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Temporal Pattern of Food Intake Not a Factor in the Retardation of Aging Processes by Dietary Restriction

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Long-term dietary restriction programs which retard aging processes in rodents usually involve meal eating rather than the nibbling pattern of food intake of ad libitum fed rodents. Thus, the possibility arises that the antiaging action may at least in part result from an altered temporal pattern of food intake. This possibility was investigated using male F344 rats maintained on the following dietary regimens: Group A rats fed ad libitum; Group B rats fed 60% the ad libitum intake in a single meal at 1500 h; Group B-2 rats fed 60% of the ad libitum intake in two meals (0700 h and 1500 h). The diurnal pattern of plasma corticosterone concentration differed among the groups as did that of the plasma glucose concentration. The median length of life and age of tenth percentile survivors were similar for Group B and B-2 rats and much greater than those for Group A rats. Both modes of dietary restriction influenced age-associated disease processes in a similar fashion. Thus, although the temporal pattern of food intake influenced circadian rhythms of food-restricted rats, it did not significantly affect the antiaging action.

RESTRICTING food intake of mice and rats (referred to as dietary restriction, or DR) below that of ad libitum fed animals increases life span and retards aging processes (Weindruch and Walford, 1988). Current evidence points to a reduction in energy intake as the major factor underlying this antiaging action (Masoro, 1988). However, DR influences circadian rhythms whether energy restriction is accomplished by limiting daily food intake or by alternate-day feeding (Nelson, 1988). Both procedures for achieving DR result in a meal-feeding pattern of food intake compared to that of the nibbling pattern of ad libitum fed animals. In the case of ad libitum fed rodents, the temporal pattern of many circadian rhythms is to a great extent determined by the daily light-dark schedule, but in diet-restricted rodents the timing of the meal also significantly influences these rhythms (Duffy et al., 1989). Thus, the question arises as to a possible role of altered circadian rhythms in the antiaging action of DR.

Nelson and Halberg (1986a, 1986b) studied groups of dietary restricted mice which varied in the number of daily meals and/or the timing of the meals but not in daily energy intake. These mouse groups did not differ from each other in longevity characteristics. However, Duffy et al. (1989) have pointed out that the level of dietary restriction used in those studies did not cause a marked increase in longevity; they suggested that investigations using a more severe level of restriction should be carried out in regard to this issue. In the study to be reported in this article, a level of dietary restriction was used which results in a marked increase in life span (Yu et al., 1982). The influence of this level of food restriction was assessed in regard to longevity, age-associated pathologic lesions, and the circadian pattern of plasma corticosterone and glucose concentrations for two groups of dietary restricted rats, one fed a single daily meal at 1500 h and the other two daily meals at 0700 h and 1500 h.

METHODS

Male F344 rats, obtained as weanlings (26–30 days of age) from Charles River Laboratories (Kingston, NY) were maintained in a barrier facility and used in accord with the guidelines of this university. They were housed one rat per cage (plastic cages, 10″ × 9½″ × 8″), with wire mesh floors suspended in a Hazleton-Enviro Rack System (Hazleton Systems, Aberdeen, MD). The operation of the barrier facility has been described (Yu et al., 1985). A 12-hour light-dark cycle was used with the light phase starting at 0400 h. Sentinel rats were killed soon after arrival and at 6-month intervals for monitoring for murine virus antibodies (Sendai, Reo-3, GP-VII, PVM, KRU, H-1, SDA, LCM and Adeno) and for Mycoplasma antibodies in serum samples sent to Microbiological Associates (Rockville, MD). The rats were negative for these antibodies throughout the study.

Until 6 weeks of age, all rats were fed a semisynthetic diet ad libitum; see Bertrand et al. (1980) for the composition of this diet. At 6 weeks of age, three dietary groups were established. Group A (61 rats) were allowed to eat this diet ad libitum. Group B (61 rats) were restricted to 60% of the mean intake of Group A rats provided in a single daily meal at 1500 h. Group B-2 (50 rats) were restricted to 60% of the mean intake of Group A provided in two daily meals at 0700 h and 1500 h. The diet of the restricted groups was the same as that of the ad libitum fed rats except for vitamin supplementation resulting in a similar intake of vitamins by all dietary groups. The amount of food eaten was measured as described by Yu et al. (1985).

