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# MODULATION OF TOXICITY AND CARCINOGENESIS BY CALORIC RESTRICTION

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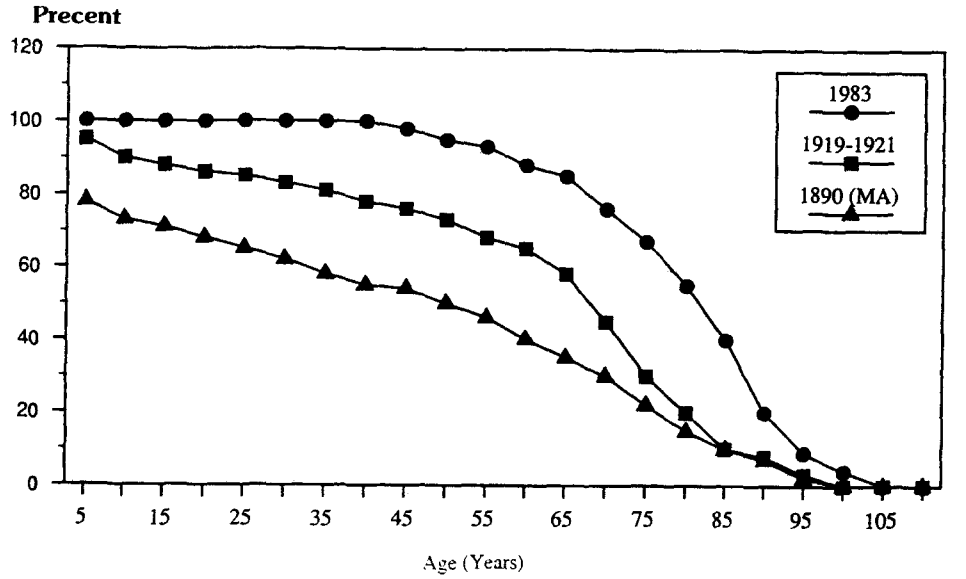
***ABSTRACT:*** *Dietary restriction (caloric restriction) is the only intervention which has been reliably shown to extend the maximum life span of warm-blooded animals and delay the many phenomena associated with aging. It is also one of the most effective modulators of toxicity, especially cancer endpoints. In spite of the known modulator effects of caloric restriction, the biological mechanisms responsible for these effects had not been investigated until recently. The National Center for Toxicological Research (NCTR), in a collaborative effort with the National Institute of Aging (NIA), initiated a project whereby nine (9) combinations of rodent species/strains and diets were fed both restricted and ad libitum. The NIA's initiative was to identify biomarkers of aging whereas NCTR's initiative was to identify the biological effects associated with the profound effects caloric restriction has in protecting against both spontaneous (age-related) and chemically-induced toxic endpoints. Independent of sex or species, caloric restriction has similar effects on body temperature, oxygen consumption and $CO_2$ production. Caloric restriction also decreased lipid glycolysis and metabolism in rats and mice, which suggest decreased production of metabolites which could lead to fatty acid epoxide formation. The age-associated loss of circadian regulation of intermediate enzymes is also significantly reduced. Moreover, caloric restriction reduced the age-associated feminization of sexually dimorphic liver isozymes, increased several glucocorticoid responsive isozymes, elevated glucagon/insulin ratios, produced less microsomal superoxide and enhanced the capacity for utilizing detoxicating metabolic pathways. Calorically restricted rats have less than half the number of aflatoxin ($AFB_1$)-DNA adducts than ad libitum animals and urinary excretion of $AFB_1$ was increased significantly. Finally, DNA repair mechanisms are enhanced and oncogene expression is decreased in calorically restricted animals.*

## INTRODUCTION

The United States and other developed countries are increasingly having to adapt to a population that has a significant percentage of individuals who are seventy-five years old or older (Fig. 1). As the population of senior citizens increases, it can be anticipated that there will be an added burden on health care systems in the U.S. and other developed countries. As reported by the Task Force for Aging Research Funding (a coalition of eleven leading health care and science groups), "Research to find cures, preventions, or postponement of the major diseases of aging may be the most effective way for the U.S. to reduce health care cost." The Task Force also recognized the role of dietary restriction in reducing many of the age related diseases as well as extending maximum life spans in laboratory animals (rodents). Indeed, dietary (or caloric) restriction is the only intervention tested which consistently reduces spontaneous and chemically-induced toxic endpoints (e.g., cancer, kidney disease) while extending the maximum achievable life spans (1). The increasing interest in the role of caloric restriction in slowing the rate of aging, plus its ability to retard and/or eliminate the onset of cancers and other diseases, coupled with the potential economic impact associated with health care, was the stimuli for the creation of the Project on Caloric Restriction (PCR).

