The Association of Early Dietary Supplementation with Vitamin E with the Incidence of Ulcerative Dermatitis in Mice on a C57BL/6 Background

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Summary

The purpose of this study was to ascertain if prophylactic ingestion of a diet rich in vitamin E would prevent or impede the development of ulcerative dermatitis in mice on a C57BL/6 background. Mice were fed after weaning a standard mouse diet, vitamin E (99 IU/kg), or a mouse diet fortified with vitamin E (3000 IU/kg). Cases of ulcerative dermatitis were recorded by individuals (i.e. aware of) the diet assignment. The incidence of ulcerative dermatitis in a retrospective cohort of mice on standard diet was compared with the group on the diet fortified with vitamin E. Age was associated with ulcerative dermatitis in standard diet and vitamin E fortified diet groups, $r = 0.43$, p-value < 0.0001 and $r = 0.18$, p-value < 0.02, respectively. The average age of incidence for ulcerative dermatitis in the mice fed the standard diet was 89 weeks and for the mice fed the vitamin E diet it was 41 weeks. The unadjusted odds ratio comparing the incidence of ulcerative dermatitis between the two diet groups was 4.6 with a 95% confidence interval of (2.44, 8.58), x^2 p-value < 0.0001. Therefore, there was an association between the diets and ulcerative dermatitis, with the mice on the vitamin E fortified diet having almost five times the odds of having ulcerative dermatitis compared with mice on the standard diet. Incidence of ulcerative dermatitis was not influenced by sex or genotype. Our study results show that a diet fortified in vitamin E initiated at weaning does not prevent or impede the development of ulcerative dermatitis in mice on a C57BL/6 background and on the contrary accelerate development when administered to young mice.

Introduction

The C57BL/6 inbred strain of mice is one of the most commonly used strains in medical research. Unfortunately, it also is the strain most susceptible to spontaneous development of ulcerative dermatitis. Ulcerative dermatitis usually appears on the back of the neck and scapula of the mouse, and often spreads to the face, throat and base of the ears (*Andrews et al., 1994*). The condition is difficult to treat effectively and often progresses to the point where the animals need to be euthanized for humane reasons before the experimental endpoint is reached (*Kastenmayer et al., 2006*). This is especially problematic with the use of this strain of mouse in aging research.

Recently, Lawson et al. (*2005*) suggested vitamin E as a treatment for established ulcerative dermatitis in mouse strains on a C57BL/6 background. In that study, mice were fed a diet fortified with vitamin E for 8 weeks after the mice had already developed ulcerative dermatitis. They found that 45% of the treated mice had resolution of their lesions. Since ingestion of vitamin E likely could affect multiple physiological processes, selective treatment of af-

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fected mice at variable ages could confound experimental design and interpretation of results. Therefore, we sought to determine if prophylactic supplementation of the diet with vitamin E could prevent or delay development of ulcerative dermatitis.

Materials and Methods

Animals

Mice (*Mus musculus*) were housed in an animal facility accredited by Association for the Assessment and Accreditation of Laboratory Animal Care, International. The colony was composed of three main genotypes of mice [wild-type, pregnancy-associated plasma protein-A-deficient (*Pappa*$^{-/-}$) and ΔH19 mutant (Δ*H19*$^{m-/+}$) mice that were also crossbred.] All mice were on a C57BL6/ x 129SV/E background as described previously (*Bale & Conover, 2005; Conover & Bale, 2007; Conover et al., 2004*). Mice fed the vitamin E-fortified diet were 21 days old at the beginning of the study. This study was approved by the Mayo Foundation Institutional Animal Care Committee and in compliance with our Animal Welfare Assurance statement through the Public Health Service.

Dirty bedding was sampled from each cage during weekly cage changing and placed in the respective sentinel cages. Sentinel serum samples, one from each side of the rack, were sent out quarterly to Charles River Laboratories, Wilmington, MA, for serologic testing. Endo- and ectoparasite screens were conducted in-house by our veterinary technicians. Bacteriological testing was not routinely done on the colony.

