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Original Article

Vitamin E supplementation fails to impact measures of oxidative stress or the anaemia of feline chronic kidney disease: a randomised, double-blinded placebo control study

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Abstract

This study was designed to test the hypothesis that supplementation with vitamin E, an antioxidant, in cats with chronic kidney disease (CKD), would reduce oxidative stress and its impact on RBC membrane fragility, resulting in these cats maintaining a greater packed cell volume (PCV) compared with CKD cats not receiving supplementation.

Thirty-six cats with CKD were randomly assigned to receive either daily vitamin E or a placebo for 3 months in a double-blinded study design.

History and physical examination, blood pressure, complete blood count (CBC), PCV, biochemical profile and urinalysis (UA) were determined. Parameters of oxidative stress and osmotic fragility were measured. Cats were administered vitamin E or placebo once daily for 3 months. Cats were then reassessed and the diagnostics were repeated.

Twenty-four cats completed the study, 11 in the vitamin E group and 13 in the placebo group. There were no significant differences between the two groups at the start, or upon completion of the study with regard to biochemical parameters, oxidative stress, erythrocyte osmotic fragility or PCV. None of these parameters changed significantly in either group over the treatment period.

Daily supplementation with 30 IU of vitamin E did not affect the measures of oxidative stress or the anaemia seen in cats with CKD.

Keywords: vitamin E, oxidative stress, feline CKD, anaemia, osmotic fragility.

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Introduction

The supplementation of vitamin E (alpha-tocopherol) in cats with chronic kidney disease (CKD) may have beneficial effects on oxidative stress and therefore, a common clinical abnormality seen in these cats, anaemia (Keegan & Webb, 2010; Bargnoux *et al*., 2013; Brown, 2008; Peuchant *et al*., 1997). As an antioxidant, vitamin E prevents the oxidation of polyunsaturated fatty acids caused by oxidative stress acting on red blood cell membranes, thereby reducing erythrocyte membrane fragility and potentially decreasing haemolysis (Jilani & Iqbal, 2011, Traber & Stevens, 2011). Vitamin E deficiency can cause RBC membrane changes and haemolysis as osmotic fragility appears to be inversely related to alpha-tocopherol concentrations in certain circumstances (Jaja *et al*., 2005; Tomten & Høstmark, 2009). Vitamin E-coated dialyser therapy in human kidney failure patients is based on these potentially beneficial properties, although thus far the results are inconclusive (Usberti *et al.*, 2002; Huang *et al*., 2015).

Oxidative stress may contribute significantly to the anaemia seen in human patients with CKD (Celik *et al*., 2011; Cruz *et al*., 2008; Usberti *et al*., 2002).

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Antioxidant capacity is significantly decreased in cats with CKD (Keegan & Webb, 2010). One manifestation of oxidative imbalance is lipid peroxidation of erythrocyte (RBC) cell membranes, which would decrease RBC lifespan and could contribute to the anaemia of conditions such as CKD (Ansari *et al.*, 2015; Gomez *et al.*, 2013). Vitamin E has antioxidant properties uniquely suited to combating cell membrane lipid peroxidation with few known side-effects, making it a potentially beneficial therapy aimed at an alternative target contributing to the anaemia of CKD (Singh *et al*., 2005). Assays of RBC osmotic fragility have been used to characterise anaemia of unknown aetiology in cats, including the anaemia of Abyssinian and Somali cats with splenomegaly (Contreras *et al*., 2015; Tritschler *et al*. 2015). Erythrocyte osmotic fragility has also been used as a quantitative measure of the effect of nutraceutical supplementation on RBCs in healthy cats (Center *et al.* 2005). The cupric iron reducing antioxidant capacity (CUPRAC) assay has been validated against atomic absorption spectrophotometry, and established as a reliable marker of total antioxidant capacity in erythrocytes under oxidative stress (Kondakçi *et al*. 2013, Soumya *et al*. 2015). Although not yet used in cats, the fact that the CUPRAC assay measures total antioxidant capacity against a water-soluble vitamin E analogue as the standard made this an ideal parameter for this study.

