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Dietary calorie restriction in the Emory mouse: effects on lifespan, eye lens cataract prevalence and progression, levels of ascorbate, glutathione, glucose, and glycohemoglobin, tail collagen breaktime, DNA and RNA oxidation, skin integrity, fecundity, and cancer

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Abstract

The Emory mouse is the best model for age-related cataract. In this work we compare the effects of feeding a control diet (C) with a diet restricted (R) by 40% relative to C animals. In the R animals, median lifespan was extended by 40%. The proportion of R mice with advanced cataract was lower than C mice as early as 5 months of age. The mean grade of

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cataract was lower in R animals, beginning at 11 months and continuing until the end of the study. Ascorbate levels in R plasma and liver were 41–56% of C animals. There was no difference between diet groups with respect to lens ascorbate. Aging was associated with a decrease in ascorbate in lenses and kidneys in C and R mice. By 22 months, R animals had 48% higher liver glutathione levels than C mice. Liver glutathione levels were maximal at 12 months. Plasma glucose levels were >27% lower in R animals at 6.5 and 22 months, and there was a 14% increase in glucose levels upon aging for both diet groups. In R mice, glycohemoglobin levels were 51% lower and tail collagen breaktime was decreased by 40%, even in younger animals. Collagen breaktime increased >360% upon aging for both diet groups. Rates of production of urinary oxo^{8}dG and oxo^{8}G were higher in R animals compared with C animals, and increased upon aging. C animals exhibited more cancer and dermatological lesions, but less tail tip necrosis and inflamed genitals than R mice. These data allow evaluation of several theories of aging.

Keywords: Mice; Diet; Aging; Antioxidants; Dietary restriction; DNA/RNA oxidation

1. Introduction

Long-term restriction of calorie intake (R) extends mean and maximum lifespan in a variety of species [1–3] and is associated with retardation of many age-related debilities including tumors and decline in immune function [4–12]. Many of these debilities and compromises of function may be associated with oxidative insult [13] and/or glycation (for review, see [14,15]). These age-related changes may reflect altered protection usually provided by antioxidants or antioxidant enzymes and/or by proteases which selectively remove damaged proteins [16,17]. More models are necessary in which to study these mechanisms. Particularly useful are models which allow study of the calorie restriction paradigm in relation to theories of aging.

A major emphasis of oxidation-based theories of aging is that free radicals escape 'useful or normal' reaction pathways [13]. These radicals oxidize many biomolecules which malfunction and/or accumulate and become cytotoxic, resulting in increased incidence of disease, aging and death [13]. Further evidence in support of oxidation-based theories of aging includes: (1) oxidative stress is associated with shortened lifespan [18]; (2) some age-related diseases are induced by oxidative challenge [19,20]; (3) oxidative stress is greater in organelles in which oxygen flux is greatest [11]; (4) some age-related diseases are delayed when antioxidant concentrations are elevated [11,17,21,22]; and (5) upon aging, some but not all defenses against free radicals are compromised [23–27].

A prediction based on the free radical hypothesis is that the increase in lifespan in R animals is conferred by enhanced protection against oxidative damage. Diminished oxidative stress in R as opposed to control (C) animals is illustrated by: (1) decreased microsomal generation of H_2O_2 in vivo [28]; (2) modulation of the age-related decline in catalase activity [26,27]; (3) enhanced preservation of P-450, a system which detoxifies many xenobiotics [28]; (4) enhanced capacity to oxidize malondialdehyde which otherwise acts to cause crosslinking of proteins, nucleic acids, and phospholipids [29]; (5) delayed accumulation of carbonyl in oxidant-ex-

posed R Fischer 344 rats [30]; and (6) delayed oxidation and insolubilization of lens crystallins in R Emory mice [31,32].

Glycation, or nonenzymatic reaction of glucose with various biomolecules, also leads to disfunction and/or accumulation of altered proteins [14,33]. Subsequent oxidation of such altered proteins would lead to further damage and another cytotoxic burden [16,34]. Damaged proteins are usually selectively removed by proteases. Age-related inactivation of proteases has been noted in several organs [34,35] and has been well documented in the eye lens [17]. Such protease inactivation would further exacerbate the load on aged cells in which the proteins are damaged. It is not surprising that age-related accumulation of damaged proteins is observed in many tissues (lens, red blood cells, retina, brain, etc.), as well as in tissues from persons with diseases of premature-aging [15,35]. Delayed accumulation of altered proteins in lenses of R mice may also be interpreted as evidence for enhanced selective protein removal in R versus C animals [31,32].

Eye lens cataract is one of the best biomarkers of aging since well over 50% of the aged human population is affected (for review, see [17]). In humans, the onset of cataract is observed at approximately 50% maximal lifespan. The lens is an appealing model of age-related diseases because lens proteins are very long-lived and are retained in an age-ordered array, like rings in a tree. This makes it possible to investigate age-related protein alterations, and to compare these alterations to observations from other tissues. Many studies document that in the lens, (photo)oxidative damage and glycation are at least in part related to protein modification and aggregation which results in insolubilized precipitates defined as cataracts. Unfortunately, research into the etiology of cataract and means to delay cataract have been thwarted by a dearth of animal models.

The Emory mouse has been bred to develop prevalent later-life cataracts ([31,36]; Taylor et al., unpublished data) which share many of the biochemical and morphological features of human cataracts. Previous work demonstrated that mild dietary restriction (21% restriction of calories) delays cataract progression in mid-age Emory mice [31]. These experiments allowed the hypothesis that consuming R diets delays cataract progression in part because of improved antioxidant status and/or enhanced protein editing. In this study, we examined lifespan and cataract at all ages. We also monitored levels of aqueous antioxidants (ascorbate and glutathione), glucose, glycohemoglobin, tail collagen breaktime, oxidation of DNA and RNA, fecundity, activity, skin condition, and cancer. These data were used to pursue relationships between diet, cataract and other age-related changes.

2. Materials and methods

2.1. Animals and diet

The Emory mouse was originally derived from CFW mice which presented with bilateral cataracts [31,36,37]. The HNRCA currently maintains a breeding colony of an inbred sub-line (brother × sister and/or father × daughter) of the Emory mouse which develops bilateral grade 3 cataracts by 11 months [31]. All animals are maintained in American Association for the Accreditation of Laboratory Animal

Care (AAALAC)-accredited facilities in an environmentally controlled atmosphere (23°C, 45% humidity with 15 air changes of 100% fresh hepa-filtered air per hour, and a 12-h light, 12-h dark cycle). All groups are monitored by an monthly animal surveillance program using sentinel mice. Mice are observed daily for activity levels, skin condition, and clinical signs of disease. Infrequent seizures were noted when they occurred, usually during handling when changing cages or ophthalmological exams. The mice for these experiments were treated in accordance with the principles identified in the NIH Guide for the Care and Use of Laboratory Animals and conformed to all federal, state, local and HNRCA standards. This investigation conformed with the ARVO Resolution on the Use of Animals in Research.

The animals were weaned 21–35 days after birth and housed in individual cages with free access to water treated by reverse osmosis, double ion exchange, acidification and UV irradiation. Cohorts of similar ages were housed in the same room, and prior to being started on their dietary regimes at 8–11 weeks of age, the mice were fed Agway High Protein Chow # 2000. Pups from the same litter were equally divided between the two dietary groups, control (C, 25 male and 25 female) and dietary restriction (R, 25 male and 25 female). The mice were weighed weekly for the duration of the experiment. Periodically throughout the study, mice from each group were placed in standard rodent metabolic cages for 24-h urine collection.

The C and R diets were prepared in the HNRCA animal facility diet kitchen and were formulated from food grade, purified nutrients, mixed at room temperature and stored at 4°C under a blanket of nitrogen. Diet constituents were as described by Weindruch et al. [1] with the substitution of torula yeast for brewer's yeast, AIN 76A vitamin mix for the ICN vitamin diet fortification mixture and the AIN 76 mineral mix for the USP XIV mineral mixture. Proximate analysis of each batch of diet was conducted (Hazelton Laboratories, Madison, WI), and the mean values from the 12 and nine batches of control and restricted diets, respectively, are presented in Table 1.