The colony was serologically negative for Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, Theiler murine encephalomyelitis virus, reovirus 3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, *Ectromelia*, K virus, polyoma virus, mouse adenovirus type 1, mouse adenovirus type 2, enzootic diarrhea of infant mice, mouse cytomegalovirus, hantavirus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, mouse parvovirus, and mouse T lymphotropic virus. The colony was also negative for endo- and ectoparasites.

Husbandry during experiment

Mice were housed in autoclaved, polycarbonate, static microisolator cages (Allentown Caging Equipment Co., Inc., Allentown, New Jersey) with woodchip bedding (Sani-Chips®, Montville, New Jersey). Up to five adult mice were housed per cage. The room was kept on a 12:12 hr light:dark cycle (lights on, 0600 h) and the temperature was maintained at 18-26°C with relative humidity between 30-70%. Room air exchanges were 10-15 per hour. Nesting material (Nestlets®, AnCare Corp., Bellmore, New York) was added as environmental enrichment to the cages.

Feeding

Control mice were fed *ad libitum* irradiated diet [PicoLab® diet 20 (5053), PMI® Nutrition International, Richmond, Indiana] and UV-treated tap water in autoclaved water bottles. Experimental mice were similarly fed, except with the Mod PicoLab Mouse 20 (58L0) w/3000 IU/kg vitamin E.

Experimental procedure

Mice, n = 196, were given a mouse diet fortified with vitamin E [Mod PicoLab Mouse 20 (58L0) w/3000 IU/kg Vitamin E] at weaning and monitored up to 45 weeks of age. Retrospective comparison data were used for analyses and were obtained from a database on a cohort of mice consuming standard diet [PicoLab® Mouse 20 (5053)]. This database information was collected on mice in ongoing aging-related research studies from July 2003 until July 2007, and included individual animal identification, gender, genotype, date of birth, date of euthanasia or death, weights (on a subset of mice), and age when ulcerative dermatitis developed.

Individuals assessing the mice for skin lesions were aware of to the diet the mice were consuming. When a mouse in either diet group showed signs of ulcerative dermatitis, the affected area was treated with 2% hydrocortisone cream every other day as palliative therapy. In addition, the mice were continued on their respective diets; mice were not switched or discontinued from the diets. Mice were removed

from the study and humanely euthanized using CO_2 (AVMA 2007) if they developed erosive/ulcerative lesions larger than 20% of body surface; lesions that interfered with eating, drinking or movement; facial dermatitis involving both eyes; lesions that led to corneal ulcers; and lesions with significant hemorrhage or serum crusting and were not healing nor responding to treatment within 14 days per the Institutional Animal Care and Use Standard.

Statistical procedures

Data were collected from a total of 1,004 mice on the standard diet (retrospective cohort) and 196 mice on the vitamin E fortified diet. Data were analyzed by using SAS® Software (SAS Institute, version 9.1, Cary, NC). Odds ratios with 95% confidence intervals and chi-square were used to analyze the data. The diets were compared using a student's t-test. Spearman correlation and ANOVA for means were used to compare weights between genotypes in the vitamin E-fortified group. A p-value of < 0.05 and a confidence interval which did not include 1.0 were considered statistically significant.

Table 1. Nutrient value of standard diet compared with diet fortified with vitamin E based on proximate analysis provided by the manufacturer.

