Effects of caloric restriction or augmentation in adult rats: Longevity and lesion biomarkers of aging

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ABSTRACT. Caloric restriction (CR) initiated in young rodents has been thoroughly documented to enhance longevity, but its efficacy when introduced at older ages has not been well investigated. Cohorts of 18- and 26-month-old male F344 x BN F1 hybrid rats were fed either: 1) NIH-31 meal (C); 2) vitamin and mineral fortified NIH-31 meal (R); or 3) vitamin and mineral fortified NIH-31 meal supplemented with corn oil and sweetened condensed milk (S). The C control rats were fed ad libitum*, R rats were restricted to 32% of the caloric intake of the controls, and S rats were allowed to consume not more than 8% more calories than C rats. After 6 weeks, the average weights were significantly different between all diet and age groups. Although calorie manipulation altered body weight, no significant effect of the dietary intervention on longevity was found. The average lesion burden, including tumor burden and prevalence of nearly all commonly occurring lesions, were comparable between the groups. Thus, the manipulation of weight at ages beyond middle age has a much less profound impact than similar interventions during growth and maturation in rats.*

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INTRODUCTION

Caloric restriction (CR) implemented at a young age is an effective means to increase longevity and decrease tumor burden in rodents (1-5). The applicability of CR to humans as a means for health maintenance/longevity extension is a topic of discussion (6), even though short-term restriction in humans has not demonstrated the anticipated modulations with regard to antioxidant capacity or oxidative stress (7). The effectiveness of CR appears to be modified both by the degree to which calorie intake is limited (8-10) and the age at which the intervention is implemented (8, 11-14). The association between increased body weight and mortality risks in humans is not well understood, especially with respect to age (15, 16). While recent data suggests that there is increased risk of death associated with excess weight, the degree to which adult obesity, as opposed to lifelong obesity, impacts on life span remains unclear (16, 17). Thus, experiments were conducted to determine the effects on life span and specific pathologies when caloric restriction or augmentation and the resulting decrease or increase in body weight are initiated in mid-aged and older rats.

MATERIALS AND METHODS

Male Fischer 344 x BN F1 hybrid rats (F3BNF1), N=245, ranging from 68 to 116 weeks of age were generously provided by Dr. DeWitt Hazzard of the National Institutes of Aging (NIA) from the NIA colony at Charles River Laboratories (Stone Ridge, NY). The animals were barrier raised, individually caged, and fed pelleted NIH-31 (Agway, Syracuse, NY) *ad libitum* prior to shipment. They were divided into mid-aged (M, 73.2±4.4 weeks, N=129) and older (O, 104.5±4.5 weeks, N=114) cohorts. These two age cohorts were then each divided into three diet groups such that within each age cohort, the diet groups were matched for both average age and weight (Table 1). The rats were individually housed in 7 × 10 in suspended wire cages at 23°C and 45% humidity, with a 12-hour light-dark cycle and *ad libitum* access to water.

The control group (C) for both age cohorts was provided *ad libitum* access to NIH-31 meal (Teklad, Madison, WI). Food intake was measured and average caloric consumption was calculated at 75 Kcal/d for both M and O cohorts after acclimation to the animal

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Table 1 - *Initial average age and body weight of age-diet cohorts.*

Age group	Diet group	Age±SD	Body weight±SD	N/group
M (mid-age)	C	73.2±4.4	512.0±49.0	43
M	R	73.2±4.4	510.0±39.5	43
M	S	73.2±4.4	514.0±35.0	43
O (older)	C	104.5±4.6	549.9±45.8	38
O	R	104.6±4.5	550.3±49.0	38
O	S	104.5±4.6	552.6±35.2	38

C: control; R: restricted; S: supplemented.