Table 1
Average proximate analysis of the diets

Nutrient	Control diet				Restricted diet			
	g/100 g	g/21.7 g diet	Cal/21.7 g diet	% of cal	g/100 g	g/13.3 g diet	Cal/13.3 g	% of cal
Protein	17.7 ± 0.8	3.84	15.4	16	31.4 ± 0.9	4.17	16.7	30
Fat	13.2 ± 1.3	2.86	25.7	28	12.9 ± 2.9	1.72	15.5	28
Carbohydrate	60.3 ± 2.1	13.09	52.4	56	43.2 ± 3.3	5.75	23.0	42
Total kcal	430.6 ± 9.5	93.44			414.4 ± 14.8	55.12		

As per Hazelton Labs; the data presented were derived from the analysis of 12 separate batches of control diet and nine separate batches of restricted diet; C animals received 21.7 g/week of the C diet; R animals received 13.3 g/week of the R diet; restricted animals received 55.12/93.44 = 59% of calorie intake. Therefore, the restriction was 41%; values are means ± S.E.M.; percentages are calculated using a conversion of 9 cal/g fat and 4 cal/g carbohydrate and protein.

In order to maximize differences between the diet groups, the R mice in this study were restricted to 60% of the calorie intake (Table 1) of the C mice [1,7,31]. Weekly aliquots of 13.3 g for R and 21.7 g for C powdered diets were weighed out for each animal, and then 1/7 of this quantity was apportioned into their bowls daily. The food bowls were weighted so that they could not be turned over by the animals, and thus food consumption could be monitored by weighing any food remaining. Initially, the C mice were fed 3.1 g/day of the C diet. This was 85% of the amount of diet they would consume if they were allowed ad libitum access to food (Hopkins, Taylor and Jahngen-Hodge, unpublished observations). Most of the C animals consumed all of the food allotted and food spillage was not observed. The R mice received 1.9 g/day of the R diet. At 7 months of age, the C mice began to lose weight. By 10 months, this was of sufficient concern to increase the amount of food fed to the C group to 26.6 g/week. Weight of the C mice was restored. However, when food was increased the C animals began to leave food, indicating that the increment was unnecessary. Other investigators indicate a similar spontaneous loss and restoration of weight which is not clearly related to dietary intake of food (R. Weindruch, personal communication).

Ophthalmologic examinations were performed monthly. Each mouse was treated topically with a 1% Tropicamide ophthalmic solution for pupil dilation, and then manually restrained for examination using a Zeiss slit-lamp biomicroscope. In keeping with prior work with the Emory mouse, a six point grading scale was utilized to monitor cataractogenesis [38].

Twenty-four hours prior to termination, food was removed from the cages. Accordingly, all plasma values presented are fasting values. For termination the mice were asphyxiated by CO_2 inhalation, and blood was collected by cardiac puncture and placed on ice. The blood was immediately centrifuged at 3000 rev./min for 15 min and the resulting plasma was decanted and immediately prepared for analysis for glucose and ascorbate.

Tail collagen fibers were removed and stored in ethanol at -20°C until analysis as described by Harrison and Archer [39]. All other tissue samples were removed immediately, frozen in liquid nitrogen and stored at -80°C.

All chemicals for analysis were obtained from Sigma Chemical Co., St. Louis, MO unless stated otherwise.

2.2. Ascorbate and glutathione analysis

Samples of plasma were combined with equal volumes of cold 0.35 M perchloric acid containing 0.1 mg/ml EDTA (PCA/EDTA), vortexed and centrifuged at 15 000 $\times$ g for 3 min at 4°C. The deproteinized supernatant was isolated and then analyzed in duplicate for total and reduced ascorbic acid. Tissues and lenses were thawed at 0°C, weighed and homogenized on ice in nine volumes of PCA/EDTA and centrifuged as described above. The resulting supernatants were also analyzed in duplicate. Ascorbate was analyzed as described in Berger et al. [40,41]. Briefly, total ascorbate was determined by a modification of an endpoint colorimetric procedure according to Roe and Keuther [42], and reduced ascorbate was determined using an isocratic reverse phase high performance liquid chromatography procedure with electrochemical detection according to Lee et al. [43].

Concentrations of glutathione (GSH) and oxidized glutathione (GSSG) in lens, liver and kidney were measured according to the procedure of Fariss and Reed [44]. Frozen tissue (50–150 mg) was homogenized in 10% perchloric acid and 1 mM 4,7-diphenyl-1,10-phenanthroline-disulfonic acid, followed by overnight derivatization with 2,4-dinitrofluorobenezene using γ-glutamylglutamate as an internal standard. Following addition of L-lysine to remove excess reagent, an aliquot was injected onto a Waters 840 gradient HPLC system with a 5-μ, 4.6 × 25 cm Waters μBondapak^{TM} NH_2 column and a LC-85 Perkin-Elmer UV detector at 365 nm. The mobile phases were A, 80:20 methanol/water and B, 64:16:20 methanol/water/sodium acetate (2.6 M, pH 5.4).

2.3. Glucose and glycosylated hemoglobin analysis

Plasma glucose concentrations were determined on a COBAS Mira Chemistry System (Roche Diagnostics Systems, Hoffman-La Roche Inc., Montclair, NJ) by an enzymatic, kinetic procedure using hexokinase coupled with glucose-6-phosphate dehydrogenase according to the Roche Diagnostic Systems technical procedure bulletin for glucose. All reagents were purchased from Roche Diagnostics Systems. A 25-μl aliquot of each sample, standard, or water was incubated with 2.0 ml of Roche reagent for glucose for 5 min at 37°C. Glucose concentration was determined by absorbance at 340 nm. Glycosylated hemoglobin levels were determined in whole blood using the Pierce Glyco-Gel Test Kit (Pierce Chemical Company, Rockford, IL) based on a boronate-affinity chromatographic method described by Malia et al. [45].

2.4. Levels of DNA and RNA oxidation

Urine was collected under oil while mice were in metabolic cages. All urine samples were frozen at −80°C until analyzed. Urine samples were thawed, diluted with an equal volume of 1 M NaCl, spiked with 10 000 counts/min [1′,2′]-[^{3}H]oxo^{8}dG and applied to a preconditioned Perkin Elmer/Analytichem (Harbor City, CA) C_{18}-OH solid phase extraction (SPE) column. The SPE column was then washed with 5 ml of 50 mM KH_2PO_4 buffer (pH 7.5) and retained compounds eluted with 3 ml of 15% methanol in the same buffer. The eluate was applied at 4°C to an immunoaffinity column prepared with either polyclonal or monoclonal antibodies raised against an oxo^{8}dG-casein conjugate. The column was washed in sequential order with 5 ml each of the following: water, 1 M NaCl, water, acetonitrile. Immediately after the acetonitrile wash, the antibody-binding compounds were eluted with methanol (5 ml), concentrated to dryness under a stream of nitrogen, resuspended to 200 μl in water and a 20- to 50-μl aliquot was analyzed by reversed phase HPLC with electrochemical detection [46–48].

Frozen liver and kidney samples were analyzed to determine levels of oxo^{8}dG in the DNA of these tissues. Aliquots (50–200 mg) were homogenized on ice in homogenization buffer (0.1 M NaCl, 30 mM Tris [pH 8.0], 10 mM EDTA, 10 mM β-mercaptoethanol, 5% Triton X-100) and the nuclear pellets recovered following centrifugation at 600 × g. The nuclear pellet was washed in 30 mM Tris (pH 8.0), 10 mM EDTA, and treated with RNase A and T (1 and 2 units, respectively) for

1 h at 50°C. Protein was digested upon incubation for 1 h at 50°C with 1% sarcosyl and 2 mg pronase in a total volume of 0.4 ml. DNA was isolated by phenol extraction, precipitated and washed with ethanol, and concentrated to dryness under vacuum. The samples were resuspended in 200 μl of 1 mM desferryl/20 mM sodium acetate (pH 5.0) and hydrolyzed to the corresponding nucleoside monophosphates at 65°C for 10 min with nuclease P1 (13.2 μg). The samples were further hydrolyzed at 37°C for 1 h with 4 units of alkaline phosphate to yield nucleosides which were subsequently analyzed by reversed phase HPLC with electrochemical detection [48]. All enzymes used for the isolation of DNA were purchased from Boehringer Mannheim.