To date the authors are aware of a single study looking at the effects of antioxidant supplementation (a combination of vitamins including vitamin E) in cats with CKD, and the results showed that the treatment reduced serum 8-OHdG levels and comet assay characteristics indicative of DNA damaged by oxidative stress (Yu & Paetau-Robinson, 2006). This study was designed to test the hypothesis that supplementation with vitamin E, an antioxidant, in cats with CKD, would reduce oxidative stress and its impact on RBC membrane fragility, resulting in these cats maintaining a greater packed cell volume (PCV) compared with CKD cats not receiving supplementation.

Materials and Methods

Selection of cases

Thirty-six adult cats diagnosed with CKD were recruited from the client-owned Colorado State University Veterinary Teaching Hospital population for this study. Inclusion criteria for study participation included a diagnosis of CKD of at least 6 months duration, based on a persistent serum creatinine >1.6 μg/dL and a urine specific gravity <1.030. Blood pressure and urine protein:creatinine ratio were obtained for all study cats for IRIS staging. Cats were excluded if they had evidence of a chronic disease other than CKD based on history, physical examination, CBC, biochemical profile, urinalysis (UA) with culture or total T4 levels. Cats were also excluded if they ever had, or were currently receiving, erythropoietin, darbepoetin, iron supplementation or antioxidants other than those already a part of their manufactured diet. Cats were given 30 IU of vitamin E (alpha-tocopherol) PO once daily for 3 months or an identical volume of a placebo (safflower oil) containing the same vehicle (tuna juice) but without vitamin E. [Stuart Products Inc., Emcelle® Tocopherol, 500 IU d-alpha-tocopherol/1 mL. Compounded: 1.2 mL into 8.8 mL tuna juice for 10 mL at 30 IU/0.5 mL in brown bottle, keep refrigerated. Administer 0.5 mL (measured syringe) per os once daily for 14 days, refill 6x]. Whether cats received the supplement or placebo was determined using a computer generated randomisation table, and the clinician, client and all involved in the running of all assays were blinded as to the group entry. Cats were excluded if unwilling to take the supplement/placebo per os or if the owner failed to administer the supplement/placebo per instructions for the duration of the study. Owners were instructed not to change their cat's diet or add any treatments or supplements during the course of the study, other than those given as a part of the study. A signed consent form was obtained and all sample acquisition and handling were in accordance with Colorado State University Animal Care and Use Committee guidelines. The cats were otherwise managed completely by the attending clinician.

Diagnostic work-up

During both the initial and final study appointments, a complete history and physical examination, including body weight, body condition score and systolic blood pressure, were performed. Samples for the CBC, PCV, biochemical profile, TT4, UA and UPC were obtained using standard venipuncture technique and cystocentesis.

EDTA-preserved whole blood was collected for the assay of RBC osmotic fragility and the 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, triethylammonium salt (DHPE) probe assay of lipid peroxidation, whereas serum was used for the CUPRAC assay of antioxidant capacity.

CUPRAC assay

The cupric ion reducing antioxidant capacity (CUPRAC) assay is a measure of total antioxidant capacity. A serial dilution of Trolox (water-soluble vitamin E analogue; .10, .08, .06, .05, .04, .02, .01, 0.0 mmol/L) was used to generate an external standard curve. For the standard curve, 50μL of a 7.5 mmol/L neocuproine hydrochloride monohydrate solution, and 50μL of a 1.0 mol/L ammonium acetate buffer, in that order (the CUPRAC solution), were added to 24 wells of a 96 well flat bottom plate. Each Trolox standard was run in triplicate. For the study samples, 200μL of each chemical, again in the specified order, was added to a 2.0 mL microcentrifuge tube. Both the plate and these tubes were incubated for 15 min at 37°C.

