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Disease incidence and longevity are unaltered by dietary antioxidant supplementation initiated during middle age in C57BL/6 mice

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Abstract

The ability of augmented antioxidant consumption to alter disease incidence, lesion burden and/or longevity was studied in adult male C57BL/6 mice. Mice were fed modified AIN76 diet or modified AIN76 supplemented with vitamin E, glutathione (GSH), vitamin E and GSH, melatonin or strawberry extract starting at 18 months of age. All the mice in this study were heavier than reference populations of male C57BL/6 mice fed NIH-07 or NIH-31, which were maintained without a mid-life change in diet. Fatty liver, focal kidney atrophy and proteinacious casts in the renal tubules were observed more frequently in this study population than in the reference populations. Lesion burden and incidence of specific lesions observed amongst the various groups in this study did not differ. There were no differences observed for longevity of any of the study groups. The longevity observed in this study was similar to that previously reported for male C57BL/6 mice. Thus, diet supplementation with antioxidants initiated during middle age did not appear to affect age-associated lesions patterns, lesion burden or longevity for ad libitum fed male C57BL/6 mice.

Keywords: Aging; Longevity; Lesions; Nutrition; Antioxidant; Mice

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1. Introduction

Nutritional supplementation, especially with antioxidants, is frequently touted as a means to enhanced wellness and increased longevity (Harman, 1962; Sies and Stahl, 1995). It has been hypothesized that much of the age-related physiologic deterioration results from an age-associated increase in free radicals generated as by-products of normal metabolism (Harman, 1956; Ames et al., 1993; Davies, 1995). There are numerous reports which demonstrate improvement in parameters, thought to be predictive of age-related functional outcome, with increased intake of either vitamins and/or fruits and vegetables (Singh et al., 1993; Davies, 1995; Tu et al., 1995; Burke et al., 1997; Meydani et al., 1997). Unfortunately, there exists only limited knowledge about the disease-preventive properties of specific micronutrients, and it remains to be determined whether the health benefits of augmented fruit and vegetable consumption (van Poppel and van den Berg, 1997) can be replicated by consumption of antioxidant supplements (Potter, 1997). Although it is unclear if supplementation during only a portion of the lifespan will effectively modulate disease processes and longevity, this is becoming a common recommendation (Ward, 1994). The understanding that antioxidants accomplish the elimination of reactive free radical molecules in-vitro, has served as a rationale to advocate for the consumption of antioxidant supplements as a means to retard aging. We have therefore sought to examine the impact of antioxidant supplementation on aging in cohorts of middle-aged mice. For the purposes of this study, we examined the effect of vitamin E, GSH, vitamin E and GSH, melatonin and a strawberry extract on lesion biomarkers of aging and longevity.

The components used to supplement the control diet in these experiments (vitamin E, GSH and melatonin) have each been demonstrated to improve parameters which undergo specific age-associated declines or are associated with specific age-related disease processes. Strawberry extract was added to the control diet for an additional set of mice based on the results of an automated assay for antioxidant activity which demonstrated that strawberries have high antioxidant capacity (Wang et al., 1996).

We have previously demonstrated that mice at 2 years of age have higher prostaglandin E_2 (PGE_2) production than young mice (Meydani et al., 1986; Hayek et al., 1997a) and that short-term consumption of diet supplemented with 500 ppm vitamin E resulted in decreased PGE_2 levels in older mice (Meydani et al., 1986). Similarly, we have recently shown that short-term vitamin E supplementation decreased lung viral titer in influenza-infected older mice (Hayek et al., 1997b). These observations suggest that consumption of vitamin E-supplemented diet elicits important beneficial changes.

Circulating levels of GSH concentrations, in-vivo, demonstrate an age-associated decrease (Julius et al., 1994; Yang et al., 1995). The age-related decrease in liver GSH concentration has previously been shown to be partially ameliorated by caloric restriction (Mune et al., 1995) and by methionine restriction (Richie et al., 1994); both dietary manipulations also increase longevity. Further, direct dietary supplementation with GSH in mice has been shown to diminish the age-associated decline in T cell-mediated immune function (Furukawa et al., 1987).

Melatonin has been reported to have anti-viral, anti-cancer and immune enhancing properties (Pierpaoli and Regelson, 1994; Mocchegiani et al., 1994), while at the same time has been found, by others, to have only limited direct antioxidant activity (Marshall et al., 1996). It is, however, of interest to establish whether long-term melatonin supplementation influences longevity.