facility. The restricted groups (R) were fed vitamin and mineral fortified NIH-31 meal, as previously described by Lipman et al. (14). The caloric intake of the R group was gradually restricted to 51 Kcal/d, i.e., 32% less than C animals, over a 3-week period. The supplemented groups (S) were fed a mix that was 47% (w/w) vitamin and mineral fortified NIH-31 meal, 8% (w/w) Mazola corn oil (Best Foods, Chicago, IL), 44% (w/w) sweetened condensed milk (Waterford Food Products, Fond Du Lac, WI), and 1% (w/w) corn starch (Dyets, Inc., Bethlehem, PA). A similarly composed diet has previously been used to induce obesity in rats (18, 19). The vitamin and mineral fortified NIH-31 diet was used to insure nutritional adequacy of the diet consumed by the S rats (20). The S rats received 81 Kcal/d to limit their consumption of this highly palatable ration to only 8% more calories than the C rats. The R and S rats were provided with their daily ration in the morning between 9:00 and 11:00. All of the rats had free access to water, and were weighed biweekly during their remaining life span.

After 60, 61, and 64 weeks, 2.5 mL blood samples were obtained *via* orbital bleed following inhalation anesthesia with methoxyflurane (Mallinckrodt Veterinary Inc., Mundelein, IL) from 7 M rats in each diet group at 7:00, 11:00, and 21:00 for assessment of circulating glucose levels. As the R and S rats consumed their rations prior to 19:00, the morning samples collected at 7:00 from these cohorts are presumed to approximate fasting glucose levels.

Rats found to be moribund, in pain or distress were euthanized with CO_2 and their organs processed for histologic examination. Rats which were found recently dead were also processed for histology. Animals were fixed by intracardiac perfusion with Tellyesniczky's fixative (70% ethanol, formalin, and glacial acetic acid at 20:2:1) after flushing the vasculature with saline. Initial animal preparation consisted of opening the skull, cutting the spine in the thoracic and lumbar regions, and removing the contents of the stomach and cecum to facilitate optimal tissue fixation. The tissues were post-fixed by immersion in the same fixative for several weeks prior to dissection of tissues, including the pituitary gland, brain, eye, salivary, thyroid and parathyroid glands, heart, lung, liver, stomach, duodenum, jejunum, cecum, kidney, adrenal gland, spleen, bladder, testes, spine, and knee. These tissues were processed for routine histology following paraffin embedding by cutting 6 µm sections stained with hematoxylin and eosin. The information obtained from each histological analysis was entered in a relational database (FoxPro™, Microsoft, Redmond, WA). The data were analyzed to determine the prevalence of individual lesions among the six age-diet cohorts; in addition, the average number of lesions per animal or lesion burden were compared within each experimental age cohort for effect of diet using STAT-SAK (21). The significance of the differences in the prevalence of individual lesions between the age and diet groups was assessed using the Pearson χ^2 statistic. Any lesion that occurred in at least 5% of the rats in any diet-age cohort was considered to be a frequently occurring lesion. The percentage of animals in each group with individual frequently occurring lesions is listed in Table 2. Additional comparisons among the various diets, ages and either life span or lesion burden involved fully factorial ANOVA analysis, and were conducted using Systat (Systat Inc., Evanston, IL).

RESULTS

Body weight

The average body weight for rats in the M and O age cohorts fed the R, C or S diets is shown in Figure 1. The S rats were significantly heavier than C controls ($p \leq 0.01$) 4 weeks after separating the rats into age-diet groups for MS *vs* MC, and after 6 weeks for OS *vs* OC. The body weights of both MR and OR were significantly lighter than their respective controls after 5 weeks of restricted caloric intake. The average