PicoLab® Mouse Diet 20 (5053) Standard Diet		Modified PicoLab Mouse 20 (50L0) with 3000 IU/KG Vitamin E Diet Fortified with Vitamin E		
Nutrients[a]	%	Nutrients	%	p value
Protein	20.0	Protein	21.6	0.80
Fat (ether extract)	5.0	Fat (ether extract)	8.9	0.30
Fat (acid hydrolysis)	5.6	Fat (acid hydrolysis)	9.9	0.30
Fiber (max)	4.7	Fiber (max)	2.4	0.39
Nitrogen-free extract (by difference)	52.9	Nitrogen-free extract (by difference)	51.7	0.91
Total Digestible Nutrients	76.2	Total Digestible Nutrients	84.7	0.51
Gross Energy, kcal/gm	4.07	Gross Energy, kcal/gm	4.60	0.86
Physiological Fuel Value[b], kcal/g	3.41	Physiological Fuel Value[b], kcal/g	3.74	0.91
Metabolizable Energy, kcal/g	3.07	Metabolizable Energy, kcal/g	3.53	0.91
Minerals		Minerals		
Ash	6.1	Ash	5.2	0.79
Vitamins		Vitamins		
Vitamin E, IU/kg	99	Vitamin E, IU/kg	3,000	<0.0001
Calories		Calories		
Protein	24.651	Protein	23.148	0.83
Fat (ether extract)	13.205	Fat (ether extract)	21.502	0.16
Carbohydrates	62.144	Carbohydrates	55.349	0.53

[a] Nutrients are expressed as a percent of ration, except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.

[b] Physicological Fuel Value (kcal/gm) – Sum of decimal fractions of protein, fat, and carbohydrate (use Nitrogen Free Extract) x 4, 9, 4 kcal/gm, respectively.

Table 2. Characteristics of mice on a standard diet compared with mice on a diet fortified with vitamin E.

	Standard Diet (n = 1,004)	Diet Fortified with Vitamin E (n = 196)
Characteristic	n (%)	n (%)
Age[a] (wks)		
16-36	267 (27)	45 (23)
37-57	69 (7)	145 (74)
58-78	206 (21)	-[b]
79-99	277 (28)	-[b]
100-120	142 (14)	-[b]
121-138	34 (3)	-[b]
139-144	9 (1)	-[b]
Disease		
Ulcerative Dermatitis	23 (2)	19 (10)
Gender		
Male	512 (51)	93 (47)
Female	492 (49)	103 (53)
Genotype		
Pappa$^{+/+}$	312 (31)	60 (31)
Pappa$^{-/-}$	308 (31)	54 (28)
Δ*H19*$^{m-/+}$	94 (9)	12 (6)
Δ*H19*$^{+/+}$; *Pappa*$^{+/+}$	144 (14)	28 (14)
Δ*H19*$^{m+/+}$; *Pappa*$^{-/-}$	74 (7)	23 (12)
Δ*H19*$^{m-/+}$; *Pappa*$^{-/-}$	72 (7)	19 (10)

[a] Six mice were missing age data for the vitamin E fortified diet.
[b] Age data not available.

Results

A comparison of nutrient values for the standard diet and fortified diet is presented in Table 1. These data were from the proximate analysis provided by the manufacturer. The vitamin E-fortified diet contained a higher percentage of fat than the standard diet, 8.9% compared with 5.0%, respectively. Consequently the mice received a larger proportion of their total daily calories from fat, 21.50% compared with 13.20% (p=0.16), respectively. However, only the amount of vitamin E was significantly different between the two diets, 99 IU/kg compared with 3000 IU/Kg, p<0.0001.

Table 2 shows the characteristics of the mice on the respective diets. Mice on the standard diet ranged in age from 16-144 weeks and mice on the vitamin E fortified diet ranged from 16-45 weeks. The incidence of eventual ulcerative dermatitis was similar between the two groups, n = 23 for the standard diet and n = 19 for the vitamin E fortified diet. Male

and female mice were similarly distributed between the two diet groups. Most mice were wild-type (*Pappa*$^{+/+}$) and *Pappa*$^{-/-}$ genotypes on a C57BL6 X 129SV/E background in both diet groups, ~30%. The remaining strains comprised 6-14% of the groups. However, the incidence was age-related. Thus the spearman rank correlation showed a strong association between age and the incidence of ulcerative dermatitis, with ulcerative dermatitis in standard diet and vitamin E fortified diet groups: $r = 0.43$, p-value < 0.0001 and $r = 0.18$, p-value < 0.02. The average age of incidence of ulcerative dermatitis in the mice fed the standard diet was 89 weeks and for the mice fed the vitamin E diet it was 41 weeks. The unadjusted odds ratio comparing the incidence of ulcerative dermatitis between the two diet groups was 4.6 with a 95% confidence interval of (2.44, 8.58), x^2 p-value < 0.0001. Therefore, there was an association between the diets and ulcerative dermatitis, with the mice on the vitamin E fortified (but higher fat) diet having almost five times the odds of having ulcerative dermatitis compared with mice on the standard diet.