Strawberries contain a variety of flavonoids, including anthocyanins, which may have measurable capacity to inhibit lipid peroxidation and scavenge free radicals (Rice-Evans et al., 1995; Tsuda et al., 1996; Cao et al., 1997) both in-vitro and in-vivo. Previously, using an in-vitro automated oxygen radical absorbance capacity assay (ORAC), the antioxidant capacity of strawberries was shown to be the highest of the 12 fruits tested (Wang et al., 1996). This suggested to us that addition of strawberry extract to the control diet would increase antioxidant intake. We sought to determine how this diverse antioxidant supplementation would compare with the other antioxidant-supplemented diets studied.

2. Materials and methods

This study utilized 460 C57BL/6 mice bred and raised under barrier conditions at our facility from stock originally obtained from the NIA colony. The protocol was reviewed and approved by the Animal Care and Use Committee of the USDA Human Nutrition Research Center on Aging at Tufts University. The mice were individually housed in autoclaved, filtered cages at 23°C and 45% relative humidity with 15 air changes of 100% fresh-filtered air per hour and a 12/12 h light/dark cycle. All animals were observed daily for clinical signs of disease. The mice were fed autoclaved chow PMI 5021 (Richmond, IN) until they reached 18 months of age. They were then fed one of seven semi-synthetic diets, control (C) which was a modified AIN76 diet, or C supplemented with one of the following: 470 ppm vitamin E (E) (Teklad, Madison), 0.5% GSH (kindly provided by Kyowa Hakko Kogyo, Tokyo), 470 ppm vitamin E + 0.5% GSH (E + GSH), 11 ppm melatonin (M) (Sigma Chemical, St. Louis) or 1% strawberry extract (S) prepared in our facility as previously described (Wang, 1996). The diets are described in complete detail in the following section. At the start of this experiment, there were 77 C-, 76 E-, 76 GSH-, 77 E + GSH-, 77 M- and 77 S-fed mice. Each mouse had ad libitum access to food and water and was weighed monthly during the course of the experiment. Caloric intake was assessed monthly for 3 days using the same mice from each diet group by providing their food in a glass feeder with a stainless steel insert to discourage spillage. During the course of the experiment, as mice died, the numbers of mice studied to establish average food intake decreased from 42 (six per diet group) down to a total of three.

Data on two distinct cohorts of male C57BL/6 mice, maintained throughout their lives without change in chow formulation at 18 months of age, were used for comparison of lifespan to the animals in this study (Goodrick et al., 1990; Turturro et al., 1994). The mice in these studies were fed NIH-31 and NIH-7, respectively; both of these are commercially available, open formulation, natural ingredient

diets. The longevity and body weight data from these two distinct colonies of C57BL/6 mice were nearly undistinguishable. Therefore, these cohorts of mice were utilized as references for the current study as a means to examine whether the weight gain of the mice in this study affected their longevity. The actual data used for the statistical analysis were obtained from the National Center for Toxicologic Research (Jefferson, AR) (NCTR) (Bronson and Lipman, 1991; Turturro et al., 1994). Previously, the pathology present in C57BL/6 mice at 6, 12, 18 and 24 months of age was determined in a cross-sectional study of rodents (Bronson, 1990). It can be used as a reference for the types of pathologies which were likely present at the time the dietary intervention was made in this study.

2.1. Mortality groupings

The mice in each diet group were divided into three mortality cohorts. These were: (1) mice sacrificed at 24 months of age; (2) mice sacrificed when the rest of their diet-date of birth cohort had reach 50% mortality and (3) those mice in the 24 months old and 50% groups which died prior to reaching either 24 months of age or the age of 50% mortality. Each of these different mortality groups provides information relevant to disease incidence and causes of death.

The first mortality group, consisted of 19–21 mice from each diet group which were sacrificed at 24 months of age. This age was chosen as an informative point near the end of their lifespan. The utility of this mortality group was that it permitted direct comparison between the various diet groups at one specific chronological age. This constituted the cross-sectional component of the study. The mice in this mortality group from each of the diets were euthanized by 100% CO_2 followed by exsanguination. The livers from these mice were perfused with cold phosphate-buffered saline, and a sample of liver was snap frozen in liquid nitrogen using pre-cooled metal sheets. The frozen liver samples were stored at -80°C until analysis of reduced and oxidized GSH as described below.