Table 2 - *Prevalence of common lesions.*

		M			O				
Organ	Lesion	C	R	S	C	R	S	M	O
Adrenal gland	Cellular nodule	9.1	4.2	3.4	3.1	12.9	12.9		
	Ectasia	0	20.8	0	9.4	0	6.5	*	
	Intracellular lipid	4.5	0	3.4	9.4	6.5	6.5		
	Pheochromocytoma	18.2	0	34.5	34.4	29.0	22.6	*	
	Vacuolization	0	0	6.9	0	0	6.5		
Any organ	Fibroma	9.1	20.8	13.8	9.4	16.1	6.5		
	Fibrosarcoma	4.5	8.3	6.9	6.3	6.5	9.7		
Bile duct	Hyperplasia	45.5	33.3	6.9	68.8	80.6	38.7	*	*
Blood vessel	Polyarteritis nodosa	22.7	12.5	10.3	15.6	22.6	6.5		
Heart	Degeneration	50.0	45.8	48.3	75.0	80.6	80.6		
Kidney	Accumulation of lymphocytes	13.6	8.3	3.4	18.8	22.6	16.1		
	Atrophy	4.5	4.2	10.3	9.4	9.7	6.5		
	Glomerulonephritis	31.8	33.3	31.0	53.1	64.5	35.5		
	Inflammation	9.1	0	6.9	6.3	3.2	0		
Liver	Accumulation of lymphocytes	0	4.2	6.9	3.1	12.9	9.7		
	Fat deposition	0	4.2	13.8	6.3	0	6.5		
	Fatty hepatocytes	0	0	3.4	12.5	6.5	16.1		
	Fibrosis	0	0	6.9	0	3.2	0		
	Necrosis	0	8.3	0	3.1	6.5	12.9		
	Nodule	0	0	6.9	0	0	0		
	Portal fibrosis	36.4	33.3	20.7	37.5	45.2	25.8		
	Vacuolated hepatocytes	13.6	0	13.8	6.3	0	22.6		
Lung	Accumulation of lymphocytes	50.0	45.8	27.6	31.3	32.3	22.6		
	Adenoma	0	0	10.3	0	6.5	9.7		
Lymph node	Ectasia	4.5	4.2	13.8	3.1	3.2	3.2		
Mammary gland	Fibroadenoma	0	0	10.3	6.3	9.7	3.2		
Pancreas	Atrophy	63.6	45.8	65.5	37.5	35.5	45.2		
	Islet cell adenoma	4.5	12.5	6.9	9.4	3.2	9.7		
	Islet cell carcinoma	0	0	0	3.1	6.5	6.5		
	Islet cell hyperplasia	31.8	20.8	24.1	21.9	29.0	25.8		
Pituitary gland	Adenoma	59.1	50.0	58.6	65.6	38.7	64.5		
Prostate	Inflammation	13.6	12.5	6.9	12.5	6.5	9.7		
Spinal root	Degeneration	36.4	20.8	48.3	31.3	41.9	41.9		
Spleen/liver	Histiocytic sarcoma	0	12.5	0	0	3.2	0		
	Leukemia	18.2	16.7	3.4	3.1	9.7	9.7		
Stomach	Papilloma	9.1	0	0	0	0	0		
	Ulcer	9.1	4.2	6.9	6.3	9.7	19.4		
Testes	Atrophy	9.1	12.5	20.7	12.5	16.1	35.5		
	Leydig cell adenoma	0	20.8	3.4	46.9	25.8	32.3		
	Leydig cell hyperplasia	13.6	8.3	3.4	6.3	16.1	9.7		
	Mineral deposition	4.5	12.5	0	6.3	0	16.1		
Thyroid gland	C-cell adenoma	0	4.2	6.9	15.6	12.9	12.9		
	C-cell hyperplasia	27.3	25.0	34.5	25.0	35.5	29.0		
	Cysts	36.4	37.5	44.8	43.8	38.7	51.6		
	Mineral deposition	4.5	0	6.9	3.1	0	6.5		
Trachea	Cysts	27.3	33.3	34.5	34.4	51.6	35.5		
Urinary bladder	Polyp	13.6	0	13.8	0	6.5	6.5		
Urothelium	Hyperplasia	36.4	37.5	37.9	31.3	45.2	45.2		