Due to the association between age and ulcerative dermatitis, the data were also age-stratified, for the 16-36 and 37-57 week age groups. Stratification of older age groups was not possible, due to the small sample sizes. For the 16-36 week old age group the odds ratio comparing the incidence of ulcerative dermatitis between the two diet groups was 18.1 with a 95% confidence interval of (0.72, 449.6), x^2 p-value = 0.16 and for the 37-57 week age group the odds ratio was 2.2 with a 95% confidence interval of (0.70, 6.68), x^2 p-value = 0.27. Therefore, there were no significant associations between diet and ulcerative dermatitis in these stratified age groups. Kaplan-Meier analysis (data not shown) demonstrated what appeared to be a difference in the diets between 34-45 weeks. However, because the vitamin E diet was terminated at 45 weeks, it is difficult to say whether this trend would have continued. Summary incidence data and effect of age is presented in Table 3.

Table 3. Incidence of ulcerative dermatitis in mice on a standard diet compared with mice on a diet fortified with vitamin E.

	Standard Diet[a]	Diet Fortified with Vitamin E
Age (wks)		
34-45	0.5%	9.9%
>45	4.7%	--[b]
Sex		
Male	3.1%	11.1%
Female	2.4%	8.8%
Genotype		
Pappa$^{+/+}$	5.0%	6.8%
Pappa$^{-/-}$	7.3%	6.5%
$\Delta H19^{m-/+}$	16.7%	27.3%

[a]See Table 1 for composition of diets.
[b]Age data not available (see Table 2).

Data for the effect of sex and genotype on incidence of ulcerative dermatitis are also presented in Table 3. There was no significant difference in incidence between the sexes. However, there was significance between the females on standard versus the vitamin E diet (2.4% and 8.8%, respectively, $p<0.007$) and males (3.1% and 11.1%, $p<0.005$). To assess whether a specific genotype may influence the incidence of ulcerative dermatitis we analyzed a subset of the data, excluding cross-bred mice. This included 375 of the 1004 mice on a standard diet and 147 of the 196 mice on the vitamin E fortified diet and showed the genotype had no significant effect on incidence of ulcerative dermatitis.

The relation of diet and weight is summarized in Table 4. For mice on the vitamin E fortified diet, the Spearman rank correlation demonstrated that genotype was significantly and negatively associated with weight, $r = -0.36$, $p<0.0001$. In general, the *Pappa*$^{-/-}$ mice were significantly lighter than wild-type mice, whether on the standard diet or the vitamin E diet, as previously reported (*Conover et al., 2004*). However, there was no significant dif-

Table 4. Comparison of body weight in mice on a standard diet compared with mice on a diet fortified with vitamin E.

	Weight (g)	
	Standard Diet[a]	Vitamin E Fortified Diet
34-45 wks		
All Mice	33.1 ± 0.63	34.9 ± 0.83
Sex		
Male	35.3 ± 0.84	37.3 ± 1.15
Female	31.2 ± 0.93	32.6 ± 1.21
Genotype		
Pappa$^{+/+}$	43.3 ± 0.97	39.9 ± 1.45
Pappa$^{-/-}$	28.7 ± 0.76	29.3 ± 1.04
Δ*H19*$^{m-/+}$	36.6 ± 2.53	47.9 ± 2.79[b]

[a]See Table 1 for composition of diets; results are mean ± Standard Deviation.
[b]$p<0.05$.

ference in weight of the mice between the standard diet and the vitamin E diet, except in the Δ*H19*$^{m-/+}$ group of mice.