The second mortality group involved following the mice longitudinally to determine when 1/2 of the mice in a given cohort had died. At this age, the remaining animals, ranging in number from 7 to 20, were sacrificed. The utility of this mortality group was to provide an estimate of the overall longevity via calculation of the age at which the group reached 50% mortality. These animals were informative for comparison of the efficacy of the various diet groups at the same point in their group mortality curves. The size of this project was such that mice born in three consecutive months were included in the experiment. Thus, for each diet, we had three independent measurements of the age at which mice fed that diet reached 50% mortality.

The last group of mice were those animals which died prior to reaching 24 months of age in the case of the cross-sectional cohort; or prior to the age at which their group attained 50% mortality. These mice, which succumbed during the course of the experiment and of which there were 28–38/group obtained for study, were valuable for the analysis of lesion incidence and for determining which lesions were lethal. This last group provides an additional longitudinal component to the

study. Its utility is in comparison not only between the diet groups but also to indicate if there were rapidly developing disease processes killing the mice. Such diseases could be expected to be under-represented in the mice sacrificed at 2 years of age and to a lesser extent for those sacrificed when groups reached 50% mortality.

2.2. Diets

2.2.1. Control (C)

The control diet (C) was a modified version of AIN76, which utilized the mineral and vitamin mix as previously reported (Meydani et al., 1987; 1992; Hayek et al., 1997b) and which contained 18% casein, 33.6% corn starch, and 33.4% sucrose, all three of which were obtained from Dyets (Bethlehem, PA); 5% cellulose (Teklad, Madison), 5% soybean oil (BioServe, Frenchtown), 0.3% DL-methionine (Teklad), 0.25% choline bitartrate (Teklad), and 3.5% AIN76A salt (Dyets) and 1% AIN76A vitamin mix (Teklad). The calculated caloric density for all the diets, using 4 kcal/g protein, 4 kcal/g carbohydrate and 9 kcal/g fat, was 3.84 kcal/g.

The other five diet groups were also fed ad libitum with the C diet supplemented with one of the following:

2.2.2. Vitamin E (E)

The E groups were fed C diet supplemented to contain a total of 500 ppm DL-α-tocopheryl acetate (Teklad). This was an increase of 470 ppm of vitamin E over the 30 ppm of vitamin E in the control diet and was calculated to increase the vitamin E intake per gram of body weight by 0.07 mg/day.

2.2.3. Glutathione (GSH)

The GSH groups were fed C diet supplemented with 0.5% GSH by weight. The calculated daily intake of GSH per gram of body weight was 0.43 mg.

2.2.4. Vitamin E + GSH (E + GSH)

The E + GSH groups received C diet supplemented with both 470 ppm vitamin E and 0.5% GSH by weight. This combination was included to determine whether, in-vivo, these antioxidants would act additively, synergistically or would negate the effects of one another. The calculated daily intake of these two nutrient equaled that of the E and GSH groups, 0.07 mg more than controls and 0.43 mg, respectively.

2.2.5. Melatonin (M)

The M groups were fed C diet supplemented with 11 ppm melatonin. The amount being consumed was calculated to result in 68 ng of melatonin/day/g of body weight.

2.2.6. Strawberry (S)

The S groups consumed C diet supplemented with 1% strawberry extract by weight. The amount of strawberry extract being consumed was calculated to be 228 ng/day/g of body weight.

2.3. Assessment of vitamin E, melatonin and GSH

Serum was obtained from 24-month-old mice after exsanguination via cardiac puncture. Not all parameters were measured in all of the diet groups due to the limited amount of serum from each mouse. Vitamin E concentration in serum was measured in the 9 C, 9 E and 9 E + GSH mice; melatonin levels were measured in serum from the 9 C and 7 M mice and the GSH levels were measured in the 4 C, 4 E + GSH and 3 GSH mice.

Quantitative levels of melatonin in serum from C- and M-fed mice were determined in accordance with the manufacturer's instructions using the radioimmunoassay Melatonin Direct Kit (Elias USA, Osceola, WI).

Liver GSH concentrations were measured by HPLC according to Viña et al. (1989)

Vitamin E concentrations in serum were measured by HPLC as previously described by Tang et al. (1993).