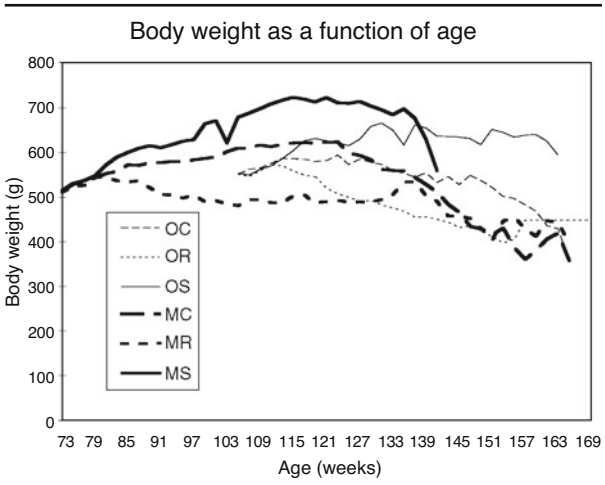

Figure 1 - *Average body weights for age-diet cohorts.*

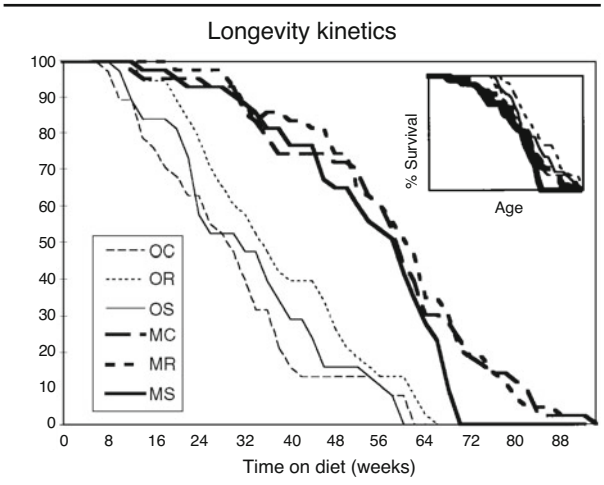

Figure 2 - *Longevity as a function of time receiving diet. Inset: Longevity as a function of age.*

weights of the various cohorts remained divergent for the remaining life span of the animals.

The average weight of the OC and MC rats reached an apex of 592.5±59.0 and 622.2±60.0 g, respectively, at 123 weeks of age. The period during which these rats continued to gain weight after acclimation and intervention was proportional to their age on arrival. There was, however, a difference between these two C cohorts as MC rats became heavier while aging in our facility than the OC rats had been upon their arrival. Thus, while the OC rats arrived at 105 weeks of age at an average weight of 549.9±45.8 g, the MC rats at 105 weeks weighed 608.7±40.4 g ($p \leq 0.0001$).

The average weight of the OS rats increased 20% relative to their starting weight; the MS had an average weight increase of 40%. This difference in percentage weight gain may be due, in part, to the different length of time during which the animals continued to gain weight, i.e., 26 and 42 weeks for the OS and MS cohorts, respectively.

Mortality kinetics

The average longevity for all the rats (Fig. 2) was similar in all the age-diet groups. Neither calorie restriction nor calorie augmentation significantly altered the average life span of the animals in either age cohort (Fig. 3). Comparison of the average age of survival for the last 10% of rats in each cohort (N=4) showed both a significant effect of diet and a diet by initial age group interaction. This was a result of the reduced longevity of the last 4 surviving rats in the MS cohort compared with the MC and MR groups. The maximum age attained by the rats in each group in

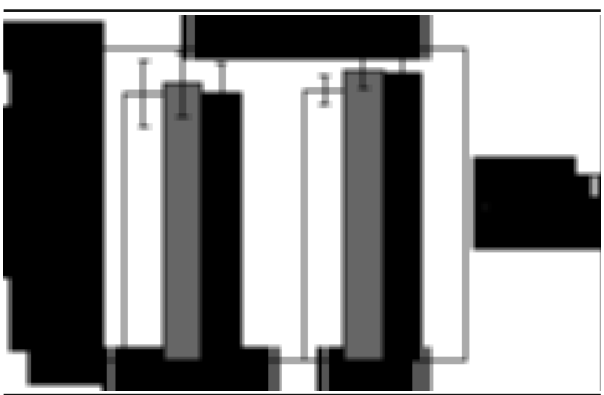

Figure 3 - *Average life span of age-diet cohorts.*

months was 45.8, 45.0, 37.8, 41.4, 41.5 and 40.3 for the MC, MR, MS, OC, OR and OS, respectively.

Circulating glucose levels

No difference in circulating glucose levels was noted between MC, MR, and MS groups, indicating that by 133-137 weeks of age, glucose levels are unaffected by diet or body weight in this rat strain (Fig. 4). Further, it appears that glucose levels at this advanced age do not vary with the time of day or proximity to food consumption. This consistency in glucose levels over time was observed for the animals in all three diet groups.