2.5. Fecundity

C (three male, four female) and R (two male, four female) mice were given free access to non-purified Agway chow at 12 months of age. One male and two females from the same diet group were housed together for breeding purposes. Males were removed from the cages after 18 days, and the pups weaned as stated above and housed in the breeding colony.

2.6. Pathology

Various disease states were evaluated by histological staining of tissue sections with hematoxylin and eosin.

2.7. Statistical analysis

Methods of analysis included univariate and multivariate analysis of variance and covariance, to adjust for the effects of diet (C and R), age, and sex. High resolution statistical graphics were employed to check for outliers and to determine the need for mathematical transformations of the response variables prior to formal analysis. Because each animal contributes two eyes, which are not independent observational units, analysis of cataract grade was carried out using either an animal's mean cataract grade or the more severe cataract grade. The difference between the cataract grades observed during the monthly ophthalmological examination of animals in both diet groups was evaluated in part by using a nonparametric repeated measures growth curve analysis [49]. Diet groups were also compared with regard to the number of animals having a specific grade of cataract by using Fisher's exact test or Pearson's chi-square test of independence in two-dimensional contingency tables, as appropriate. Multiple comparison techniques, such as the Bonferroni adjustment, were used when many age groups were analyzed separately. Effects were judged to be statistically significant if two-tailed observed significance levels (P-values) were no greater than 0.05.

For ascorbate, urine volumes, and oxo^{8}dG and oxo^{8}G, the logarithmic transformation was applied to the data prior to analysis because groups with larger mean levels also had larger within group standard deviations. The data presented in the tables were not transformed.

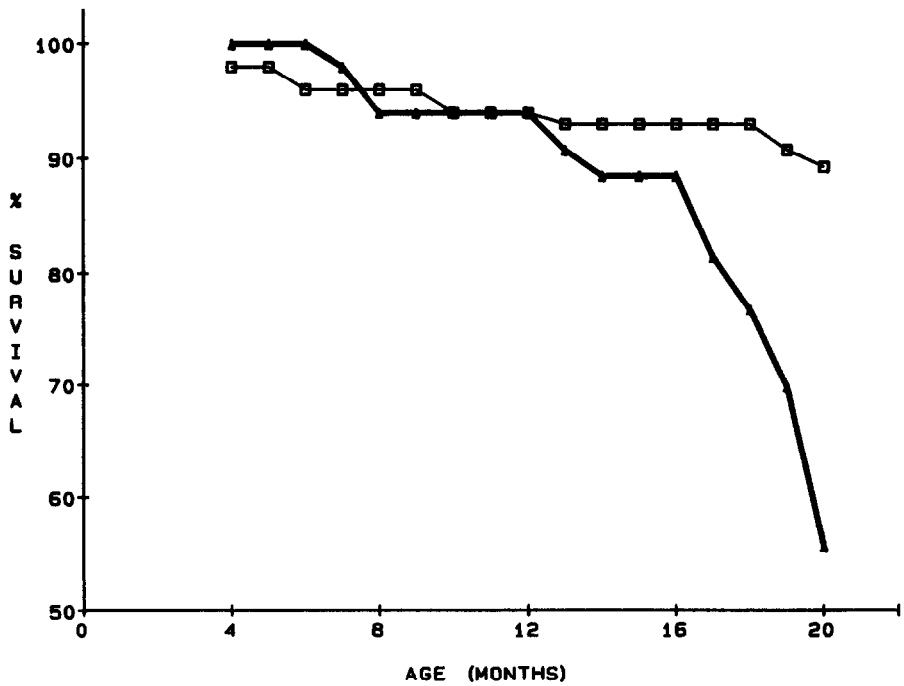

Fig. 1. Percent survival versus months of age. At the start of the experiment, there were 50 animals per diet group. For further details, please see the text; △, C; □, R.

3. Results

3.1. Growth, weight gain, and survival

The weights of the male and female R mice diminished during the first 6 weeks after the animals were fed the R diet. After that time, weights of the R mice stabilized at 20.8 ± 0.2 g (S.E.M.) for males and 19.2 ± 0.2 g for females. For C mice, maximal weights were 47.1 ± 1.5 g for males (observed at 73 weeks) and 40.0 ± 1.1 g for females (observed at 65 weeks). The maximum weights of the R male and female mice were 44% and 50% the weight of the C animals, respectively.

Mortality of 50% was observed in the C mice at 22 months of age. This median lifespan is within the lifespan range reported by a commercial vendor of CFW mice, the strain from which the Emory mice were derived [50]. In contrast, only 10% mortality was observed in the R mice at this age (Fig. 1).

3.2. Cataract prevalence and progression

The initial ophthalmologic examination at 3.5 months of age indicated that the two groups were equally affected by cataract. At this time, approximately half of the lenses in both diet groups had early cataractous changes (cataract grade < 1). Mean grades of cataract progressed but were not significantly different for C and R animals until 11 months (Fig. 2). Although the mean cataract grade for both groups were indistinguishable, the number of lenses with grade 5 cataracts was significantly

greater for C animals beginning after 5 months on the diet. This difference was maintained until the end of this study. At 11 months of age, there were 39 C and 27 R mice with grade 4 cataracts ($P < 0.007$). Eight C and 20 R animals were still without a grade 4 cataract (not shown). After 11 months and until termination of the experiment, the mean grade of cataract for C mice was significantly greater than that of the R mice ($P = 0.001$, Fig. 2).

Mouse lenses are small. Nevertheless, the ophthalmologic exam was sufficiently detailed to assess different types of cataracts (i.e. nuclear, subcapsular, cortical, mixed) in these mice. Differences in localization in C and R mice were not observed.

3.3. Ascorbate and glutathione levels

Total ascorbate was measured in plasma, liver, and kidney because these comprise the circulating pool and sites of ascorbate synthesis and catabolism, respectively (Fig. 3). There were independent diet and age effects with respect to ascorbate levels. Ascorbate levels in plasma and liver of R mice were 41–56% of the values obtained for the C mice ($P = 0.001$ and $P = 0.003$, respectively; Fig. 3A,B). A similar trend for lower ascorbate levels was also noted in the kidneys of R mice compared with C mice at 6.5 months of age ($P = 0.08$, Fig. 3C). The ascorbate levels in the livers and kidneys of the C mice were within ranges of levels previously reported in mice [53], guinea pigs [40,41], and humans fed diets sufficient in vitamin C [54].