2.4. Statistical analysis

The body weight data were analyzed (on a Power Macintosh 8100 with the program Systat 5.2.1) by ANOVA followed by Post Hoc Analysis using Tukey's HSD. Comparisons between groups for the presence and absence of specific lesions were made by Pearson's χ^2 analysis using Stat-SAK, "The Statistician's Swiss Army Knife" (Dallal, 1986).

2.5. Pathology

The mice in the longitudinal and cross-sectional study groups were euthanized by CO_2 inhalation followed by exsanguination. Samples of heart, lung, liver, kidney, adrenal gland, pancreas, spleen and testis were taken from each animal and were fixed in 10% neutral buffered formalin before being processed for routine histology. The livers of the mice in the cross-sectional groups were perfused with ice-cold phosphate buffered saline (PBS) prior to being fixed. The mice in the longitudinal group were perfused through the heart with saline to flush the vasculature and then with 10% formalin for fixation. The whole bodies from the longitudinal groups, including those animals dying spontaneously, were fixed for several weeks prior to dissection and routine processing. Sections of tissue samples were cut at 6 μm and stained with hematoxylin and eosin. Lesions were considered to occur commonly if they were observed in $\geq$ five individuals in any diet/mortality cohort.

3. Results

3.1. Body weight and caloric intake

The average weight for each of the diet groups was the same at the start of the experiment. This body weight, 41.2 ± 0.5 g, was comparable to that reported for other populations of 18-month-old male C57BL/6 mice (Goodrick et al., 1990; Turturro et al., 1994). Since 18 months of age is when body weight normally reaches its apex for male C57BL/6 mice (Goodrick et al., 1990; Turturro et al., 1994), it was of interest that all of the mice in this study continued gaining weight until they reached 26 months of age (Fig. 1). There were no significant differences among diet groups for caloric intake during the course of the experiment (data not shown). The average daily caloric intake of 14.5 kcal/day/mouse demonstrated that the mice in this study were not hyperphagic.

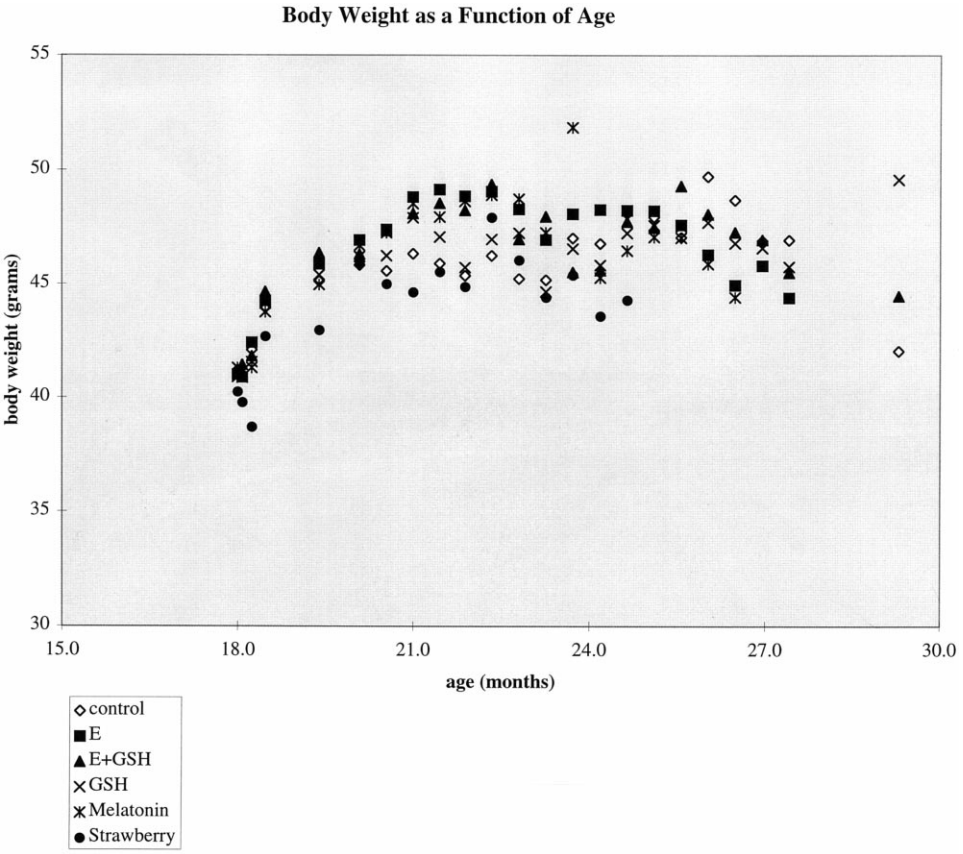

Fig. 1. Average body weight as a function of age for all the mice (cross-sectional and longitudinal) in each diet group.