Discussion

This study indicates that a prophylactic diet enriched with vitamin E does not prevent or delay ulcerative dermatitis; rather, when introduced at weaning it appears to accelerate the onset of the disease. Ulcerative dermatitis is generally associated with aging mice on a C57BL/6 background, with an average age of onset at about 80 weeks (*Andrews et al., 1994*). In our study, ulcerative dermatitis occurred in mice on the standard diet at an average age of 89 weeks, whereas, the average age of incidence of ulcerative dermatitis for the vitamin E fortified mice was 41 weeks. Because of this unexpected increase in onset of the disease, which occurred in both male and female mice of all genotypes on the C57BL/6 genetic background, the study of the effect of vitamin E in these mice was not continued. Because the study was terminated, we cannot draw conclusions about disease incidence with statistical confidence beyond 45 weeks of age. In addition, only the incidence of ulcerative dermatitis was recorded in these mice to compare with findings in mice fed the standard diet and concurrently in an aging protocol. Therefore, we do not know whether or not the vitamin E diet could ameliorate the severity of the ulcerative dermatitis at a later time point. However, there did not appear to be a difference in response to hydrocortisone treatment in the two diet groups.

Previously, treatment of established ulcerative dermatitis by putting mice on a vitamin E diet showed some success in resolution in the ulcerative dermatitis. Lawson et al. (*2005*) suggested that a diet fortified with vitamin E lessens the severity of ulcerative dermatitis, possibly due to its antioxidant nature. It may be that a high concentration of vitamin E in the diet at a young age is more harmful than helpful. It is possible that a diet fortified with vitamin E when started in adult mice could have beneficial effects.

It was also noted that the diet with added vitamin E contributed increased calories to the diet due to the fat content. This can negatively affect research on mice, especially in aging and obesity studies. Because vitamin E is a fat soluble vitamin, increased levels of fat are necessary to adequately incorporate the vitamin into the diet. Unfortunately, this change in caloric content of the diet may also have an unknown impact on the incidence of ulcerative dermatitis. However, we are unable to separate these effects in this study. The diet with added vitamin E increased the weight only of the Δ*H19*$^{m-/+}$ strain of mice significantly. However, it is unclear as to why this particular genotype of mouse gained more weight than other genotypes on the same diet. It may be due to the over-expression of insulin-like growth factor (IGF-II) in the Δ*H19*$^{m-/+}$ strain. IGF-II is a growth-promoting factor and Δ*H19*$^{m-/+}$ mice are 120% the size of a standard mouse (*Bale & Conover, 2005; Devedjian et al., 2000*). This, and the vitamin E diet containing nearly 10% fat, could have possibly led to the significant increase in weight between the standard diet and vitamin E diet in Δ*H19*$^{m-/+}$ mice.

Our study design was somewhat unique in the field of laboratory animals. Mixed cohorts with both non-concurrent and concurrent follow-up components are more common place in human epidemiology studies (*Szklo & Nieto, 2004*). There are pros and cons to the use of this research method in animal research. Although there are several advantages to this method, such as conservation of animal resources and decrease of study duration, there are also potential biases, including relying on existing records, which may be less complete and accurate than the prospective data being collected on the mice on the vitamin E fortified diet (*Greenberg et al., 2006*). For example, we did not collect weight data on the retrospective cohort, therefore comparisons could only be made between the strains of mice on the vitamin E diet. However, we were primarily interested in finding a scientifically rational means to reduce the observed incidence of ulcerative dermatitis in our aging studies, so this retrospective cohort is an appropriate control. Secondly, individuals assessing the mice on the fortified vitamin E diet were aware of the treatment, which may result in observer bias. Therefore, perceptions of the individuals assessing these mice may have influenced the outcome, thereby reducing the certainty in the study results (*Greenberg et al., 2006*). Despite the limitations, these data assist in the understanding of strain susceptibility and potential preventative measures for this common disease problem in C57BL/6 mice.

In conclusion, a prophylactic diet containing vitamin E and high fat (which itself may be a confounding factor) is not a suitable preventative measure for ulcerative dermatitis. This diet appears to accelerate the onset of ulcerative dermatitis in mice on a C57BL/6 background when started at weaning.

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