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EFFECT OF DIETARY VITAMIN E SUPPLEMENTATION AND ROTATIONAL STRESS ON ALVEOLAR BONE LOSS IN RICE RATS

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Summary—The effect of this supplementation on bone loss (distance from the cementum enamel junction to the alveolar crest measured at the midline of the lingual aspect of each of the mandibular molar roots) was studied in rats that were either not stressed or stressed on a rotational device for 90 days. In the first study, neither vitamin E nor stress condition had statistically significant effects but there was substantial bone loss and bone-loss variability in all groups. Before the start of the second study, to reduce differences in bone loss that might otherwise exist before introduction of the treatments, rats received an antibiotic in their drinking water. In addition, rotational stress was introduced more abruptly than in the first study to reduce the likelihood of adaptation. Bone loss and bone-loss variability were substantially reduced in the second study. Analysis of these data indicated that vitamin E supplementation had a statistically significant protective effect, which was most pronounced at sites most susceptible to loss. Stressed subjects tended to lose more bone, but this effect was not significant. These findings suggest some role for vitamin E supplementation in the maintenance of periodontal health but also a sensitivity in this effect to initial periodontal status.

Key words: alveolar bone loss, vitamin E, stress, psychological.

INTRODUCTION

Vitamin E functions as a free-radical scavenger that inhibits lipid peroxidation and inflammation, protects ischaemic and hypoxic tissues, and is immunostimulating (Crary, 1984). Because of this spectrum of activities, the relation between vitamin E and periodontal health and disease has been studied; findings tend to support a therapeutic role for the vitamin but the evidence is inconsistent.

Periodontal tissues of albino rats were not affected by long-term vitamin E deficiency despite early European reports of successful treatment of periodontitis with this substance in man (Nelson and Chaudhry, 1966). However, vitamin E deficiency had negative impact on periodontal health in rats in another study (Schneider and Pose, 1969). Ligature-induced periodontitis in rats was not affected by vitamin E supplementation (Parrish, DeMarco and Bissada, 1977), but careful inspection of the data suggests that supplementation resulted in greater numbers of inflammatory cells with, simultaneously, less alveolar bone loss. This would be consistent with vitamin E's immunostimulant and antioxidant properties, even though sample sizes and methods of analysis did not have sufficient power to detect these shifts at statistically significant levels. Dietary vitamin E supplementation was shown to accelerate gingival wound healing in albino rats (Kim and Shklar, 1983).

In man, greater dietary intake of vitamin E has been associated with fewer reported oral symptoms

(Cheraskin and Ringsdorf, 1970), but circulating concentrations of the vitamin were the same in patients with and without periodontitis (Slade *et al.*, 1976). Patients with periodontal disease who were given vitamin E daily for 21 days to swish in their mouths and swallow exhibited a significant decrease in fluid flow from the gingival sulcus than in controls with disease but no vitamin E supplementation (Goodson and Bowles, 1973). Also, subjects given vitamin E supplementation for 12 weeks exhibited a reduction in Russell's Periodontal Index (Cerna *et al.*, 1984). However, topical vitamin E did not reduce gingivitis over a 4-week period relative to a placebo (Cohen *et al.*, 1991), but the method appeared insensitive in that chlorhexidine similarly had non-significant effects on gingivitis.

Lack of consistent benefits associated with vitamin E may be due, in part, to the absence of physiological stress manipulation. During stress, vitamin E stores are depleted and, once depleted, tissues are at greater risk. Without stress, vitamin E may remain at effective concentrations so that there may be no relation between it and periodontal destruction. One may argue that in man, insufficient vitamin E and a subsequent increase in the likelihood of periodontal destruction occur during brief intervals associated with stressful life-events. Therefore, the cross-sectional analysis of Slade *et al.* (1976) might not identify a relation.

A further problem with many rat studies is that this species is not usually susceptible to periodontal destruction, except when this is ligature induced or is the result of other extraordinary interventions. It would be difficult, therefore, to observe a beneficial

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Abbreviations: AN(AC)OVA, analysis of (co-)variance; CEJ, cementum–enamel junction.

effect of vitamin E unless it is superimposed on an adverse initial state such as healing of experimental gingival wounds, vitamin E deprivation (as contrasted to supplementation), or stress.