Comparison of body weights for the mice in this experiment using fully factorial multivariate analysis of variance, followed by Tukey's mean comparison demonstrated an interaction between weight and diet. Post hoc testing using Tukey's highly significant differences for multiple comparison showed that the S diet fed mice were, on average, significantly lighter than the controls ($P = 0.016$). While the average daily caloric intake of the S fed mice (13.6 kcal/day $\pm$ 2.6) was somewhat less than that of the controls (14.1 kcal/day $\pm$ 1.1), the difference was not statistically significant.

3.2. Serum levels of vitamin E and melatonin

Measurement of serum concentration of vitamin E demonstrated that both the E- and E + GSH-fed mice had significantly greater levels of circulating vitamin E than controls (data not shown, $P < 0.002$). The average levels of vitamin E in the serum of the E- and the E + GSH-fed mice did not differ from each other. Supplementation of the diet with melatonin resulted in significantly higher serum melatonin levels for the M mice than for the controls (data not shown, $P = 0.002$). The average levels of liver GSH for GSH- and E + GSH-fed mice were not different than those of the controls (data not shown).

Thus, consumption of increased amounts of vitamin E and melatonin resulted in augmented levels of these compounds in the serum, as expected, compared with controls.

3.3. Longevity

There were three measures for each diet of the age at which mice consuming that diet reached 50% mortality because this study utilized three cohorts of mice born in consecutive months. None of the five experimental groups differed from the controls as seen in Fig. 2. The average age at which 50% mortality was attained ranged from 25.0 to 27.4 with variability within diet groups of $\pm$ 2.5 months. This is comparable to the 50% mortality age of 26.1 $\pm$ 1.0 months reported by Goodrick et al. (1990), though perhaps less than the 30 months reported from the NCTR (Turturro et al., 1994).

A second measure of longevity was taken from the average age at which mice in each diet cohort died prior to the group reaching 50% mortality. As shown in Fig. 2, there was no difference in the average age at which these early deaths occurred in the various diet groups compared with the controls.

3.4. Pathology

All of the individual mice which were sacrificed at 24 months of age or when 50% mortality had been attained for their group were properly preserved for histologic study. Several individuals within the group of mice which died of natural causes were either too completely autolyzed for study or had selected organs in too poor a state to yield information regarding the presence or absence of lesions. These

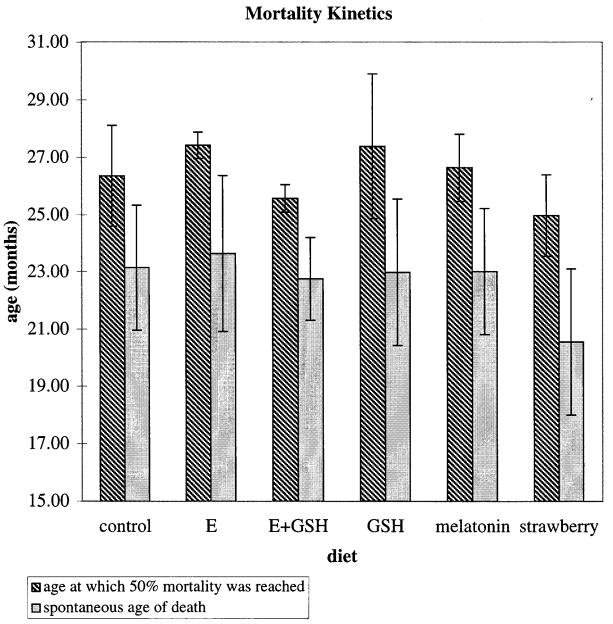

Fig. 2. Mortality kinetics: The average age at which 1/2 of the animals in each group had died ± S.D. is presented as the first bar for each diet group. The second bar for each diet group shows the average age of death ± S.D. for those animals succumbing prior to the group attainment of 50% mortality.

animals were not included in the analysis of lesion incidence, but were included for the calculation of longevity data. Table 1 lists the commonly observed lesions in the various diet-mortality groups.