Study samples were thawed at room temperature and vortexed before diluting 1:50 with 50% acetone (i.e. 10μL sample mixed with 490μL 50% acetone). These diluted study samples were vortexed and 400μL added to one microcentrifuge tube of CUPRAC solution immediately following incubation. The CUPRAC reaction occurs almost immediately. The tubes were then briefly vortexed and centrifuged at 2360g for 3 min at room temperature. Of the resulting supernatant, 250 μL was added to empty wells of the previously prepared 96 well plate. The study samples were run in triplicate. For each Trolox dilution, 100 μL was added to the previously prepared standard wells containing the CUPRAC solution. Again, the CUPRAC reaction in the Trolox standard wells occurs almost immediately, as seen by the production of a yellow colour.

The plate was read once at 450 nm on a Biotek Synergy HT plate reader. The triplicates of the Trolox standards and study samples were averaged and normalised against the 0 mmol/L Trolox control. The Trolox standard curve was created by plotting normalised absorbances vs. Trolox concentration (mmol/L). A linear equation was fitted to the data and used to interpolate the mmol/L concentration of Trolox in the study samples. The final concentration of antioxidant capacity as measured by mmol/L Trolox concentration in the study samples was determined by multiplying by the dilution factor.

Osmotic fragility assay

EDTA-preserved blood samples were run within 24 h. From the EDTA blood 100μL was added to 2 mL microcentrifuge tubes (2 tubes per sample). Two microlitres (2 μL) of 0.80% cumene hydroperoxide (an oxidising agent) was then added to one of the two samples containing tubes and vortexed. The cumene-treated blood was incubated at room temperature for 15 min.

Two sets of serial dilutions of sodium chloride (NaCl) were created per study sample using distilled water (0.90%, 0.75%, 0.70%, 0.65%, 0.60%, 0.55%, 0.50%). For one set of serial NaCl dilutions, 10μL of cumene-treated blood was added to 1 mL of each dilution tube and mixed. For the second set of serial NaCl dilutions, 10 μL of non-treated blood was added to 1 mL of each dilution tube and mixed. Both sets were incubated at room temperature for 15 min. All tubes were centrifuged at 380g for 3 min. Two hundred microlitres of supernatant from each tube was added to a 96-well flat bottom plate in triplicate.

The plate was read immediately at 540 nm on a Biotek Synergy HT Plate Reader. The NaCl concentration at 50% haemolysis was calculated by taking the average of the highest and lowest absorbance reading and interpolating between these two absorbance readings.

DHPE Probe

The DHPE probe [DHPE probe, Fluorescein DHPE (N-(Fluorescein-5-Thiocarbamoyl)-1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine, Triethylammonium Salt), ThermoFischer Scientific, Waltham, MA.] was prepared with 0.5% ethanol to make a 5 mmol/L concentration and stored at −20°C. EDTA-preserved whole-blood samples were run within 48 h of acquisition. The PCV and RBC concentration ($\times 10^6$ cells/μL) were recorded for each sample. From the study sample 50 μL EDTA blood was added to each of two 2 mL microcentrifuge tubes. One microlitre of 0.80% cumene hydroperoxide was added to one of the two tubes and vortexed. The cumene-treated blood was incubated at room temperature for 15 min.

All samples were washed three times with 1 mL of PBS and centrifuged at 2500 rpm for 3 min. Each sample was suspended in PBS such that the total cell count was 2×10^7 cells per mL PBS. One microlitre of this diluted blood was added to a new 2 mL microcentrifuge tube with 1 μL DHPE probe and mixed well. Samples were incubated at 37°C for 1 h. Excess diluted blood was saved and kept at room temperature for later use during flow cytometry.

The incubated samples were centrifuged at 4000 rpm for 3 min and the supernatant was discarded. The remaining pellet was washed once with 500 μL PBS and transferred to a flow cytometry tube. The saved diluted blood sample of 500 μL with no DHPE probe was used as a control.

