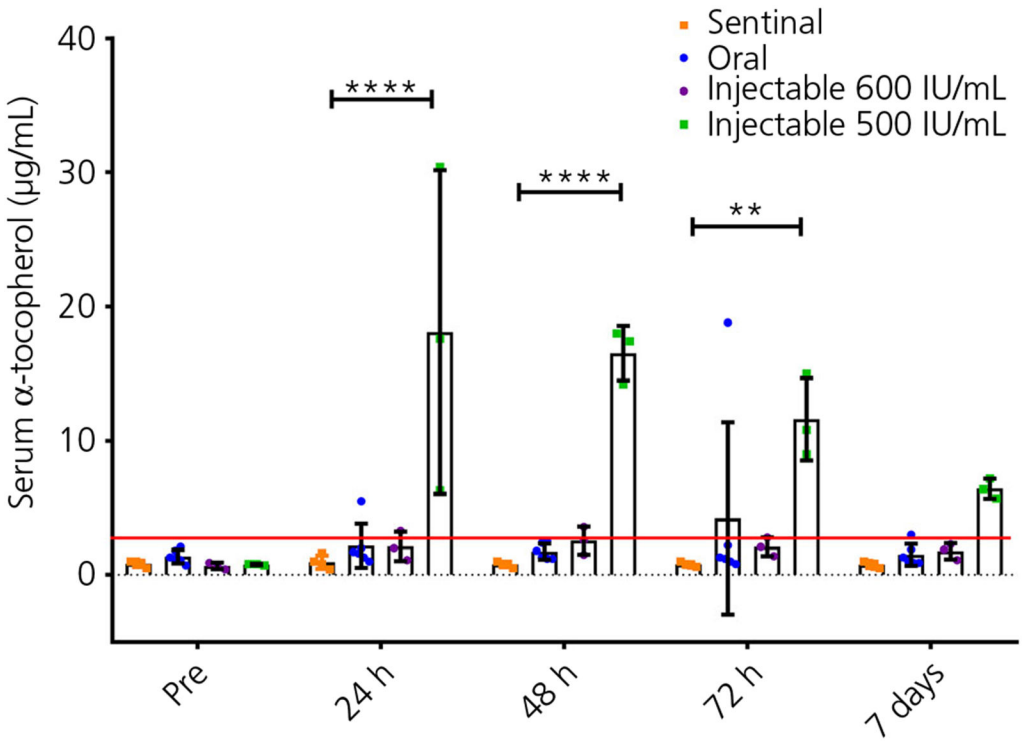

Fig 1:

Serum α-tocopherol concentrations with individual animals plotted by treatment group. Significant increase in concentration in 500 IU/mL injection compared to sentinel, oral and 600 IU injections at 24 h ($P<0.0001$), 48 h ($P<0.0001$) and 72 h ($P<0.05$). Red line denotes the serum normal threshold 2 µg/mL