Lesions observed in only the animals that died but not those that were sacrificed may be lethal lesions or alternatively may represent postmortem artifact. Glomerulonephritis, a potentially lethal lesion, was frequently observed in mice from every diet group which had died. A second kidney lesion, dilated renal tubules, was also seen in mice which had died. A third, relatively common problem observed in the kidney was scattered areas of atrophy and degeneration. Atrophy was common to all mortality groups; the kidney degeneration was seen only in the mice that died and those which survived to the 50% mortality level. However, the common incidence of several disease processes in the kidney is suggestive of renal problems that have an impact on the lifespan of the mice in this study.

Lesions which were commonly seen in the mice which died and those which were sacrificed when their group reached 50% mortality are also suggestive as lethal lesions. In this study these included myocardial degeneration, severe fatty liver and lymphoma. These occurred with equal frequency in all of the diet groups, but were not, however, commonly observed in the mice sacrificed at 24 months of age.

Lesions which were commonly observed in all three mortality groups may not be lethal disease processes and may have resulted from the increased body weight maintained by all the mice in this study, the particular basal diet consumed, or

Table 1
Common lesion incidence for each diet-mortality group

Common lesions (% incidence)	Diet					
	Control	Vitamin E	Vitamin E+GSH	GSH	Melatonin	Strawberry
Mice which died						
Fatty liver	26.3	14.3	30.3	30.6	46.7	28.6
Glomerulonephritis	31.6	21.4	24.2	27.8	13.3	17.9
Heart degeneration	23.7	25.0	36.4	36.1	40.0	32.1
Kidney atrophy	26.3	35.7	21.2	41.7	36.7	10.7
Kidney lymphoid nodule	21.1	14.3	21.2	33.3	23.3	21.4
Liver lymphoid nodule	15.8	10.7	21.2	33.3	10.0	17.9
Lung lymphoid nodule	5.3	7.1	15.2	25.0	10.0	14.3
Lymphoma	21.1	21.4	24.2	13.9	23.3	25.0
Normal liver (no fat deposition)	39.5	35.7	30.3	36.1	33.3	21.4
Renal tubule dilation	10.5	17.9	3.0	5.6	6.7	0
Testicular atrophy	10.5	7.1	9.1	22.2	6.7	7.1
50% *mortality cohort*						
Adrenal gland pigment deposition	0	35.3	21.4	18.2	23.1	15.0
Heart degeneration	28.6	0	14.3	9.1	0	30.0
Kidney atrophy	28.6	94.1	71.4	63.6	53.8	70.0
Kidney lymphoid nodule	85.7	76.5	85.7	72.7	69.2	60.0
Liver leukocyte clusters	0	70.6	57.1	45.5	38.5	20.0
Liver lymphoid nodule	28.6	88.2	64.3	54.5	53.8	20.0
Lung lymphoid nodule	28.6	29.4	71.4	45.5	23.1	40.0
Lymphoma	28.6	29.4	7.1	9.1	76.9	35.0
Normal liver (no fat deposition)	42.9	11.8	14.3	0	23.1	30.0
Pancreas lymphoid nodule	14.3	29.4	21.4	9.1	15.4	10.0
Testicular atrophy	28.6	11.8	35.7	9.1	15.4	15.0
24 *month old mice*						
Adrenal gland pigment deposition	20.0	20.0	23.8	19.0	20.0	21.1
Heart leukocyte clusters	0	0	4.8	4.8	25.0	26.3
Kidney atrophy	55.0	85.0	66.7	71.4	90.0	68.4
Kidney lymphoid nodule	85.0	60.0	90.5	81.0	85.0	57.9

Table 1
Common lesion incidence for each diet-mortality group

Common lesions (% incidence)	Diet					
	Control	Vitamin E	Vitamin E+GSH	GSH	Melatonin	Strawberry
Liver leukocyte clusters	45.0	50.0	52.4	47.6	65.0	68.4
Liver lymphoid nodule	65.0	50.0	47.6	76.2	50.0	57.9
Liver, fatty	75.0	65.0	76.2	81.0	80.0	63.2
Lung lymphoid nodule	20.0	30.0	14.3	23.8	45.0	52.6
Normal kidney	25.0	10.0	4.8	9.5	5.0	21.1
Pancreas lymphoid nodule	20.0	30.0	19.0	23.8	30.0	10.5
Pancreatic islet lymphoid nodule	25.0	10.0	14.3	19.0	20.0	10.5
Testicular atrophy	25.0	25.0	19.0	9.5	20.0	26.3