All samples, including the cumene-treated blood, the cumene-treated blood with DHPE probe, the blood with DHPE probe and the untreated blood sample were read on the CyAn ADP Flow Cytometer using the FL1 fluorescence channel. The resulting scatter plot was gated using appropriate forward- and side-scatter coordinates to capture the RBC population and record the fluorescence produced by the DHPE probe. Fifty microlitres of each treated sample was added to the flow cytometer tube containing the untreated blood sample so that the signal from both treated and untreated RBCs could be quantified off the same scatter plot. Approximately 200,000 events were collected for analysis, such that the standard deviation of the recorded signal became minimised and stable.

Statistical analysis

The CSU electronic medical record database was searched for cats with CKD over a period of 1 year (2011). From those cats for which the PCV was recorded on two separate occasions, the mean and standard deviation for the decrease in PCV was determined. These numbers were used in a two-sample one-sided power calculation to determine group size ($n = 16$) for a power of 0.8 and a Type I error rate of 5%. A computer software program [GraphPad Prism software, GraphPad Software, Inc. La Jolla, CA.] was used for statistical analysis. Gaussian distribution was confirmed with the Kolmogorov and Smirnov test, and the difference in the means between groups was tested using a two-tailed unpaired *t*-test assuming unequal variance with the Welsh correction. A paired *t*-test was used to compare means before and after supplementation with vitamin E or placebo. Statistical significance was set as $P < 0.05$. All data were expressed as the mean $\pm$ the standard deviation.

Results

Cats

Thirty-six cats diagnosed with CKD and meeting the inclusion criteria were recruited into the study. Twenty-four cats completed the study, 11 in the vitamin E supplemented group and 13 in the placebo group. Nine cats were killed prior to completion of the study because of a progression of clinical signs and owner's perception of poor quality of life, six of those cats were from the vitamin E group and three were from the placebo group. Three cats were lost to follow-up with no explanation. None of the study participants reported any non-compliance in supplement/placebo administration, any change in diet, or any other changes to the treatment regimen for their cats during the 3-month study period, other than the administration of the study treatment. No owners reported any adverse side-effects with administra-

tion. One cat initially refused per os administration, but became easily compliant following further instruction of the owner.

The vitamin E group included four neutered males and seven spayed females: eight domestic short-haired cats, one domestic long-haired, one Siamese and one Persian cat. The placebo group contained seven neutered males and six spayed females: 11 domestic short-haired cats and two domestic long-haired cats. The mean age for the two groups was not significantly different, 13.5 ± 1.0 and 14.9 ± 1.0 years for the vitamin E and placebo groups respectively. The vitamin E group contained five IRIS Stage 2 cats and six IRIS Stage 3 cats; five of these cats were non-proteinuric and six were borderline; four cats were normotensive, one was borderline, two were hypertensive and four cats were considered severely hypertensive. The placebo group contained nine IRIS stage 2 cats and four IRIS stage 3 cats; four cats were non-proteinuric, seven were borderline and two cats were proteinuric; eight cats were normotensive, one was borderline, two were hypertensive and two cats were considered severely hypertensive. Table 1 compares the clinical parameters between groups at the start of the study. There were no statistically significant differences in any of these parameters between the two groups at study entry.

There was tremendous variability in the diets of both groups of study cats, and only one cat from each group was being fed a single diet, which in both cases was Hill's® k/d canned. All other study cats were reportedly fed multiple different foods. Four cats from the vitamin E group and five cats from the placebo group were not being fed any food specifically marketed or formulated for cats with CKD. Of these cats, one from each group was being fed Fancy Feast® canned, whereas all others were being fed "various [commercial] brands", both canned and dry. For all other study cats at least some portion of their diet was composed of a prescription feline CKD food. The distribution of brand and consistency was very similar between the two groups: three vitamin E and two placebo received Purina NF® canned, one vitamin E and two placebo received Purina NF dry, five vitamin E and six placebo received Hill's k/d canned, two vitamin E and three placebo received Hill's k/d dry and two vitamin E and two placebo received Royal Canin® Feline Renal Support canned.