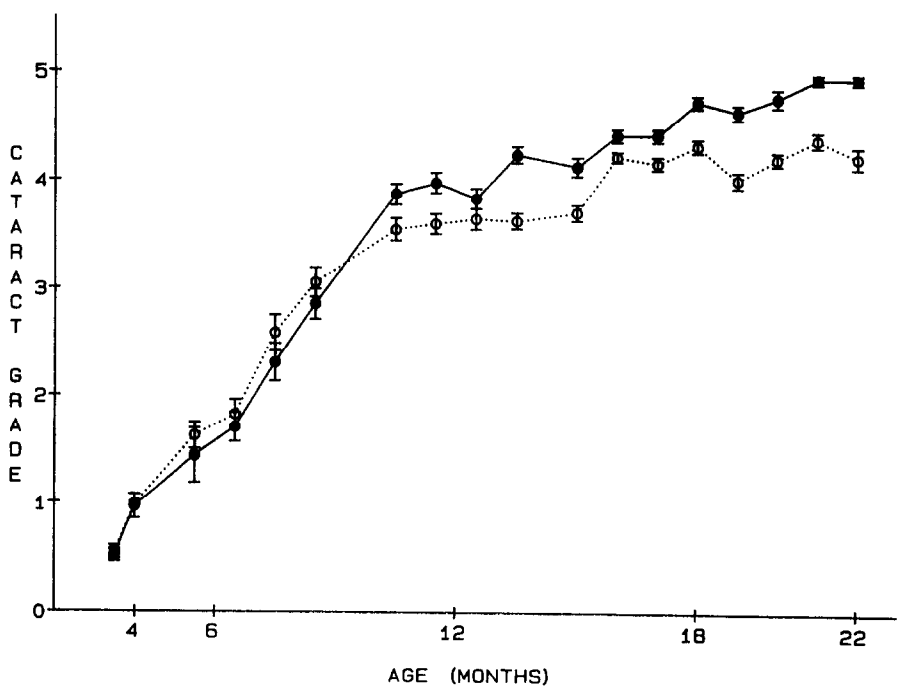

Fig. 2. Mean cataract grade for C and R Emory mice versus age. The severity of lens cataract was graded using a 0–5 scale [31,38]. Cataract grade expressed as mean ± S.E.M.; ●, C; ○, R.

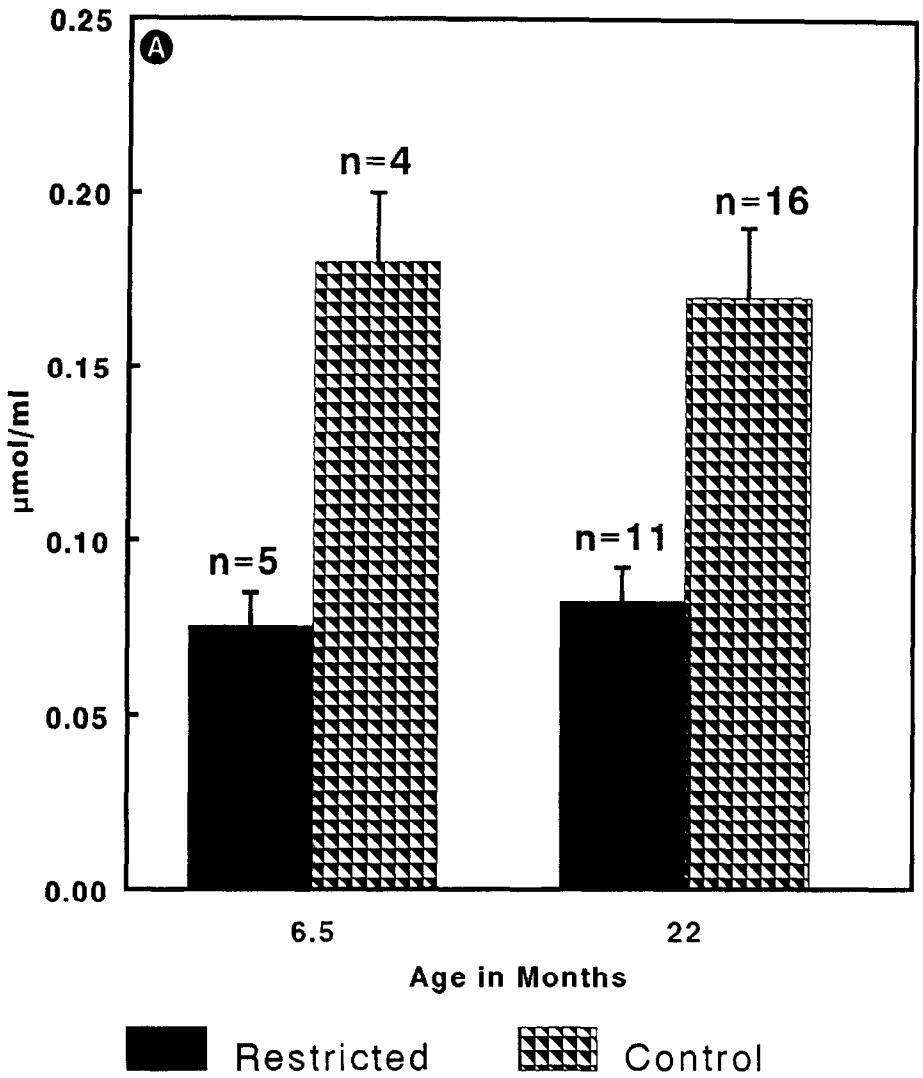

Fig. 3(A)

Fig. 3. Total ascorbate in plasma (A), liver (B), and kidney (C) of 6.5- and 22-month-old C and R mice, and reduced ascorbate in lenses (D) of 4.5- and 22-month-old Emory mice. Values indicate mean ± S.E.M. The number of animals analyzed is indicated above the bars.

Levels of ascorbate in the lens were 5- to 10-fold greater than in plasma and were comparable to ascorbate levels in other tissues which concentrate ascorbate (Fig. 3D; see [41,54,55] for data regarding other species). However, there was no statistically significant difference in lens ascorbate concentrations due to feeding R or C diets to these mice.

Age was associated with a decrease in ascorbate level ($P = 0.05$, both diet groups considered together; see Methods section) in kidneys (Fig. 3C) and lenses (Fig. 3D).

Reduced ascorbate values in Emory mouse lenses were at least 85% of total ascorbate values and confirm the age-related decrease ($P = 0.003$, data not shown). A similar trend ($P = 0.07$, both diet groups considered together) for a reciprocal relationship between age and ascorbate level was also found in the livers (Fig. 3B). This is consistent with the age-related diminution in ascorbate values observed in lenses of guinea pigs [41].

At 12 months, liver GSH levels were highest in both dietary groups, although there was no statistical difference between diet groups (Fig. 4). By 22 months, there was a diet-related difference in liver GSH levels (R mice: 32.8 $\pm$ 5.1 μmol/g protein; C mice: 22.1 $\pm$ 8.3 μmol/g protein; $P < 0.01$). These levels were significantly lower than at 12 months (for R mice, $P < 0.05$; for C mice, $P < 0.001$). The decrease between 12- and 22-month C mice (70%) was almost twice the