Effects of stress on periodontal destruction have been documented and are consistent with generally held concepts of the relation between stress and disease resistance (Troxler, 1986; Dantzer and Kelley, 1989; Ballieux, 1991). In a series of experiments on rodents, increased rates of periodontal destruction (Ratcliff, 1956; Fedi Jr, 1958; Shklar and Glickman, 1959; Gupta, Blechman and Stahl, 1960; Gupta, 1966) and decreased rates of gingival wound healing (Stahl, 1961) were shown to accompany the presence of stressful stimuli. While similar experimental designs are not possible in man, there is evidence that the same relations hold. Early anecdotal reports (Moulton, Ewn and Thiemman, 1952) have been supported by correlational studies relating life-stress events to rates of periodontal destruction (Manhold, Doyle and Weisinger, 1971; Haskell, 1975; Green et al., 1986).

Although stress will affect a variety of physiological processes, activation of the sympathetic–adrenal–medullary and hypothalamic–pituitary–adrenocortical systems (Axelrod and Reisine, 1984) appears particularly important in terms of defining explanatory mechanisms for enhancement of disease progression. Exposure of animals to stressful stimuli is accompanied by increases in the synthesis, storage and release into the circulation of catecholamines and glucocorticoids. The effects of these events and subsequent enzymatic shifts, as indexed by a variety of measures, can include: salivary and small-vessel vasoconstriction, release of cytotoxic free radicals, increased membrane permeability, impaired wound healing, decreased resistance to infection, and suppressed cellular immune responses (Matheny, 1988; Weiss et al., 1989).

In addition to studies relating stress to periodontal health, there are others in which stress-related hormones have been measured or manipulated directly. Administration of cortisone caused osteoporosis of alveolar bone in mice (Glickman, Stone and Chawla, 1953) and patients with acute necrotizing ulcerative gingivitis were found to have higher levels of overnight urinary cortisol (Cohen-Cole et al., 1981). Catecholamines have been shown to reduce gingival circulation (Ito et al., 1973; Clarke, Shephard and Hirsch, 1981) and to enhance the virulence of gingival crevicular bacteria (Courant and Gibbons, 1966).

The presence of oxygen radicals has been suggested as playing a central part in tissue damage associated with chronic inflammation (in general) and periodontal disease (Hoffeld, 1982). Based on vitamin E's ability to protect tissue from oxidative damage, Goodson (1975) predicts therapeutic value but recognizes that there has been an insufficient number of studies in this area. Hence we have now sought to evaluate the ability of vitamin E supplementation to prevent periodontal destruction in a species inherently susceptible to such destruction, and while exposed to environmental stress that could affect the supplement's physiological value.

MATERIALS AND METHODS

Study 1

Rice rats (*Oryzomys palustris*) were chosen because of susceptibility to periodontal destruction (Leonard, 1979). The study (protocol reviewed by a Laboratory Animal Use Committee) had a two-by-two factorial design with two levels of dietary vitamin E supplementation and either a control or high-stress environment.

Thirty-two male rice rats (57–84 days of age) were switched from standard rodent diet to a synthetic test diet (modified Purina Basal Test Diet 5755). This diet contains 44% dextrin, 21% casein, 15% sucrose, 5% lard, 5% corn oil and the remaining percentage is comprised of other necessary additives. One half of the animals received a synthetic control diet, which included a standard 50 IU of vitamin E per kg feed (35 IU attributable to d-α-tocopherol acetate oil supplement and approximately 15 IU due to other components, principally corn oil). The remaining animals received a synthetic test diet that contained 5000 IU of the vitamin per kg feed (4985 IU due to the tocopherol additive). This IU/kg dose (60 IU/day based on a 12 g daily food ingestion) had been used in a similar long-term study of periodontal destruction in rats, without reported side-effects (Parrish et al., 1977). In general, vitamin E is not considered toxic, mutagenic, carcinogenic, or teratogenic, even at high doses (Bendich and Machlin, 1988).

After 35 days of feeding on the synthetic diets, rats were assigned to normal or high-stress environments. Assignment to both diet and stress conditions involved grouping animals by weight and then randomly assigning them within groups to the various treatment conditions.

All rats were housed individually in plastic cages (standard multi-mouse cages, approx. 7 in. wide × 12 in. long × 5 in. high) with compressed, shreddable wood shavings as bedding material. All groups were housed in the same quiet room with daily 12-h light/12-h dark cycles. Non-stressed animals were housed in standard racks, with diet groups in counterbalanced order relative to location.