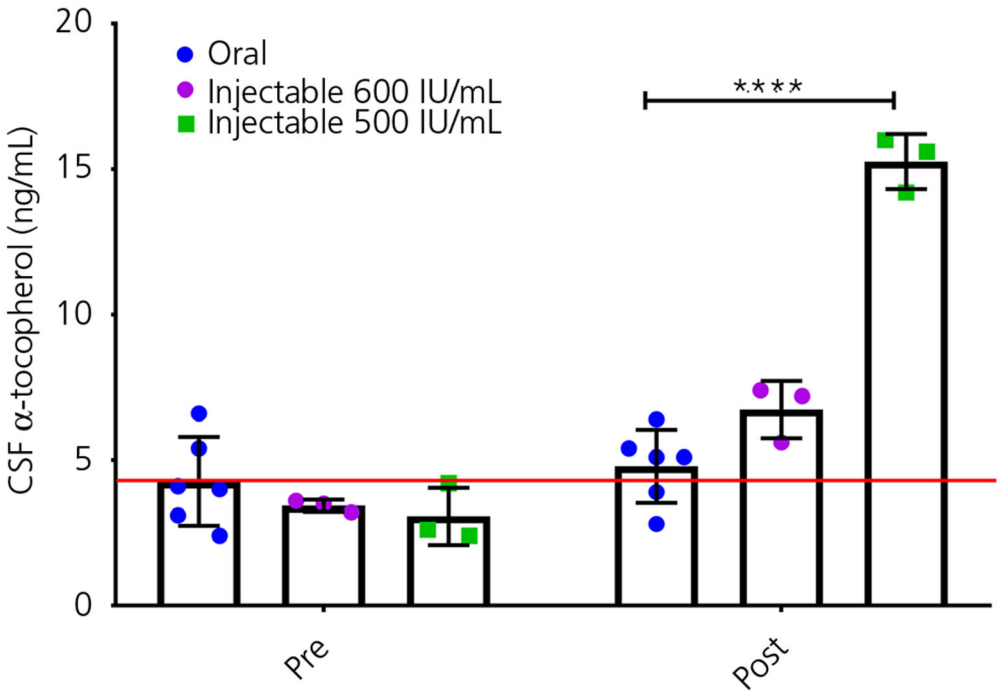

Fig 2:

CSF α-tocopherol concentrations pre- and 7-day postadministration with individual animals plotted by treatment group. Significant increase in concentration in 500 IU/mL injection compared to oral and 600 IU injections at 7 days ($P<0.0001$). Redline denotes the CSF normal threshold 4 ng/mL

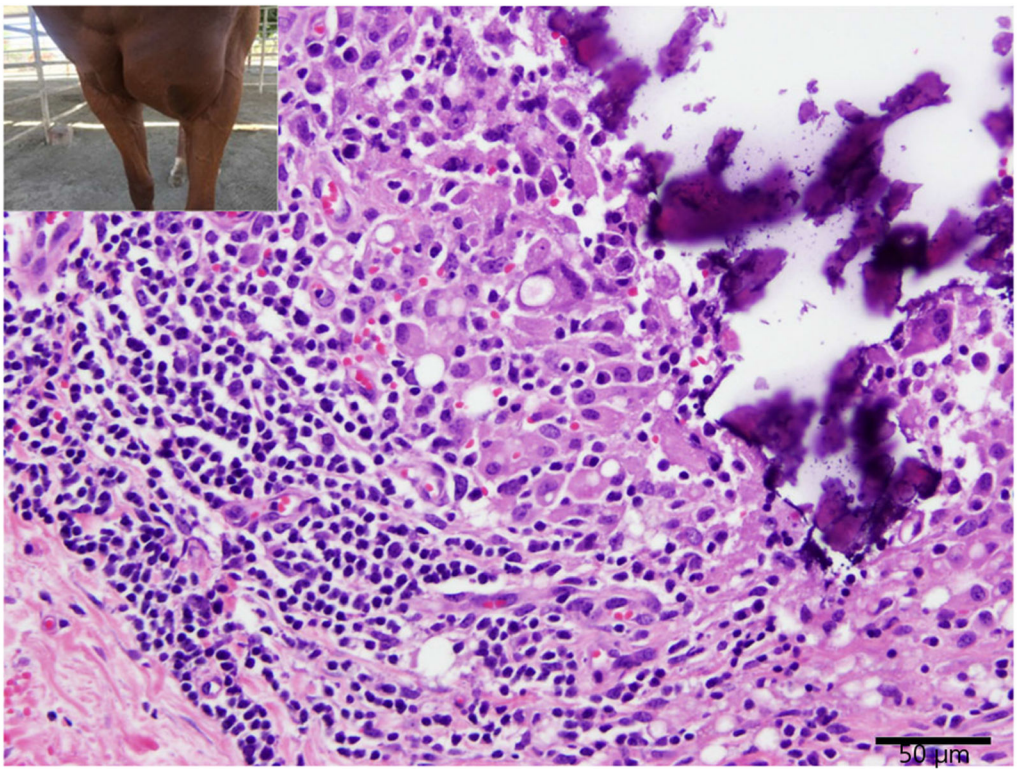

Fig 3:
Histological section at 200× magnification showing granulomatous inflammation surrounding a mineralised core and surrounded by a rim of lymphoplasmacytic inflammation and fibrosis. Numerous macrophages are multinucleated and often contain clear, distinct vacuoles consistent with lipid. Clinical image of gross tissue reaction 24 h postinjection (inset)