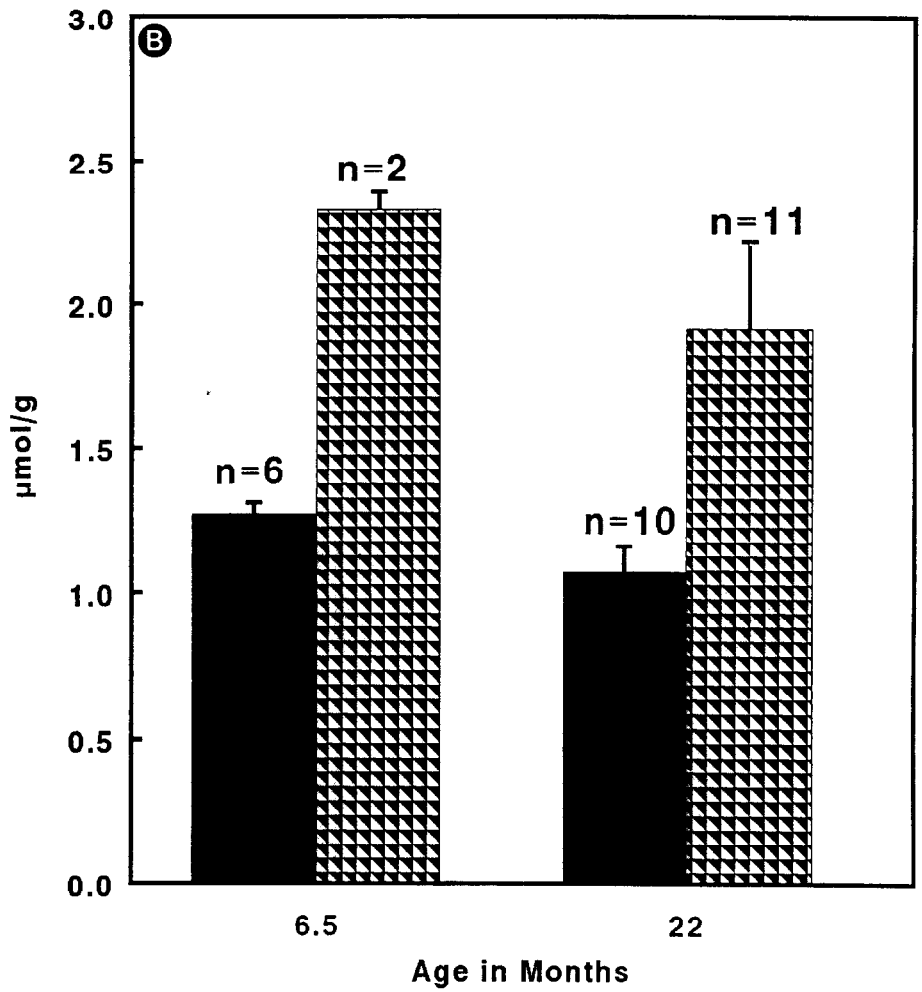

Fig. 3(B)

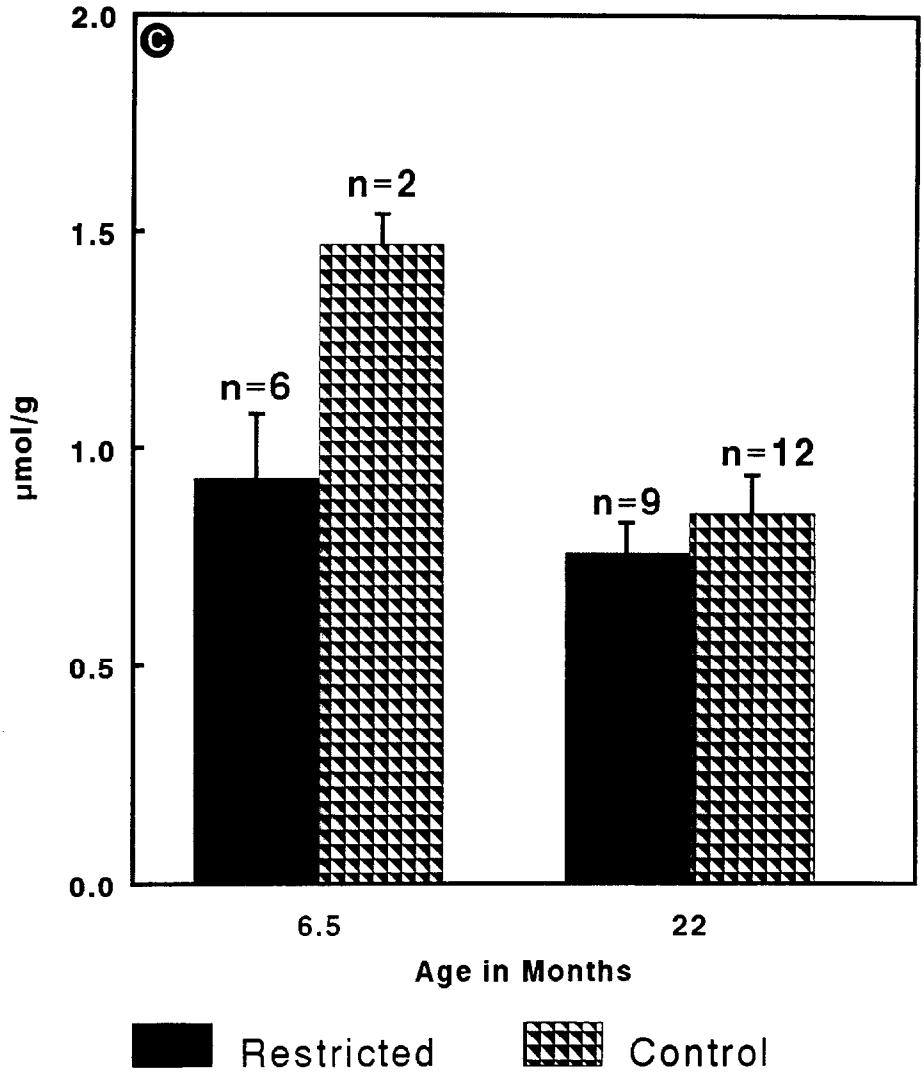

Fig. 3(C)

decrease in R mice (38%). Less than 10% of the glutathione was present in the oxidized form (GSSG), and it is plausible that some of this was formed during sample preparation. Liver GSSG was similar in C and R mice at 12 months, but at 22 months, the level of GSSG was significantly higher in R mice (5.43 $\pm$ 1.48 μmol/g protein) than in C mice (3.22 $\pm$ 1.02 μmol/g protein, $P < 0.01$). Total GSH in liver from 22-month R mice was higher than in C mice of the same age (43.6 $\pm$ 5.9 and 28.6 $\pm$ 9.0 μmol/g protein, respectively, $P < 0.001$). Overall, these data indicate that consuming the R diet minimizes the age-related decline in reduced and total GSH.

GSH levels in C and R mouse lenses were ≈ 1 μmol/g lens protein ($n = 13$). This is lower than GSH levels noted previously in Emory mice given free access to food [56]. Diet and age effects on lens GSH levels were not obvious in the samples tested. Kidney levels of GSH and GSSG were approximately 1.5 and 0.1 μmol/g protein, respectively, and showed no age or diet effects.

3.4. Plasma glucose, glycohemoglobin, and tail collagen break time

Fasting glucose levels were more than 27% higher in C than in R mice at both ages examined; however, the difference was significant in 22-month-old animals only (Table 2). There was also a trend for an age-related increase ($> 14\%$) in plasma glucose for both dietary groups. Preliminary data indicate a sex-age interaction with respect to glucose, i.e. the increase in glucose upon aging is

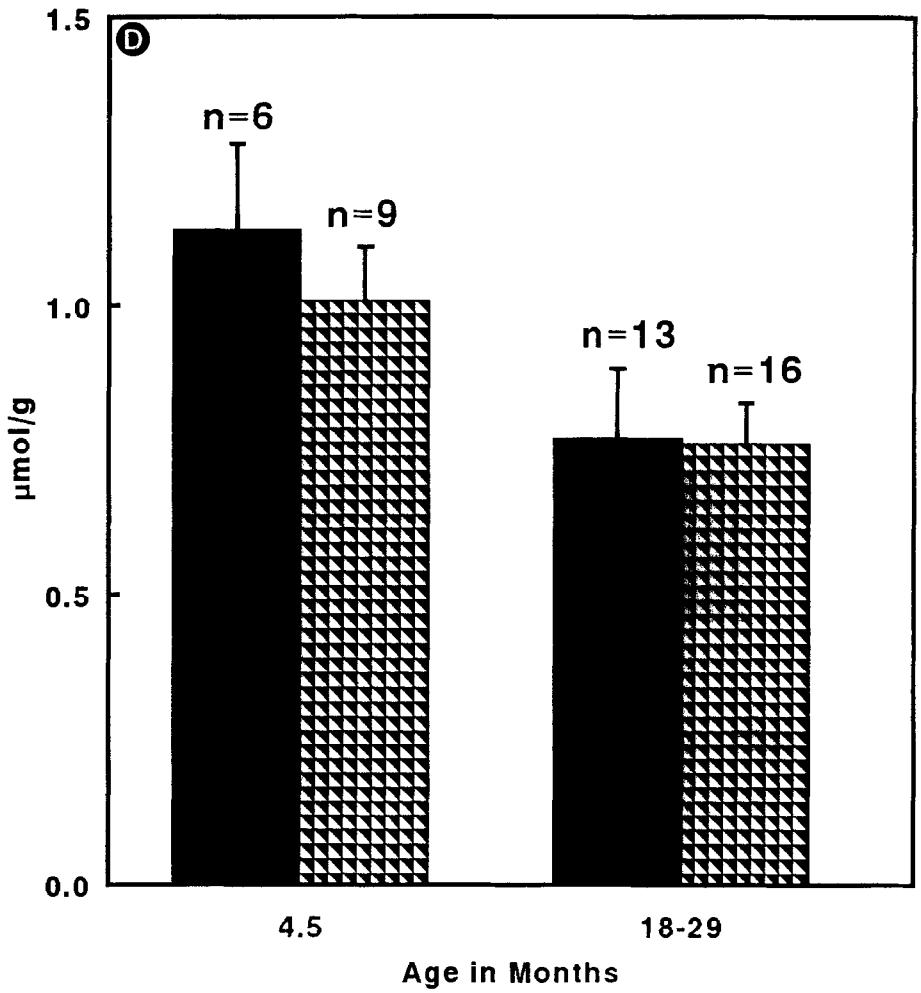

Fig. 3(D)