The percentage of mice in each diet-mortality group with each of the commonly observed lesions is presented. Lesions were deemed common for each of the mortality cohort if they occurred in at least 10% of the mice in one diet group.

simply the physiology of older male C57BL/6 mice. Lesions for which the incidences in all of the mortality groups did not differ were: lymphocytes in the kidney, liver or lung; fatty liver; testicular atrophy and kidney atrophy as previously mentioned. These were also observed to have equal prevalence in all of the diet groups.

We compared the frequently observed lesions and the variety of lesions for the mice in this study with a previously described (Turturro et al., 1994) population of 24-month-old C57BL/6 mice (Bronson and Lipman, unpublished data) obtained from the NCTR. At 24 months of age, there were 12 lesions observed to occur in at least 10% of the mice sacrificed at 24 months of age in this study, as shown in Table 1. Of these, only lymphocytes in the kidney, liver and lung were also commonly observed in the 24-month-old NCTR mice (Bronson and Lipman, unpublished data). It is interesting that the incidence of lymphocytes in the kidney, while common both to the mice in this experiment and those from the NCTR colony, was 4 times more common to the mice in this experiment ($P = 0.01$). Perhaps there is some link between this lesion and the other kidney pathology observed in these mice. There were three additional lesions commonly observed in the NCTR mice; however, these were found in tissues which were not harvested from the mice in this study, and so no comparison between the studies can be made. Limiting our assessment to lesions occurring in organs collected from both sets of animals, the variety of lesions found in the two populations did appear to be comparable (37 distinct lesions in the NCTR mice vs. 26 for the mice in this study).

Total lesion burden, defined as the number of lesions present, has been suggested for use as a biomarker of aging (Bronson, 1990). The lesion burden for the mice in this study is documented in Table 2. There was no effect of diet observed in any of the mortality groups. The mice which died during the course of the study had significantly less lesion burden per mouse than either the mice sacrificed at either 24 months of age or when their birth date-diet cohort reached 50% mortality ($P < 0.004$). This decreased lesion burden may be correlated with the decreased average age for this group of mice. In addition, this was the only group of animals for which tissues were lost to analysis due to autolysis, the impact of which would be to decrease the number of lesions observed.

4. Discussion

The diets used in this work were nutritionally adequate, although perhaps not optimal. The nutritional requirements for long-term study of rodents have never been documented (McDonald, 1997). The basal diet used in this study was, however, consistent with diets used previously in our laboratories as well as those of other researchers. A unique aspect of this study was that dietary intervention occurred when the study animals were middle-aged.

It is interesting that the kidney lesion, glomerulonephritis, which occurred frequently in mice which died during the course of study, has previously been reported to occur frequently in F344 rats and Wistar rats fed a similar semi-purified diet utilizing casein as its protein source (Maeda et al., 1985; Williams et al., 1987; Iwasaki et al., 1988; Bertrand et al., 1992; Rao et al., 1993). It is well documented that the renal lesions of the F344 rat are reduced when fed less than 14% casein (Maeda et al., 1985; Williams et al., 1987; Bertrand et al., 1992) or eliminated when soy protein is substituted for casein (Williams et al., 1987; Iwasaki et al., 1988; Bertrand et al., 1992). The kidney damage we observed may be a result of the relatively high level of protein (20%) in the diet. Although a reduction in the

Table 2
Average lesion burden for each diet-mortality group

Mortality group	Mice which died	50% mortality cohort	24 month old mice
Diet			
Control	3.82 ± 1.81 (38)	4.86 ± 2.48 (7)	5.95 ± 2.16 (20)
Vitamin E	4.14 ± 1.92 (28)	7.35 ± 1.73 (17)	5.60 ± 1.70 (20)
Vitamin E+GSH	3.39 ± 1.43 (33)	7.71 ± 2.13 (14)	5.29 ± 1.38 (21)
GSH	4.67 ± 2.08 (36)	5.36 ± 1.36 (11)	5.57 ± 1.54 (21)
Melatonin	3.93 ± 1.96 (30)	5.85 ± 3.21 (13)	6.35 ± 2.03 (20)
Strawberry	3.29 ± 1.56 (36)	4.80 ± 2.19 (20)	5.58 ± 1.95 (19)