Profoundly painful or exhausting stimuli were not used as stressors. Rather, rats were subjected to cage rotation. This method of stress induction is considered relatively benign but nevertheless is associated with shifts in physiological status (Shipov et al., 1985) including circulating concentrations of stress-related hormones (Riley, 1981). Stressed animals were housed in cages mounted in a star pattern on a large platter that was rotated by a quiet electric motor controlled by a computerized timing device. The platter was approx. 120 cm in diameter and the radius of a circle formed by the outer edges of the cages was 55 cm. Two of these devices (henceforth called wheels), each holding eight subjects (four from each of the two diet groups), were used.

Immediately after their assignment to high-stress conditions, these animals were subjected to a 1-min period of 30 rev/min rotation once every 4 h. When wheels were spinning, rats preferred to place themselves at the far end of their cages, approx. 50 cm (radius, r) from the center of the wheel. Therefore, 30 rev/min corresponds to a gravitational (g) force of 0.5

[where a is acceleration and r is velocity, $g = a/980$; $a = r^2/r$; $r = (2\pi r)$ (rev/min 60^{-1})].

Each wheel was rotated on an independent, continuous (24 h/day) variable-interval schedule (i.e. the location of the 1-min rotational period within the 4-h time interval was random). As rotational stress may reduce feeding and cause weight loss, subjects were weighed at least once per week. A protocol for temporary termination of rotation because of catastrophic weight loss was in place but never needed to be implemented.

Over a period of approx. 2 months, rotations were increased from the initial 1 min at 30 rev min every 4 h to 15 min (in a single segment) of 42 rev/min (1.0 g) every 30 min. Ninety days after initiation of stress manipulations, animals were anaesthetized, orbital blood samples drawn to assay for vitamin E, and the animals killed by nitrogen asphyxiation.

Jaws were dissected out and defleshed by boiling. Mandibles were dried and mounted on modelling clay on small dishes, and lingual alveolar bone loss was evaluated using a standard approach (Keyes and Gold, 1955; Gupta and Shaw, 1956). Seven measurements taken on each jaw side reflected the distance from the CEJ to the alveolar bone crest at the midline of each root (three roots for the first molar and two roots each for the second and third molars). Measurements were made through a dissecting microscope fitted with an optical caliper eyepiece. This method yielded a measurement precision of approx. 0.01 mm and allowed for minor adjustments in light intensity and angulation to improve visualization. Nevertheless, the CEJ was sometimes difficult to visualize, even after application of eosin stain. In such cases, an approximation was made based on other anatomical landmarks. To decrease variability and to preclude a potential for bias, these measurements were made by one person who was blind as to experimental condition. The primary dependent variable of interest was the CEJ–bone crest distance averaged across 14 sites (seven sites on each of two sides).

RESULTS

Study 1

One animal in the low vitamin E, high-stress group died during the course of the study and was not included in the analysis. ANACOVA was made on body weight. The covariate was weight on stress day 0, the dependent variable was weight at study termination, and the between-subjects variables were stress and vitamin condition. Over the 90 days, rats gained 6.0 g (from a mean weight of 87.6 g to one of 93.6 g), but there was no effect of the independent variables, or their interaction, on the covariate adjusted weights.

Of 31 blood samples drawn for vitamin E assay, one sample was lost and six were combined into three sets of pairs (within treatment groups) to achieve a minimum assay quantity. ANOVA with the between-subject factors of dietary vitamin E and stress revealed only a significant effect for diet condition ($F = 222.99$, d.f. = 1, 23, $p < 0.001$). Animals given high dietary vitamin E had more than four times the

Table 1. Mean distance, in mm, and SD in parentheses from CEJ to alveolar crest as a function of dietary vitamin E and rotational stress in Study 1

	Dietary vitamin E		
	Standard	Supplemented	Total
Stress			
No	0.711 (0.160)	0.737 (0.239)	0.724 (0.197)
Yes	0.806 (0.087)	0.833 (0.260)	0.820 (0.240)
Total	0.756 (0.197)	0.785 (0.246)	0.771 (0.221)

circulating vitamin E than those given low amounts (10.55 versus 2.44 mg/l).

Table 1 shows mean alveolar bone loss for the four groups. Effects of diet and stress level did not approach statistical significance (ANOVA $Fs = 0.10$, 1.37 and 0.00, d.f. = 1, 27, for vitamin E stress, and interaction, respectively).