Forty rats were randomly chosen from each group for the

measurement of longevity characteristics of an unperturbed sample. Twenty-one Group A rats, 21 Group B rats, and 10 Group B-2 rats were randomly chosen for a longitudinal study of the circadian patterns of plasma total corticosterone and glucose concentrations.

Blood sampling. — Blood samples were drawn into heparinized capillary tubes from the tail by the method of Keith et al. (1978). Sufficient blood for both the plasma glucose and corticosterone assays was obtained in less than 60 seconds from initial contact with the rat. Time course measurements indicate that the concentrations of plasma glucose and corticosterone do not change significantly during the blood sampling procedure.

The blood sample was immediately centrifuged for 5 minutes. Four 5 μl aliquots of plasma were removed and stored at −20 °C pending measurement of glucose and corticosterone concentrations.

Assessment of the circadian concentration pattern of plasma glucose and corticosterone. — The rats were sampled at 6-month intervals for the longitudinal life span assessment of the circadian pattern of plasma glucose and corticosterone concentrations. Measurements were started at 3 months of age for 6 Group A, 6 Group B, and 4 Group B-2 rats, at 4 months of age for 5 Group A, 5 Group B, and 2 Group B-2 rats, at 5 months of age for 5 Group A, 5 Group B, and 2 Group B-2 rats, and at 6 months of age for 5 Group A, 5 Group B, and 2 Group B-2 rats. Blood samples were collected for all groups at 0400, 0800, 1200, 1600, 1700, 2000, and 2400 h, also at 1500 h for Groups B and B-2 and at 0700, 0900, and 1800 h for Group B-2. Only one blood sample was taken from a rat in a 24-hour period, and at least 72 hours elapsed between samples from the same rat. The circadian pattern was constructed from blood samples collected over approximately a one-month period.

Plasma analyses. — Duplicate 5 μl samples of plasma were assayed for corticosterone concentration by a RSL ^{125}I corticosterone kit for rats and mice (ICN Biochemicals, Carson, CA). The sensitivity of the assay (88% of total binding) was 25 ng/ml and the range was 25–1000 ng/ml. The intraassay and interassay coefficients of variation were 4.2% and 7.6%, respectively.

Duplicate 5 μl samples were analyzed for glucose concentration by Sigma Chemical Co. (St. Louis, MO) Procedure No. 315. This glucose oxidase method utilizes the Trinder reaction (Trinder, 1969).

Pathologic analysis. — Rats were inspected at least twice daily (at 0700 to 0800 h and 1500 to 1600 h), and dead rats were either necropsied immediately or refrigerated for a brief time. Autolysis almost never was severe enough to prevent grading of lesions. The brain, pituitary gland, hearts, lungs, trachea, aorta, esophagus, stomach, small intestine, colon, liver, pancreas, spleen, kidneys, urinary bladder, prostate, testes, epididymis, seminal vesicles, thyroid gland, adrenal glands, parathyroid glands, psoas and thigh muscles, sternum, femur and vertebrae were excised, fixed in 10% formalin, and examined histologically as described previously (Iwasaki et al., 1988). Other tissues with gross lesions were also examined histologically.

Nephropathy was graded by the system described previously (Yu et al., 1982) and designated in the order of increasing severity as Grade 0 (no lesions), Grade 1, Grade 2, Grade 3, Grade 4, Grade E. Photomicrographs of each grade have been published (Maeda et al., 1985). Only rats with Grade 4 and Grade E lesions have elevated levels of serum creatinine and blood urea nitrogen.

Cardiomyopathy was graded in order of increasing severity as Grade 0 (no lesions), Grade 1, Grade 2, Grade 3. This grading system has been described previously including a photomicrograph of Grade 3 lesions (Maeda et al., 1985).