The lesion burdens for each diet-mortality group are presented $\pm$ S.D.
Figures in parentheses indicate the number of mice (n) in each estimation.

contribution of casein from 20 to 14% was made when the AIN76A was reformulated to AIN-93 (Reeves et al., 1993; Reeves, 1997), its concentration may still be too high for long-term studies using genotypes predisposed to develop this lesion. We hypothesize that for some genotypes of rodents, there is a generalized susceptibility to renal damage due to long-term consumption of greater than 14% casein. Alternatively, there may phytochemical components in grain-based diets that may be especially beneficial for some strains of mice and rats. The wisdom of not substituting soy protein for the casein in the AIN formulation has been questioned by others (McDonald, 1997).

It has previously been demonstrated, in human patients with a variety of glomerular diseases, that oxidative stress is present during disease development (Turi et al., 1997). The lack of effect of antioxidant supplementation on the incidence of glomerulonephritis in this study suggest four possibilities or some combination of these: (1) the dosages of the various antioxidants used in the study were insufficient to elicit change in the renal disease process(es); (2) the severity of the renal insult was such that antioxidant supplementation was insufficient to result in any change; (3) the particular antioxidants tested in this study were ineffective in combating glomerulonephritis in mice and (4) the optimal age for initiating antioxidant treatment in order to affect glomerular pathology was much earlier than when the antioxidant supplemention was started for the mice in this study.

In light of the increased body weight of the mice in this study, it is noteworthy that the age at which the mice in this study reached 50% mortality did not differ from other colonies of ad libitum-fed male C57BL/6 mice (Goodrick et al., 1990; Turturro et al., 1994). It is supportive of the hypothesis that manipulation of body weight later in life has little impact on longevity (Lipman and Grinker, 1996; Lipman et al., 1995, 1996). Although it is well documented that calorie restriction enhances longevity, the lack of effect of later life weight gain may simply suggest that there is a limit to the age at which body weight manipulation affects the lifespan of an animal.

The lack of impact on longevity, lesion incidence or lesion burden by any of the antioxidants tested may have resulted from several factors. The weight gain of these mice which occurred simultaneously with their antioxidant consumption, may have masked any effect of the antioxidants they consumed. Another possibility is that the antioxidant formulation of the diet that these mice were consuming was optimal and that additional antioxidant supplementation is not beneficial. Alternatively, antioxidant supplementation may be ineffective in altering these parameters when introduced later in life. Similarly, there may be disease process(es) which, once established, are not amenable to antioxidant intervention. The observation that supplementation of 18-month-old mice with antioxidants had no effect on longevity is consistent with a recent report in which mice that were 16 months old or older also failed to demonstrate enhanced longevity when supplemented with a mixture of antioxidants (Bezlepkin et al., 1996).

Although it is clear that antioxidants protect biologically important molecules from oxidative damage in-vitro, it is also true that antioxidants can also function as pro-oxidants under certain conditions (Kontush et al., 1996; Schwartz, 1996; Cao et

al., 1997). There may also be protective properties that are specific to individual antioxidants. Therefore, dietary supplementation with a particular antioxidant may be insufficient to modulate the range of deleterious processes which accompany the transition from middle to older age.

Our animals were housed in a specific pathogen-free, barrier facility. By design, then, their immune function was not stressed. It would, therefore, be premature to extrapolate the lack of observed effect of antioxidant supplementation on longevity beyond a similarly controlled environment. Studies conducted on these and additional animals fed similarly supplemented diets have indicated that the immune function and resistance to viral infection was enhanced in mice supplemented with vitamin E (Han et al., 1997; Hayek et al., 1997b). Enhanced immune response does not always lead to increased longevity in laboratory settings (Hsu et al., 1997); however, it is generally accepted that maintenance of immune function is essential for healthful aging. Our inability to demonstrate differences between the supplemented and control diet groups may be the result of the protected environmental conditions in which the mice were maintained. It is possible that differences between the groups would be observed if they had been subjected to oxidative insult, immune suppression or other challenges.

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