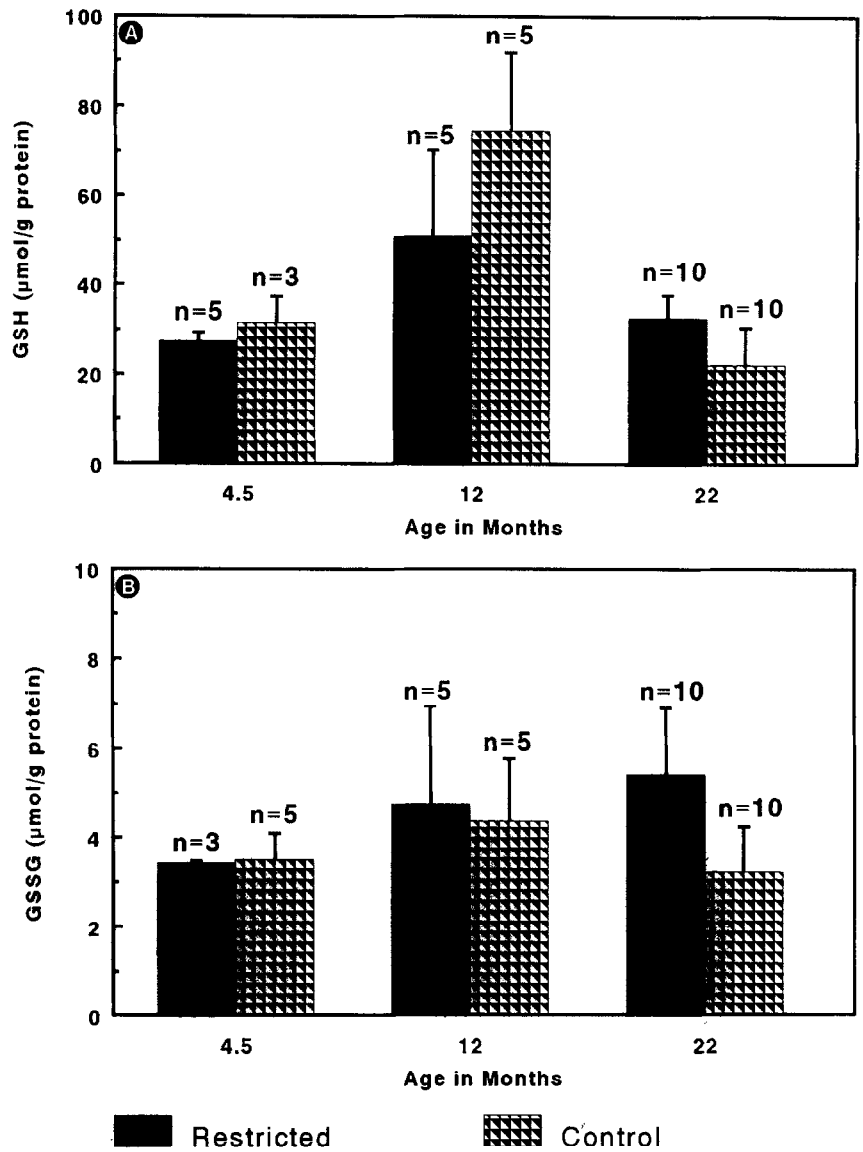

Fig. 4. Glutathione (A) and glutathione disulfide (B) in livers of C and R Emory mice. Values indicate mean ± S.E.M. The number of animals analyzed is indicated above the bars.

observed primarily in females. There may also be a sex-diet interaction, i.e. glucose is elevated in C males compared with R males, but not in females.

By 22 months of age, glycohemoglobin in C mice was 51% higher than in R mice ($P < 0.01$, Table 2). A marginally significant elevation (> 35%, data not shown) in glycohemoglobin in the C versus R animals was also observed at 9.5 months.

While these trends are consistent with prior reports, the levels of glycohemoglobin observed in this work were somewhat lower than levels obtained by Masoro et al. [57] and Kato et al. [58].

Another biomarker of aging, tail collagen breaktime was measured at 6.5, 13, 20.5, and 22 months of age (Table 2). Increased tail collagen breaktime is indicated by a decreased rate of denaturation of the tail tendon fiber in a concentrated urea solution. This is thought to be related to enhanced intermolecular cross-linking of collagen. Enhanced crosslinking, as indicated by significantly longer breaktimes in C compared with R Emory mice, was observed even in the youngest groups ($P < 0.05$ for C versus R mice at all ages). The ratio of breaktimes in R versus C mice (R/C $\approx$ 0.6) remained fairly constant throughout life.

Between 6.5 and 20.5 months of age, tail collagen breaktime increased in both the R ($\approx 375\%$, $P = 0.008$) and C ($\approx 360\%$, $P = 0.004$) mice. The age-related increase in tail collagen breaktime appeared to reach a maximum for both diet groups by 20.5 months (66 min in C and 42 min in R mice). However, the breaktime maximum which was observed in the R group at 20.5 months, was observed in the C group 7 months earlier. Similar absolute values and age-related increases in breaktime were observed in B6 mice of comparable ages given free access to food, but 2-year-old B6CBAF1 male mice had tail collagen breaktimes which were 2.5- to 3-fold these levels [51]. Rates of collagen aging are genetically controlled, but do not necessarily predict lifespans of inbred strains since B6CBAF1 mice outlive B6 mice. On the other hand, food restriction greatly retarded collagen aging and increased the longevity of B6CBAF1 mice [51].

Table 2
Plasma glucose concentration, percent glycohemoglobin and tail collagen breaktime in R and C Emory mice of different ages

Parameter	Age (months)	Restricted	Control
Fasting plasma glucose (mg/dl)	6.5	126.26 ± 13.50^{a} ($n = 5$)	160.24 ± 31.59^{a} ($n = 5$)
	22	143.75 ± 6.43^{a} ($n = 22$)	186.41 ± 19.40^{b} ($n = 13$)
% Glycohemoglobin	22	0.89 ± 0.06^{c} ($n = 22$)	1.34 ± 0.14^{d} ($n = 13$)
Tail collagen breaktime	6.5	8.75 ± 4.28^{e} ($n = 6$)	14.28 ± 5.55^{f} ($n = 6$)
	13	30.04 ± 3.96^{g} ($n = 7$)	42.14 ± 5.14^{h} ($n = 7$)
	20.5	41.18 ± 5.24^{g} ($n = 4$)	66.20 ± 5.55^{i} ($n = 7$)
	22	38.93 ± 2.24^{g} ($n = 22$)	63.17 ± 3.63^{i} ($n = 14$)

Values are means $\pm$ S.E.M; values with different superscript letters are statistically significantly different at $P = 0.05$ using two sample t-test using summary statistics.