Hepatic bile duct hyperplasia and hepatic fatty change were graded in order of increasing severity as Grade 0 (no lesions), Grade 1, Grade 2, and Grade 3. The grading systems and photomicrographs of Grade 3 lesions have been published previously (Maeda et al., 1985).

The procedure of Peto et al. (1980) was used to assess the prevalence and severity of leukemia/lymphoma. Categories 1, 2, 3, and 4 of this classification were viewed as increasing grades of severity based on the likelihood of a role of the neoplasm in the death of the rat [Categories: (1) definitely incidental; (2) probably incidental; (3) probably fatal; (4) definitely fatal].

The following procedure was used to assess the prevalence and severity of pituitary lesions. Hyperplastic nodules less than 1 mm in diameter were classified as hyperplasia and those greater than 1 mm as adenoma. Grades 1, 2, 3, and 4 of adenoma indicate the order of increasing severity based on the likelihood of a role of the tumor in the death of the rat.

Statistical analysis. — The survival curves were estimated using product limit estimates and the curves were compared using Wilcoxon test (Gross and Clark, 1975). The median and 10th percentile survival times of the dietary groups were compared using the quantile test (Conover, 1971).

The total frequency of a pathologic lesion or grade of lesion was analyzed with a chi-square test (Siegel, 1956). When the expected frequencies were too small for the chi-square tests, the data were analyzed with Fisher's exact test for 2-by-2 tables (Siegel, 1956).

The controls for the corticosterone assays varied with the kit. A kit with values near the center of the distribution was selected as a standard. The ratio of control values with each kit to control values of the standard kit was used to adjust for different kits (Snedecor and Cochran, 1967). The diurnal patterns were compared using multivariate analysis of variance (Morrison, 1967). Only the points common to all three diet groups were used (i.e., 0400, 0800, 1200, 1600, 1700, 2000, and 2400). The 24-hour averages of glucose and corticosterone were assessed by analysis of variance for repeated measures (Winer, 1971).

RESULTS

Data on the 3 to 7 month and 15 to 19 month age range from the life span longitudinal study of the diurnal pattern of plasma total corticosterone concentration for Groups B and

B-2 are presented in Figure 1. In the age range of 3 to 31 months, the diurnal pattern differed for the two groups ($p < .02$). Some change with age occurred in the diurnal pattern in both groups ($p < .003$).

The mean 24-hour plasma total corticosterone concentration did not differ between Group B and B-2 rats at any age (Table 1). In the 15 to 19 month age range the Group A rats had a marginally higher plasma total corticosterone concentration than Group B rats ($p < .05$) and in the 21 to 25 month age range a marginally higher concentration than Group B-2 rats ($p < .05$).

Data on the 3 to 7 month and 15 to 19 month age range from the life span longitudinal study of the diurnal pattern of plasma glucose concentration are presented in Figure 2 for Groups A, B, and B-2. In the age range of 3 to 25 months, the diurnal pattern differed for all three groups ($p < .008$) and in the 27 to 31 month age range differed between Groups B and B-2 ($p = .001$). Some change with age occurred in the diurnal pattern in all dietary groups ($p < .02$).

In the age range of 3 to 25 months, the mean 24-hour plasma glucose concentration (Table 2) was higher for Group A rats than for Group B and B-2 rats ($p < .05$). Mean 24-hour plasma glucose concentration did not differ between rats of Group B and B-2 at any age.

Table 1. Mean ± *SE* 24-hour Plasma Total Corticosterone Concentration (ng/ml)

Age Range, Months	Rat Dietary Groups					
	A[b]	*n*	B[b]	*n*	B-2	*n*
3–7	102 ± 6	21	117 ± 6	21	119 ± 9	10
9–13	100 ± 6	21	99 ± 6	21	81 ± 9	10
15–19	138 ± 6	21	94 ± 6	21	101 ± 9	10
21–25	128 ± 7	15	101 ± 6	19	80 ± 9	9
27–31	[a]		117 ± 8	13	109 ± 10	7

[a]Four Group A were studied but three were suffering from terminal disease and died shortly after sampling was completed. Therefore, a mean for these rats is not reported.