MATERIALS AND METHODS

Study 2

As described above, there was high variability in bone loss within all groups in Study 1. This may be attributed to animals being 57–84 days old before the diet variable was introduced and 92–119 days old before the stress variable. Rice rats can have substantial bone loss before these ages (Leonard, 1979) and this 'noise' may have overwhelmed the diet and stress effects.

To evaluate this possibility, the study was repeated with two changes. First, starting from 18 to 31 days post-weaning, rats received 1.35 g/l tetracycline hydrochloride in their drinking water (Vetquamycin-324^{tm}, Phizer, 1.902 g/l) on an approx. 2 weeks on/2 weeks off schedule. Antibiotics substantially reduce bone loss in rice rats (Gupta, Auskaps and Shaw, 1957) and so should reduce pre-study, between-subject variability. Based on estimated daily water intake of 6.4 ml for an 80-g rat, tetracycline intake was (1.35 mg/ml)(6.4 ml) = 8.64 mg, and dosage was 8.64 mg/0.08 kg = 108 mg/kg per day. However, giving the antibiotic orally can reduce absorption by more than 50% (Plumb, 1991). Recommended oral dosages of tetracycline are about 33–110 mg/kg per day (Huber, 1982; Kirk, 1992; Plumb, 1991) and 5 mg/ml (Kohn and Barthold, 1984). In general, tetracycline is considered relatively non-toxic, with oral doses of 75–465 mg/kg per day for 8 weeks being well tolerated by dogs without evidence of toxicity (Huber, 1982). Possible interaction between tetracycline (toxicity) and Vitamin E supplementation was considered unreasonable given the clinical acceptance of these concentrations, the lack of observed toxic effects, and the ending of antibiotic supplementation 1 week before the introduction of test diets.

Second, in Study 1 the stress had been instituted gradually over a period of 2 months. This may have attenuated the stress variable by allowing animals to adapt. Therefore, in Study 2 the rotational schedule reached the end-stage of 15 min rotation at 42 rev/min per 30 min after 3 days.

The rats in Study 2 were between 76 and 97 days old when they were switched to the synthetic diets

Table 2. Mean distance, in mm, and SD in parentheses from CEJ to alveolar crest as a function of dietary vitamin E and rotational stress in Study 2

| | Dietary vitamin E | | |
	Standard	Supplemented	Total
Stress			
No	0.420 (0.082)	0.359 (0.044)	0.391 (0.072)
Yes	0.451 (0.102)	0.379 (0.023)	0.417 (0.083)
Total	0.436 (0.091)	0.369 (0.036)	0.404 (0.077)

(antibiotics had been discontinued 1 week previously), the stress manipulation was instituted 35 days later, and again they were killed after 90 days.

RESULTS

Study 2

One stressed subject and one non-stressed subject, both vitamin E supplemented, died in the course of the study and their data were not analysed. Over the 90 days, subjects gained 5.1 g (from a mean weight of 89.5–94.6 g). ANACOVA on body weight, similar to that in Study 1, revealed a significant main effect for stress condition ($F = 10.97$, d.f. = 1, 25, $p = 0.003$). Covariate adjusted terminal weights were 97.6 g for non-stressed and 91.5 g for stressed animals.

Table 2 shows the results of Study 2. Total bone loss and bone-loss variability were substantially reduced as compared to Study 1. ANOVA revealed a statistically significant main effect for vitamin E ($F = 6.51$, d.f. = 1, 26, $p = 0.017$) but no effect due to stress ($F = 0.97$, d.f. = 1, 26, $p = 0.337$) or to a

vitamin–stress interaction ($F = 0.04$, d.f. = 1, 26, $p = 0.850$). A second ANOVA was done with the tooth root at which measurements were taken (seven levels corresponding to mesial of the first molar through distal of the third molar, averaged across the left and right sides) added as a within-subject variable. The effect of 'root' was statistically significant ($F = 224.29$, d.f. = 6, 156, $p < 0.001$) with a pattern consistent with published descriptions (Gupta and Shaw, 1956), and the root–vitamin E interaction might be considered significant ($F = 2.66$, d.f. = 6, 156, $p = 0.018$; but $p = 0.066$ and $p = 0.052$ with Greenhouse–Geisser and Huynh–Feldt adjustments, respectively). The data are shown in Fig. 1 and suggest that vitamin E exerts its protective effects at those sites that are most susceptible to bone loss.