Following 3 months of either vitamin E supplementation or placebo, the cats were reassessed using the same parameters as were measured upon study entry. Table 2 shows these parameters for each group. The only statistically significant difference was in total protein. There were no statistically significant changes in any of the other measured parameters for either group, comparing before and after

Table 1. Comparison of clinical parameters between the vitamin E group and the placebo group upon study entry

Parameter	Group		*P* value
	Vitamin E	Placebo	
Body weight (kg)	4.1 ± 0 .4	4.5 ± 0.3	0.42
Body condition score	4.6/9 ± 0.7	4.5/9 ± 0.5	0.99
Blood Pressure (mmHg)	168 ± 12	147 ± 7	0.14
Creatinine (mg/dL)	3.2 ± 0.3	2.8 ± 0.3	0.36
Potassium (mEq/L)	4.2 ± 0.2	4.4 ± 0.2	0.41
Phosphorus (mg/dL)	4.9 ± 0.3	4.9 ± 0.4	0.99
Urine Specific Gravity	1.017 ± 0.001	1.015 ± .002	0.48
UPC	0.2 ± 0.03	0.3 ± 0.08	0.16
Packed cell volume (%)	31.5 ± 1.0	30.9 ± 1.1	0.69
Total Protein (gm/dL)	7.9 ± 0.2	7.7 ± 0.2	0.47
IRIS Stage	2.5 ± 0.2	2.2 ± 0.2	0.14

UPC, urine protein–creatinine ratio; Blood Pressure, systolic.

Table 2. Comparison of clinical parameters between the vitamin E group and the placebo group at the conclusion of the 3-month study period

Parameter	Group		*P* value
	Vitamin E	Placebo	
Body weight (kg)	4.4 ± 0.6	4.2 ± 0.3	0.81
Body condition score	4.5 ± 0.5	4.6 ± 0.5	0.92
Blood Pressure (mmHg)	159 ± 9	146 ± 5	0.20
Creatinine (mg/dL)	3.3 ± 0.3	2.8 ± 0.3	0.35
Potassium (mEq/L)	4.2 ± 0.1	4.5 ± 0.2	0.10
Phosphorus (mg/dL)	4.5 ± 0.4	6.3 ± 1.0	0.12
Urine Specific Gravity	1.016 ± 0.001	1.017 ± 0.002	0.80
UPC	0.14 ± 0.02	0.27 ± 0.07	0.16
Packed cell volume (%)	31 ± 1	31 ± 2	0.77
Total Protein (gm/dL)	8.0 ± 0.2	7.5 ± 0.1	0.03
IRIS Stage	2.7 ± 0.15	2.2 ± 0.17	0.06

UPC, urine protein–creatinine ratio; Blood Pressure, systolic.

Table 3. Comparison of antioxidant capacity parameters for both groups before and after the 3-month treatment with vitamin E supplementation or placebo

Group	CUPRAC (Trolox Equivalent)		DHPE (Fluorescence)	
	Pre-	Post-	Pre-	Post-
Vitamin E	1.24 mmol/L ± 0.07	1.28 mmol/L ± 0.06	1068 ± 94	961 ± 79
Placebo	1.27 mmol/L ± 0.10	1.33 mmol/L ± 0.04	1227 ± 118	1031 ± 88

Table 4. Comparison of Osmotic Fragility (%NaCl producing 50% haemolysis) for both groups before and after the 3-month treatment with vitamin E supplementation or placebo, with and without the cumene challenge

Group	Osmotic fragility		Osmotic fragility (Cumene Challenge)	
	Pre-	Post-	Pre-	Post-
Vitamin E	0.62% ± .005	0.62% ± .006	0.65% ± .014	0.62% ± .008
Placebo	0.62% ± .012	0.61% ± .009	0.65% ± .017	0.67% ± .025

treatment values using a paired *t*-test (data not shown).