[b]The data for Groups A and B have been published previously (Sabatino et al., 1991).

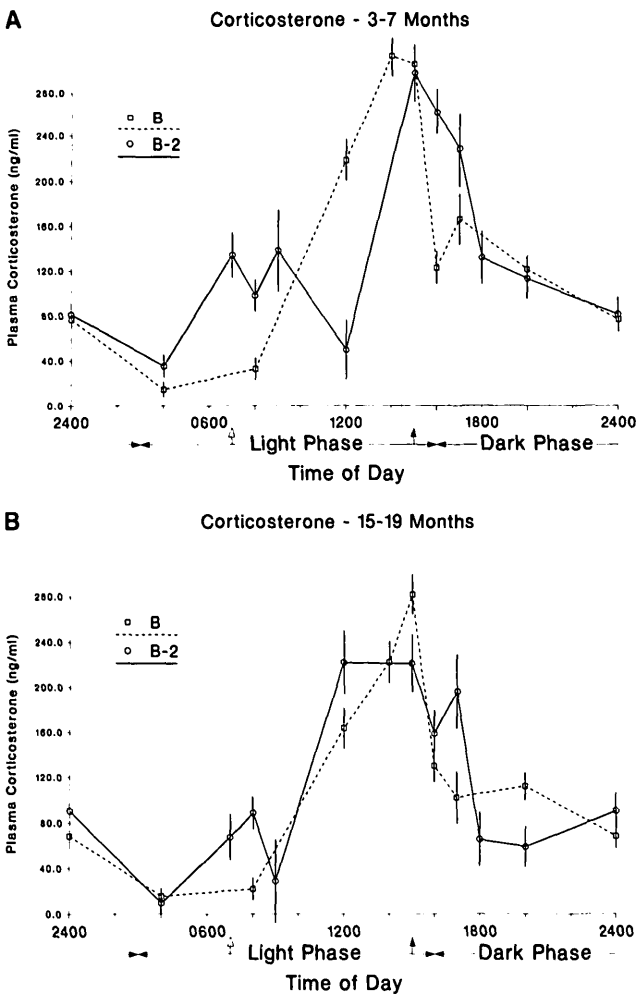

Figure 1. Circadian pattern of plasma total corticosterone concentrations in Group B and Group B-2 rats. **A**. Age range 3 to 7 months, mean ± *SE* for 21 Group B and 10 Group B-2 rats; **B**. Age range 15 to 19 months, mean ± *SE* for 21 Group B and 10 Group B-2 rats. The circadian patterns of the Group B rats have been published previously (Sabatino et al., 1991).