DISCUSSION

The findings of Study 2 support the hypothesis that dietary vitamin E supplementation can decrease alveolar bone loss. This study does not define the protective mechanism of vitamin E, which may be related to its antioxidant, immunostimulant or other activities. The study does not provide sufficient information to assess the relative contributions of infective, inflammatory and hormonal mechanisms on observed bone loss, and does not define the therapeutic route, which may be either topical or systemic. As an exploratory study it does suggest, however, that despite inconsistent findings in the literature, this may be a productive area for research.

The absence of an observed vitamin E effect in Study 1 had been attributed to variability in bone loss
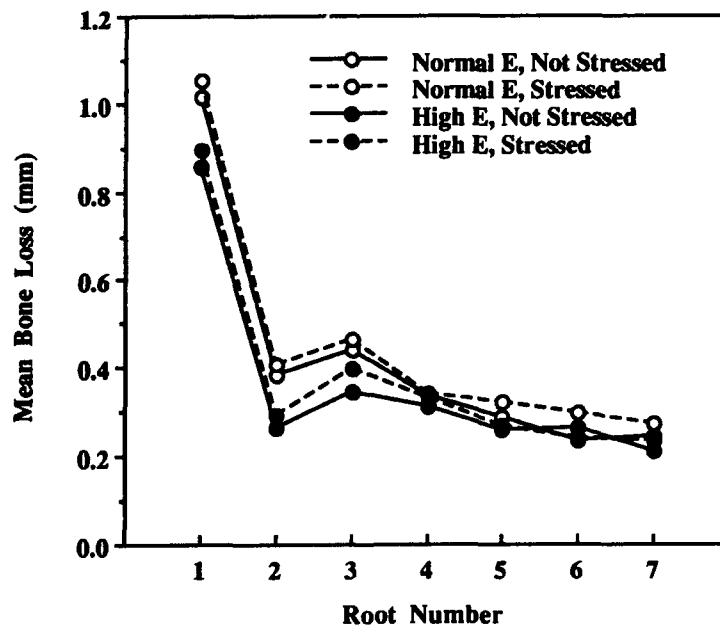

Fig. 1. Distance from the CEJ to the alveolar crest measured at the midline of the seven mandibular roots found in rats and averaged across the left- and right-hand sides. Roots 1–7 correspond to the mesial, central, and distal roots of the first molar (1–3) and the mesial and distal roots of the second (4 and 5) and third (6 and 7) molars, respectively. The protective effects of dietary vitamin E supplementation are most apparent at those locations at most risk for bone loss.

before the start of the study. Decreased bone loss and bone-loss variability in Study 2, which incorporated antibiotic prophylaxis, supports this contention and suggests that a vitamin E effect may be relatively sensitive, at least in the rice rat, to initial periodontal status.

It had been anticipated that vitamin E effects would be increased among stressed animals. This was not observed. Although they tended to lose greater amounts of bone, this effect was not statistically significant and stress level did not interact with vitamin condition. It is possible that for rice rats the 'non-stressed' environment may have been stressful d le to solitary caging and small cage size. Because of this, the stress manipulation may not have been as dramatic as had been anticipated. An appropriate non-stress control group might require large, communal cages.

It is also possible that, while cage rotation might be an effective acute stressor (Riley, 1981), the relevant hormones may adjust in animals subject to chronic stress (McCarty, Horwatt and Konarska, 1988). Thus, there may be a reduction in some stress-related effects. Weight loss was not affected by stress level in Study 1 but was in Study 2, suggesting a more effective stress manipulation. Nevertheless, more definitive evaluation of stress effects over time would require repeated assays of blood hormone concentrations.

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The effect of this supplementation on bone loss (distance from the cementum-enamel junction to the alveolar crest measured at the midline of the lingual aspect of each of the mandibular molar roots) was studied in rats that were either not stressed or stressed on a rotational device for 90 days. In the first study, neither vitamin E nor stress condition had statistically significant effects but there was substantial

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bone loss and bone-loss variability in all groups. Before the start of the second study, to reduce differences in bone loss that might otherwise exist before introduction of the treatments, rats received an antibiotic in their drinking water. In addition, rotational stress was introduced more abruptly than in the first study to reduce the likelihood of adaptation. Bone loss and bone-loss variability were substantially reduced in the second study. Analysis of these data indicated that vitamin E supplementation had a statistically significant protective effect, which was most pronounced at sites most susceptible to loss. Stressed subjects tended to lose more bone, but this effect was not significant. These findings suggest some role of vitamin E supplementation in the maintenance of periodontal health but also a sensitivity in this effect to initial periodontal status.