Lesion burden and prevalence

Although only 154 (63%) of the rats were recovered for the study of pathology, the rats in each diet cohort assessed for lesions were of comparable ages

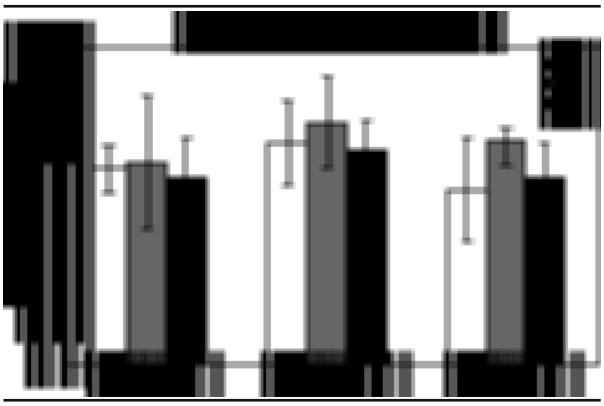

Figure 4 - *Circulating glucose levels for MC, MS, and MR rats.*

(Table 3). The incomplete recovery of animals resulted from rats which died early in the evening being too autolyzed by morning to yield tissue useful for histopathology. Using Pearson's correlation matrices, no significant correlations between life span, diet or initial age group and lesion burden were observed.

Distinctions between initial age group or diet cohort and the prevalence of individual lesions were generally not observed. However, an effect of age at which calorie restriction was started was noted for two adrenal gland lesions. Using the significance levels corrected for the comparison of three diet groups with a Bonferroni adjustment, pheochromocytoma was less commonly observed in MR than MC rats (p=0.03), while no significant difference was found between OR and OC rats; at the same time, adrenal gland ectasia was more commonly observed in the MR than MC rats (p=0.02), but there was no difference between OR and OC rats.

The prevalence of bile duct hyperplasia showed a consistent effect of diet in both age groups. The prevalence of this lesion was decreased in both the MS and OS rats as compared with either the C or R rats in their respective age group after Bonferroni adjustment ($p \leq 0.03$).

DISCUSSION

Cheney et al. (8) reported that the increase in life span was greater for mice that were calorie restricted as neonates than for those which began calorie restriction at weaning. Similarly, Weindruch et al. (11) demonstrated that caloric restriction started at 12 months significantly increased longevity, but not to as great an extent as when started at an earlier age. Nonetheless, initiating calorie restriction in late life in mice at 22 months has been reported to enhance *in vitro* mitogen-stimulated proliferation of splenic lymphocytes (22).

Calorie restriction initiated at 12 months has also been shown to have a beneficial impact on immune function (23). Thus, although introduction of calorie restriction at mid-age and late life in rodents can enhance some immune responses, only initiation of calorie restriction before 18 months has been clearly demonstrated to increase longevity (24).

The question of whether late-life calorie restriction significantly affects the health span or longevity in a fashion similar to that observed with intervention at earlier ages has received little attention. Nonetheless, the efficacy (or lack thereof) of late-life intervention may help to elucidate the mechanism(s) of calorie restriction, and indicate its value in the elderly, a group characterized by relatively low intake of calories and many essential micronutrients (25, 26). We previously reported that late-life (18 months) calorie restriction by 33% in Long Evans rats had no impact on maximal life span, and suggested that the effect of calorie restriction on life span may be titrated by the age at which the intervention is initiated, with a threshold past which dietary change no longer modulates longevity (14).