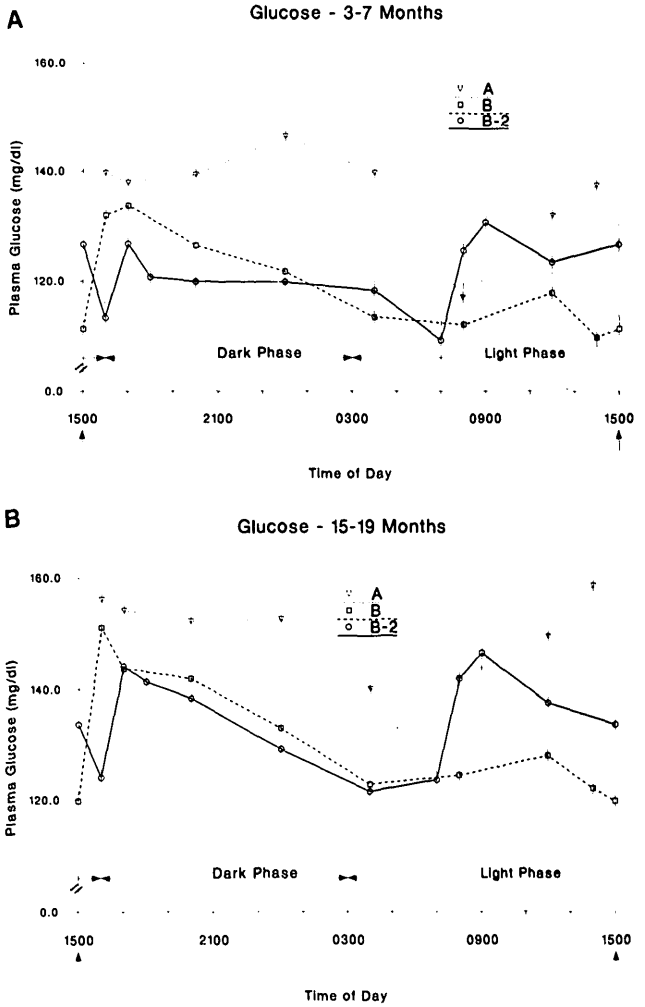

Figure 2. Circadian pattern of plasma glucose concentrations in Group A, Group B, and Group B-2 rats. **A**. Age range 3 to 7 months, mean ± *SE* for 21 Group A, 21 Group B, and 10 Group B-2 rats; **B**. Age range 15 to 19 months, mean ± *SE* for 21 Group A, 21 Group B, and 10 Group B-2 rats. The circadian patterns of Group A and Group B rats have been published previously (Masoro et al., 1992).

Survival curves for Groups A, B, and B-2 are presented in Figure 3. Data used in generating these curves included the longevity groups and the rats used for the longitudinal study of plasma total corticosterone and glucose levels. The periodic blood sampling did not appear to influence longevity characteristics. The survival curve of Group A rats differed from those of the rats in Groups B and B-2 ($p = .001$). The survival curve of rats in Group B did not differ from that of rats in Group B-2. Results on the median length of life, age of tenth percentile survivors, and maximum length of life are reported in Table 3. The median length of life and the age of the tenth percentile survivors were less for the rats in Group A than for those in Groups B and B-2 ($p = .0001$). Rats in Group B and those in Group B-2 did not differ in regard to either.

The severity of nephropathy (Table 4) at the time of spontaneous death was much less in rats of Groups B and B-2 than in rats of Group A ($p = .0001$). The rats in Groups B and B-2 did not differ in regard to the severity of this lesion.

At the time of spontaneous death, the dietary groups did not differ in the severity of cardiomyopathy or bile duct hyperplasia (Table 4). The severity of hepatic fatty change (Table 4) was less in the rats of Groups B and B-2 than in the rats of Group A ($p < .03$), but rats in Groups B and B-2 did not differ from each other in this regard.

The prevalence of other non-neoplastic lesions at the time of spontaneous death is reported in Table 4. The rats of Groups B and B-2 had a lower prevalence of cardiac and

Table 2. Mean ± *SE* 24-hour Plasma Glucose Concentration (mg/dl)

Age Range, Months	Rat Dietary Groups					
	A[b]	*n*	B[b]	*n*	B-2	*n*
3–7	136 ± 2	21	119 ± 2	21	122 ± 3	10
9–13	147 ± 2	21	126 ± 2	21	128 ± 3	10
15–19	149 ± 2	21	131 ± 2	21	133 ± 3	10
21–25	147 ± 2	15	134 ± 2	19	136 ± 3	9
27–31	[a]		126 ± 3	13	130 ± 3	7

[a]Four Group A were studied but three were suffering from terminal disease and died shortly after sampling was completed. Therefore, a mean for these rats is not reported.

[b]The data for Groups A and B have been published previously (Masoro et al., 1992).