Table 3
Urinary oxo^{8}dG and oxo^{8}G in R and C Emory mice

Parameter	Age (months)	Restricted	Control
Urine volume* (ml/day)	9.5	7.86 ± 6.77 ($n = 6$)	1.25 ± 0.28 ($n = 4$)
	22	3.59 ± 3.04 ($n = 10$)	1.64 ± 0.53 ($n = 4$)
Oxo^{8}dG* (pmol/kg·day)	9.5	1155 ± 464 ($n = 6$)	597 ± 151 ($n = 4$)
	22	1651 ± 1408 ($n = 10$)	1001 ± 306 ($n = 4$)
Oxo^{8}G** (pmol/kg·day)	9.5	3108 ± 1046^a ($n = 6$)	1229 ± 273^b ($n = 4$)
	22	3736 ± 1175^a ($n = 10$)	2885 ± 847^a ($n = 4$)

Values are means ± S.D.; one oxo^{8}dG residue per 10 dG residues is equivalent to 6.5 fmol oxo^{8}dG/μg DNA.

*Significant differences ($P < 0.05$) in urine volume and oxo^{8}dG are related to diet only, as determined by analysis of variance.

**Values with different superscript letters are statistically different at $P < 0.05$ as determined by Tukey's honestly significant differences after significant age by diet interaction was determined by analysis of variance. Data presented are not logged (see Methods section).

3.5. Urinary excretion rates of oxo^{8}dG and oxo^{8}G in Emory mice

Measures of rates of production of urinary oxo^{8}dG and oxo^{8}G provide non-invasive means of assessing oxidative DNA and RNA turnover or damage. Rates of urine production were 2- to 6-fold greater in R animals ($P = 0.003$, Table 3). Oxo^{8}dG, a product due, in part, to repair of oxidized DNA, was found at lower levels in C animals ($P < 0.05$). The RNA oxidation product, oxo^{8}G, was found at significantly lower levels in the younger C animals than in the other three groups ($P = 0.019$, followed by Tukey's honestly significant differences). In addition, mean oxo^{8}G levels were lower in C animals at both ages, but the difference did not achieve statistical significance (at the $P = 0.05$ level) at 22 months. These data indicate that DNA and RNA oxidation products are found at lower levels in urine from C versus R mice.

The urinary excretion rate for oxo^{8}dG in the Emory mice is approximately 2- and 3.5-fold higher than the urinary excretion rates observed in Fischer 344 rats and humans, respectively. Urinary excretion rates of oxo^{8}G can also be compared to urinary excretion rates of oxo^{8}G in humans, rats, and mice of approximately 950, 4600, and 6550 pmol/kg/day, respectively (Shigenaga, unpublished observations). Elucidation of the reasons why the Emory mice show lower oxo^{8}G than other mice will have to await further experimentation.

Levels of oxo^{8}dG in livers and kidneys were in the range of 4.3–6.3 fmol oxo^{8}dG/μg DNA, respectively. These values serve to establish norms for this parameter in Emory mice and are comparable to oxo^{8}dG levels in Fischer 344b6cb rats [59,60].

3.6. Skin integrity, seizure, and activity levels

Mice in both diet groups were housed in the same room and therefore had equivalent environments, presumably including comparable exposure to parasites. While there were no observations of R mice with significant alopecia, pruitis, self-inflicted wounds (i.e. patchy depilation of the head and neck that may progress to ulceration and pyoderma) or other associated dermatological lesions, 17 out of 50 C mice (34%) demonstrated such manifestations. *Radfordia* spp. (fur mite) was identified in nine of 50 C mice (18%). All mice demonstrating clinical signs associated with pruitis were treated by moving each animal to a microisolator cage and suspending 1" × 1" sections of Vapona™ (Dichlorvos [DDVP], Shell) at the top of the cage for 7 days. No animal demonstrated any adverse reaction to this treatment, and most lesions and scratching behavior subsided as a result of the treatment.

Hair coat is another indicator of dermatologic integrity. The hair coat was scruffier in some C animals after 4 months, and in all C animals after 9 months. Two skin lesions were exacerbated upon feeding the R diet. Inflamed genitals were observed in 9% of the R male mice as compared with only 2% of the C mice. Tail tip necrosis was also more prevalent in R mice which affected 11% of the R mice and none of the C animals.

Seizure was observed in 7% of the C mice but not observed in any of the R mice. Such transient seizure activity is not uncommon in mice following handling. Age-related compromises in involuntary neuromuscular function were also observed in the B6 and B6CBAF1 mice, but neuromuscular function was prolonged by feeding the R diet [51].

Visual observation during the light phase of the illumination cycle indicated that the R mice were more active than the C mice at all ages. This is consistent with reports of elevated physical activity in other R animals [52]. However, we did not attempt to monitor activity of the animals during the dark period.

3.7. Fecundity

Emory mice in this colony show generally limited production of progeny after approximately 6 months of age. However, when these mice were fed R diets and then returned to ad libitum food, breeding occurred in R mice up to 17 months of age. One male R mouse fathered offspring until 19 months of age. No control animals were productive. Extended fecundity has been observed in other R animals upon ad libitum feeding [12].

3.8. Pathology

Differences in pathology between R and C animals were obvious after approximately 22 months. The major difference between C and R mice was in the incidence of malignant tumors, 82% in C and 25% in R after 22 months. All tumors were adenocarcinomas, most commonly identified in the lung. From their distribution within the pulmonary parenchyma, they appeared to be metastatic, but no obvious non-pulmonary sources were identified. The incidence of other findings varied between the groups. For example, focal calcification of renal tubules was seen in

87% of the R mice but only in 36% of the C mice. These renal tubular changes were seen without any apparent associated glomerular or vascular changes.

4. Discussion

Our ability to delay the severity of post-maturity cataract in 14-month-old Emory mice using mildly calorie-restricted diets [31] encouraged us to determine if death and other age-related debilities might also be delayed in Emory mice by consuming calorie-restricted diets. Moreover, it appeared that similar etiology might pertain to some of these age-related phenomena and that the Emory mouse would be a useful animal model in which to investigate these phenomena and the effects of diet on them. Here we discuss the effects of feeding a R diet on lifespan, prevalence and progress of eye lens cataract, levels of plasma, liver, kidney and lens ascorbate, glutathione, plasma glucose, and glycohemoglobin, tail collagen breaktime, stability of DNA and RNA, skin integrity, activity levels, and cancer in Emory mice. These data also allow examination of several theories of aging.

4.1. Relationships between diet, survival and cataract

To enhance chances of observing differences between diet groups, the carbohydrate in the diet consumed by the R animals in this study was restricted by 40% relative to the control diet. Extrapolation of the data in Fig. 1 indicates that feeding the R diet results in an $\approx 40\%$ extension of lifespan in Emory mice. This is similar to the greatest lifespan extensions noted for other strains of mice and other animals (reviewed in [12]).

Our previous study was terminated when the animals were only 14 months old [31]. The data in Fig. 2 extend the prior results and demonstrate that the mean cataract grade was significantly lower in R than in C mice starting at 11 months of age and continuing until 22 months of age. Thus, progression of cataract was retarded over the entire second half of life by consuming the R diet. R mice also had a lower prevalence of earlier grades of cataract at younger ages. This treatment provides the first means to delay progress of 'senile' cataracts in vivo.

The absence of a significant difference in mean cataract grade between C and R animals at younger ages does not preclude influences of R on lens composition at earlier stages [32]. It is probable that in younger C and R animals, differences in early cataractous changes are too subtle to be distinguished with the ophthalmologic techniques employed. Biochemical changes which are usually associated with cataract have also been delayed in Emory mice by consuming the R diet. These include delayed postsynthetic protein modification including loss of γ- (and, to a lesser extent, α-) crystallins from the water-soluble fraction, transfer of protein to the water-insoluble fraction, accumulation of protein aggregates, and loss of protein from the water-insoluble fraction [31,32,61]. These biochemical data suggest that R animals sustain less stress or that they are better able to cope with it. Enhanced proteolytic capability may provide one means of eliminating damaged proteins. In support of this notion are our observations in R Emory mice of prolonged function of ubiquitination, a process required for marking proteins for removal (Jahngen-Hodge and Taylor, unpublished observations).