Antioxidant capacity

Antioxidant capacity was assessed using the DHPE and CUPRAC assays. Table 3 shows these parameters for each group before, and after the 3-month treatment period. There were no significant differences in these parameters between groups either before or following the 3-month treatment period, and there were no significant changes in either of these antioxidant parameters within either group, comparing values before and following the 3-month treatment period.

RBC Osmotic Fragility

RBC osmotic fragility was assessed using a series of NaCl concentrations and identifying the per cent NaCl concentration at which there was 50% haemolysis of the sample. Table 4 shows that there were no significant differences in the Vitamin E supplementation and placebo group prior to treatment, or following the 3-month treatment protocol. There was also no significant difference within groups between the pre- and post-treatment RBC osmotic fragility (data not shown).

Discussion

Oral supplementation with 30 IU of vitamin E (alpha-tocopherol) once daily for 3 months did not significantly change any of the measured parameters of oxidative stress or osmotic fragility in cats with CKD. This vitamin E protocol also failed to impact the PCV of the supplemented group compared with placebo control. The power of the study was decreased by the death of study participants, and although the group means appear similar, it cannot be ruled out that this represents a Type II error.

Vitamin E is an antioxidant that is theoretically well suited to protect RBC membranes from lipid peroxidation. The study dose of 30 IU alpha-tocopherol per cat per day was taken from a standard veterinary formulary and appears consistent with the clinically recommended dose from a number of sources (Williams, 2000; Scherk & Center, 2005; Halliwell & Gutteridge, 2007). The absence of a significant increase in antioxidant capacity in these cats suggests that the study dose was insufficient, the supplement was not absorbed, or the supplement was not utilised by the patient. In one study, increasing the vitamin E content in a formulated diet resulted in an increase in the skin and serum levels of vitamin E in dogs and cats, but our study is the first to use a direct oral vitamin E supplement (Jewel *et al*., 2002).

It may be that vitamin E supplementation is most beneficial when combined with other antioxidants or vitamins, creating a potentially synergistic effect (Azari *et al*., 2015). A previous study showing a beneficial effect of vitamin E supplementation in CKD cats also used dietary intervention, and the supplement consisted of multiple vitamins including vitamin E (Yu & Paetau-Robinson, 2006). In human dialysis patients the combination of vitamin E and alpha-lipoic acid supplementation failed to produce a significant decrease in biomarkers of lipid peroxidation, but did result in a significant decrease in interleukin-6 concentration (Ahmadi *et al*., 2013). The length of the study period may have been too short to capture progression of disease, although in preparation for this study a review of records suggested that a 3-month duration would be sufficient to see a significant drop in PCV in CKD cats. The progression of anaemia is likely different for different IRIS stages, with the cats in this study being predominantly IRIS stage 2 or 3.

A limitation of the study was the non-uniformity of the diets consumed by the cats, and the potential impact diet may have had on the baseline plasma tocopherol concentrations between the two groups and within each individual cat. Qualitative comparison of the diets used in each group failed to reveal any systematic differences. Unfortunately a more quantitative comparison was impossible for a variety of reasons, including the fact that the vast majority of study cats were being fed multiple diets, each of an un-specified amount. Ideally the plasma tocopherol concentration would have been determined for each cat at the start and conclusion of the study, but the quantitative measures were oxidative stress and anaemia, not the pharmacokinetics of vitamin E supplementation. Plasma tocopherol levels would also have served as an independent measure of client compliance, an important factor that was only addressed through client communication at study entry and client reporting at study conclusion.

The oxidative stress in CKD cats may not be of the same magnitude or impact as seen in human kidney disease patients on dialysis (Kumar *et al*., 2014). Although an earlier study found a decrease in total antioxidant capacity of CKD cats, a more recent study failed to find significant differences in a variety of antioxidant parameters between CKD and normal cats (Keegan & Webb, 2010; Krofič *et al*., 2014). The study population of CKD cats may also have been heterogeneous in ways that would only have been identified through imaging or histopathology. Both the degree of oxidative stress and the need for antioxidant supplementation in cats with CKD requires further study and clarification.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Contributions

The authors have no additional contributions to declare.

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