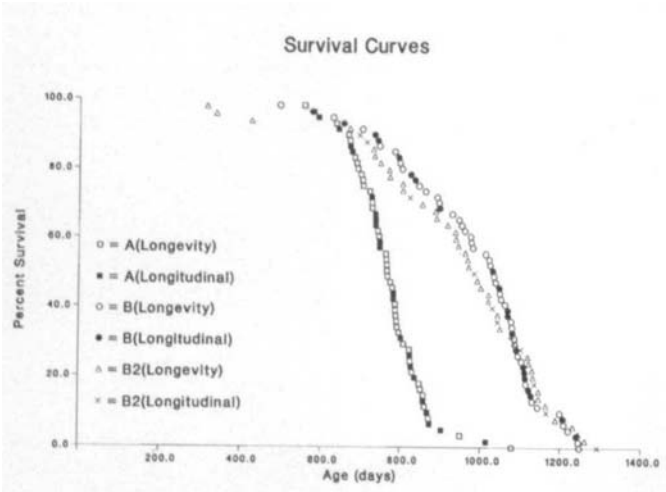

Figure 3. Survival curves for rats in Groups A, B, and B-2. Longevity refers to rats set aside for the purpose of assessing survival characteristics in unperturbed rats. Longitudinal refers to rats used in the longitudinal study of plasma total corticosterone and glucose concentrations.

Table 3. Longevity Characteristics

Dietary Group	*n*[a]	Median Length of Life, Days	Age of 10th Percentile Survivors, Days	Maximum Length of Life, Days
A	61	768 (744–790)[b]	867 (849–982)	1079
B	61	1035 (966–1075)	1195 (1117–1244)	1248
B-2	50	989 (918–1044)	1167 (1136–1266)	1292

[a]Includes rats set aside for the purpose of assessing survival as well as rats used in the longitudinal study of plasma glucose and total corticosterone concentration.

[b]95% confidence intervals in parentheses.

Table 4. Pathologic Lesions at the Time of Spontaneous Death

Lesion	Number of Rats in Group		
	A	B	B-2
Chronic nephropathy			
Grade 0	0	15	17
1	0	37	22
2	10	5	4
3	12	1	6
4	11	2	1
E	28	1	0
Cardiomyopathy			
Grade 0	0	2	3
1	15	18	19
2	41	38	23
3	5	3	5
Bile duct hyperplasia			
Grade 0	13	9	10
1	25	19	16
2	18	25	18
3	5	7	6
Hepatic fatty change			
Grade 0	22	30	30
1	21	22	13
2	13	6	7
3	5	2	0
Pituitary			
No lesions	10	25	28
Hyperplasia	15	19	11
Tumors			
Grade 1	19	12	6
2	6	2	1
3	5	0	0
4	4	1	3
Cardiac thrombus[a]	2	8	7
Cardiac mineralization[a]	26	3	1
Esophageal hyperkeratosis[a]	42	20	19
Gastric hyperkeratosis[a]	18	11	12
Osteodystrophy[a]	30	7	8
Skeletal muscle mineralization[a]	26	10	4
Skeletal muscle degeneration[a]	29	19	14
Skeletal muscle atrophy[a]	8	24	9

[a]The number of rats examined were 61 for Group A, 61 for Group B, and 50 for Group B-2.

skeletal muscle mineralization ($p < .003$), of esophageal hyperkeratosis ($p < .002$), and of osteodystrophy ($p < .0003$) than rats of Group A but did not differ from each other. However, rats of Group B had a higher prevalence of skeletal muscle atrophy than the rats of Group A or Group B-2 ($p < .03$).

The prevalence and severity of leukemia (data not shown) at the time of spontaneous death did not differ among dietary groups. Pituitary lesions (hyperplasia and tumors) at spontaneous death (Table 4) were more common in Group A rats than in those of Groups B and B-2 ($p < .005$) but there was no difference in this regard between rats of Group B and those of Group B-2. No significant difference was found among dietary groups at the time of spontaneous death in regard to heart tumors, kidney tumors, liver tumors, intestinal tumors, mammary gland fibroma and fibroadenoma, auditory sebaceous gland tumors, testicular interstitial cell tumors, thyroid adenoma, adrenal medullary hyperplasia, pheochromocytoma, pancreatic islet hyperplasia, and adenoma (data not shown).