Lipman et al. (27) found that mid-aged C57BL/6 mice gained weight in response to either increased caloric density or increased diet palatability, and suggested that the propensity for diet-induced obesity is similar in young and older C57BL/6 mice. The older animals maintained their gained weight even when subsequently fed regular chow. This maintenance of weight by older mice is consistent with the results ob-

Table 3 - *Average lesion burden.*

Average	MC	MR	MS	OC	OR	OS
Lesions/rat	8.32	9.15	9.12	10.38	11.29	10.79
±SD	4.13	5.26	3.57	3.87	4.19	4.35
N	22	20	26	29	28	29
Age (days)	889.00	896.93	869.88	951.34	1004.22	1000.48
±SD	131.00	121.60	121.56	106.87	116.30	94.18
N	22	20	26	29	28	29

tained in a human study where older men were significantly slower than young men to return to baseline weight following over-feeding (28). The mortality rate and tumor prevalence of the heavier mice reported by Lipman et al. (27) did not differ from normal weight mice. This lack of effect of late-life obesity is consistent with the lack of effect of late-life calorie restriction on longevity both in this and a previous study (14, 27). Thus, the potential for a robust beneficial impact of dietary intervention on longevity appears limited to ages younger than 18 months in rodents.

Fernandes et al. (29) have reported that early life (14 weeks) calorie restriction by 40% in BNF3F1 rats significantly increased longevity. In contrast, our study demonstrates no benefit when calorie restriction is initiated at 73 or 105 weeks in the same rat strain. Thus, the lack of effect of the intervention on life span appears to be a function of the age at which the restriction was started, and not a genetic predisposition of this strain. Calorie augmentation had no effect on the average longevity, although it did decrease the survival time for the oldest 10% of the MS rats. This may suggest that the age at which weight gain affects longevity is older than for CR. However, consistent with the idea that there are limitations on the age at which calorie and/or weight manipulation influence life span, neither calorie augmentation or restriction affected average survival time, or survival of the oldest 10% in the older cohort of rats.

The lack of diurnal variation in glucose levels and postprandial differences between the restricted and *ad libitum* fed rats, as well as the lack of effect on the prevalence of the majority of lesions, further demonstrate that the effects of calorie restriction in young animals are different than those in older animals (30). The similarity in glucose levels for the R and C rats also contrasts with the significant differences in glucose levels between restricted and *ad libitum* fed adult (8-14 years) rhesus monkeys (31).

Although no generalized effect of dietary intervention on tumor burden was observed, calorie restriction initiated at 73 weeks did reduce the prevalence of pheochromocytomas, a late-life tumor common to this genotype (32). We have previously reported the presentation of pheochromocytomas in male BNF3F1 at an average age of 119 weeks, but at 130 weeks in the reciprocal cross (32). Thus, there may be an age-specific trigger for tumorigenesis occurring between 73 and 105 weeks resulting in increased propensity to pheochromocytoma.

The difference in body weight between the MC and OC cohorts may be a consequence of the change from pelleted chow to meal, providing an easier form of food to consume. Chewing force has been reported to alter collagen deposition and chondrocyte numbers (33), as well as parotid gland weight (34). However, we did not observe any unusual parotid gland morphology or pathology.

Interventions other than calorie restriction designed to enhance longevity have demonstrated a correlation between age of introduction and efficacy. Bezlepkin et al. (35) reported that dietary antioxidant supplementation enhanced life span in mice when the treatment was initiated at 6 or 12 months, but not at 16 months or older. Similarly, we have found that feeding C57BL/6 mice vitamin E (470 ppm), glutathione (5% w/w) or the combination of both antioxidants beginning at 18 months did not affect life span or tumor prevalence (36), although *in vitro* immune responses were enhanced in animals receiving vitamin E supplementation (37). Viidik et al. (38) compared the effect of treadmill running (800 m/d) initiated in 18-month and 20-month-old rats, and found only a transient increase in spontaneous activity, suggesting a declining efficacy of this intervention with age. Fiatarone et al. (39) reported no effect of a dietary (supplemental calories and micronutrients) intervention on exercise training in very old people, although high-intensity, progressive resistance exercises did counteract physical frailty (39). Recent findings by Kalu et al. (40) document the lack of effect on both longevity and pathology of long-term administration of growth hormone begun at 18 months of age in F344 rats and 17 months of age in Balb/c mice. This lack of effect on these two parameters for an intervention initiated in older animals is consistent with our lack of effect for dietary intervention at this age. In conclusion, changes in environmental factors (diet and exercise) which have been demonstrated to have a beneficial impact on aging may be attenuated when introduced late in life.

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