4.2. Ascorbate, glutathione, glucose, glycohemoglobin and tail collagen breaktime

Aging/death and cataractogenesis are thought to be at least partially related to oxidative damage in many species ([11,62–66]; Shang et al., unpublished data). This is corroborated by prior studies which indicate that cataract is delayed in conditions in which antioxidant concentrations are likely to be elevated [17,64]. These precedents, the data discussed above, as well as data which indicate that ascorbate is a primary aqueous antioxidant [67], allowed anticipation that R mice would have higher tissue ascorbate levels. This prediction is consistent with reports of a (nonsignificant) trend for elevated ascorbate in R Fischer 344 rats, and with the observation that R animals accumulate fewer oxidatively-induced lesions than C animals [30,68].

In contrast with expectations, ascorbate levels were significantly lower in plasma, liver and kidney of R mice than C mice at young and old ages (Fig. 3). Lens ascorbate levels in R and C Emory mice were indistinguishable, suggesting that there is an enhanced ability to sequester ascorbate in the lens in R animals. Nevertheless, since the lens represents a minor proportion of the total body ascorbate pool, the data regarding ascorbate in plasma, liver, and kidney indicate that the life extension and the delay in cataract or other debilities observed in R Emory mice is not associated with elevated levels of ascorbate. A lack of association between elevated lens ascorbate and delayed cataract in mice is also suggested by the observation that ascorbate levels in these Emory mice were slightly higher than in eyes of Swiss Webster mice which do not develop opacities ([53]; also, see below).

Why are ascorbate levels higher in C animals? While a clear answer to this question must await further experiments, it is probable that consumption of the reduced calorie diet by the R mice results in utilization of some of the ascorbate that they synthesize as an energy source. This results in diminished ascorbate pools in the R animals. The enhanced tendency of R animals to tail tip necrosis may be related to prior observations of impaired wound healing in R animals [51]. This might be related to diminished ascorbate reserves in R animals.

The GSH and GSSG data serve to establish norms for reduced and oxidized GSH, respectively, in these tissues in dietary-restricted Emory mice. We are unaware of other reports regarding oxidized glutathione levels in R and C animals. Several observations regarding the GSH data are analogous to data from R and C Fischer 344 rats [27]. These include the similarity of the GSH levels at 12 months in both diet groups and the enhanced decline of GSH in older C animals, resulting in a significantly greater GSH level in 22-month-old R mice than in C mice. In contrast with prior observations [27], we noted that absolute levels of GSH in livers of these animals were 50–60% of the levels noted in Fischer 344 rats and that GSH levels in young (4.5-month-old) C and R Emory mice were indistinguishable from the GSH levels found in the 22-month-old animals. Thus, liver GSH levels were highest at 12 months, mid-life in these Emory mice.

There appears to be an age-related decline in ascorbate (> 6.5 months) and GSH (> 12 months). An age-related decline in ascorbate has previously been noted in guinea pigs and in preliminary information regarding humans [25,40,41]. The

age-related decrease in total GSH and ascorbate is consistent with putatively causal relationships between oxidative stress and aging, but more data are required. An explanation of these results with respect to the relationship between antioxidant enzyme activities and diet will also have to await further experimentation.

Glycation has also been proposed as an insult which may be causally related to aging [14]. Glycation is due to reaction of carbohydrates with proteins, including lens proteins and collagen [14,33,63,69–76]. Glycation is often followed by crosslinking of the glycated proteins. Glucose is the most common carbohydrate identified as a glycating agent. Ascorbate has also been shown to be a glycating agent, particularly under oxidizing conditions [72].

Consistent with the hypothesis that glycation is associated with aging are observations that plasma glucose levels in C Emory mice were more than 127% the level found in R mice at young and older ages. Furthermore, there is an age-related increase in plasma glucose (Table 2). This suggests impaired ability to control glucose in C compared with R animals, even at a young age and continuing throughout life. Elevated glucose in C mice at older ages is corroborated by higher glycohemoglobin values in C animals (C/R $\geq$ 150%). An analogous elevation in glycohemoglobin in C animals is also seen with C versus R (< 6-month-old) rats [57] and in other C versus R Emory mice (data not shown). It is plausible that, in C animals, the elevated glucose is involved in the formation of glycoproteins which in the lens might be cataractogenic.

Lens crystallins and collagen share many similarities. Both are long-lived proteins. As with age-related lens rigidification, rigidification of collagen is due in part to crosslinking, possibly involving glycation and oxidation [58]. Age-related increases in collagen breaktime were obvious early in life in C and R animals, and this increase in breaktime continued well into adulthood of the Emory mice. However, breaktimes for the R mice were consistently lower than those observed for age-matched C mice, and the ratio of breaktimes in R versus C (0.6) was relatively constant throughout life. These data are the first to show that tail collagen breaktimes differ between groups as early as 6.5 months, and they are consistent with data regarding 1- and 2-year-old mice [51,77]. They also support the hypothesis that enhanced glucose, glycation, and collagen rigidification are associated with attenuated glucose level regulation in C Emory mice relative to R Emory mice, as well upon aging.

Might higher glycation in C mice be related to the generally higher levels of ascorbate and is this associated with enhanced cataractogenesis in C mice? The following observations do not support conjecture that ascorbate is a primary glycating agent associated with cataractogenesis: (1) levels of ascorbate in the C and R Emory mouse lenses were indistinguishable; (2) levels of ascorbate are only slightly greater in cataractous Emory mice than in strains of mice which do not get cataracts (Taylor et al., unpublished data) [53]; (3) lens ascorbate levels in these C and R animals are not in excess of lens ascorbate levels in cataract-free guinea pigs (guinea pigs have 1.25 mM ascorbate; [40,41]); (4) guinea pigs fed 50 mg ascorbate/day ($\approx$ 20-fold the daily requirement) showed no cataracts (Berger and Taylor, unpublished observations); and (5) mice fed a diet containing 8% (by weight) ascorbate

did not develop cataracts [74]. Direct evidence of differential covalent binding of ascorbate or ascorbate derivatives in C and R animals is required to test the hypothesis further.

4.3. Oxidation of DNA and RNA

There have been few reports regarding measures of DNA and RNA oxidation in R and C animals. The values presented herein serve to indicate ranges of concentrations of these moieties in C and R Emory mice (Table 3). As with other parameters, there are both dietary and age effects. Generally, rates of oxo^8dG and oxo^8G excretion appear lower in C than in R animals. The rates also appear to increase upon aging, significantly for C but not R mice.

Increased DNA and RNA oxidation rates of R animals would also appear inconsistent with prior reports of enhanced antioxidant function. They have been rationalized to be associated with an elevated metabolic rate in tissues (e.g. muscle) involved in spontaneous activity, a behavioral parameter that is elevated in these and other R animals [78–81]. Additional explanations for this include: (1) elevated rates of DNA repair (oxo^8dG) or RNA turnover (oxo^8G) in the R mice; and (2) decreased rates of autoxidation of oxo^8dG and oxo^8G in R mice. The latter is consistent with increased antioxidant defenses [26], but would appear inconsistent with the diminished ascorbate levels observed in these R animals.

The increased rates of urinary excretion of oxo^8dG and oxo^8G upon aging in R Emory mice are opposite to the age-related decrease in urinary oxo^8dG excretion rates of Fischer 344 rats given free access to food [48].

5. Conclusion

Overall, these data clearly demonstrate many health advantages of dietary restriction in Emory mice. Some of these are completely novel. Elucidating mechanisms by which calorie restriction elicits these health benefits takes on added importance since Walford and colleagues obtained data indicating that humans may also benefit from such dietary management [12,83]. The data note several common features in aging tissues (i.e. accumulation of damaged/altered proteins [11,15,16,34,35]) and suggest renewed investigation to elucidate causes of aging. Future areas of research which should be particularly revealing are relationships between the diets, age, physiological function and antioxidant function, proteolytic capabilities and glycation. It is probable that several of these variables are related [17].

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