Discussion

Providing DR rats their daily ration of food in two allotments, one in the early morning and the other in the late afternoon, clearly altered the circadian rhythm of plasma total corticosterone concentration compared to rats receiving food in a single allotment in the late afternoon. Specifically, the Group B-2 rats exhibited two daily peaks in plasma total corticosterone concentrations, the smaller between 0600 and 0800 h and the larger between 1300 and 1700 h; in contrast, Group B rats had a single daily peak in plasma total corticosterone concentration between 1300 and 1700 h. These findings suggest the likelihood that other circadian rhythms are also different between Group B-2 rats and Group B rats. Indeed, such was found to be the case for plasma glucose concentration. It is striking, however, that in spite of the difference in circadian patterns between Groups B and B-2 rats, the mean 24-hour plasma glucose concentration did not differ between the two groups and was significantly below that of the Group A rats. Masoro et al. (1992) have suggested that the ability to maintain lower levels of plasma glucose throughout the life span may be a factor underlying the antiaging action of dietary restriction.

Although circadian rhythms are influenced in DR rats by the temporal pattern of food intake, Groups B and B-2 did not differ from each other in regard to survival curves and in the increase in median length of life and age of tenth percentile survivors compared to Group A rats. Thus, it appears that the temporal pattern of food intake does not play a major role in the antiaging action of dietary restriction.

Consistent with this view, pathologic assessment showed that both modes of dietary restriction influenced most age-associated disease processes in a similar way. In both Group B and B-2 rats, a marked reduction was observed in the occurrence of severe nephropathy, a major contributor to the death of Group A rats (Shimokawa et al., 1993a). None of the Group B-2 rats and less than 2% of Group B rats had the most severe (Grade E) lesions compared to 46% of the Group A rats.

Both modes of dietary restriction also retarded to a similar extent several other non-neoplastic disease processes: hepatic fatty change; cardiac and skeletal muscle mineralization; esophageal hyperkeratosis; osteodystrophy. Only in the case of one non-neoplastic disease did Group B and Group B-2 rats differ; Group B rats had a higher prevalence at the time of spontaneous death of skeletal muscle atrophy than Group B-2 or Group A rats.

Comment is in order in regard to the finding of no difference in severity of cardiomyopathy between dietary groups at the time of spontaneous death. The fact that dietary restricted rats live longer may be the reason for this lack of difference. Indeed, in a cross-sectional study in which rats were sacrificed at various ages, Maeda et al. (1985) reported that the progression in severity of cardiomyopathy was slowed by dietary restriction.

The major neoplastic contributor to the death of males of the F344 strain is leukemia/lymphoma (Shimokawa et al., 1993a). The prevalence of this neoplasm did not differ among dietary groups at the time of spontaneous death. Again, this lack of difference appears to relate to longer length of life of Group B and Group B-2 rats compared to Group A rats. Recent analyses by Shimokawa et al. (1991, 1993b) have revealed that dietary restriction delays the age of occurrence of leukemia/lymphoma but not the progression in its severity after occurrence.

Pituitary adenoma is the other tumor that is a major contributor to the death of male F344 rats (Shimokawa et al., 1993a). In Group B and Group B-2 rats, the prevalence of pituitary adenoma at the time of spontaneous death was similar and much less than in Group A rats.

In conclusion, the temporal pattern of food intake played little or no role in the antiaging actions of a dietary restriction regimen which in the present study was sufficient to markedly extend life span. Thus, altered circadian rhythms do not appear to significantly underlie the retardation of aging processes by dietary restriction. These conclusions confirm those of Nelson and Halberg (1986a, 1986b), which were based on studies in which the level of restriction was not great enough to cause a marked increase in longevity. On the basis of the present study, as well as earlier studies carried out by our and other laboratories (Masoro, 1988), it appears that it is the reduction of energy intake and not of a particular dietary component or a change in temporal pattern of food intake which is responsible for the antiaging action of dietary restriction.

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