PCR is one of the most monumental research studies ever undertaken by the Public Health Service (PHS). It is a collaborative effort of the National Institute on Aging (NIA) and the National Center for Toxicological Research (NCTR). The study has the following objectives: 1) establish a colony of aged, closely controlled and tightly regulated rodents, both calorically restricted and *ad libitum* cohorts, for research pur-



poses (Table 1); 2) create a scientific interchange promoting integration of the extensive research data bases in toxicology and aging; 3) design studies that will bridge the whole animal and biochemical/molecular data bases providing for more realistic interpretation of experimental results; 4) identify and evaluate biomarkers of aging (predicting length of life); 5) establish rodents for each genotype, species, and diet to provide age-specific pathology profiles for animals maintained on *ad libitum* and diets and on calorically-restricted diets; and 6) determine maximum life spans for *ad libitum* and calorically restricted rodents for each genotype, species, and diet.

The PCR study was divided into three basic modules: 1) study design, 2) biomarkers of aging, and 3) mechanistic toxicology. The NIA, through a granting mechanism, is supporting module two, "biomarkers of aging." NCTR provides the aged animals for successful investigators. From a colony of some 20,000 rodents (rats and mice), researchers from across the U.S. and Canada are being provided aged calorically-restricted and *ad libitum* animals. The NCTR, on the other hand, is conducting the mechanistic toxicology studies, i.e., determining the physiological and biochemical processes that account for reductions in the expression of spontaneous and chemically-induced toxic endpoints (e.g., cancer).

It has been known for some time that nutritional manipulation can alter chemically-induced and spontaneous toxicity (1). As early as 1908, it was demonstrated that the growth rate of transplanted tumor cells in rodents was inhibited by extreme food restriction (2). This finding has been repeated in other systems using more moderate underfeeding. Numerous studies have demonstrated the inhibitory effect of food restriction on chemically-induced carcinogenesis (1,3,4). Compounds studied include benzo(a)pyrene and methylcholanthrene in skin, diethylnitrosamine in liver, and dimethylbenz(a)anthracene in mammary gland (reviewed in Reference 1). Moreover, ultraviolet light-induced (5) and ionizing radiation-induced (6) tumors are also reduced in incidence by food restriction. Other diseases have been less studied, but dietary restriction has been shown to positively effect such things as survival after viral challenge (7) and induced end-stage renal pathology (8).

Underfeeding effects all nutrients. Numerous studies (9-11) have shown that the most important component of underfeeding for positively altering toxic effects appears to be caloric (or energy) restriction (CR). Various levels of CR have been used, but restriction of approximately 40% of *ad libitum* levels with the addition of vitamins to pre-

**Table 1.** Genotypes and diets

| Mouse | Diet | Rat | Diet |
|---|---|---|---|
| B6D2F1 | NIH-31 | Fischer-344 | NIH-31 |
| DBA/2NNia | NIH-31 | Fischer-344 | Masoro* |
| B6C3F1 | NIH-31 | Brown-Norway | NIH-31 |
| C57Bl/6NNia | NIH-31 | Brown-Norway X Fischer-344 | NIH-31 |
| C57Bl/6NNia | EM-911A** | | |

NIH-31 is the autoclavable form of NIH-06. EM-911A is Emory-Morse 911A developed by the Emory Morse Company, Guilford, CT. Masoro is Masoro Diet C from Ralston Purina.

vent deficiencies, has been shown to be adequate for long-term maintenance in a number of different strains of mice and rats.

### Animals and Diets

Given these unique findings, and because there was a paucity of scientific data addressing the reasons for such observations, the NCTR has developed a comprehensive project to study the effect of caloric restriction on spontaneous and chemically-induced toxic endpoints. The experimental design of PCR is fairly straightforward. The project uses 4 genotypes of mouse and three of rat. These are listed in Table 1. Since different pathologies arise in the different strains and species, using different genotypes helps address strain- and species-specific pathologies that may be independent of the experimental conditions studied or unique to that strain or species, addressing the question of whether genotype-specific pathology accounts for the effects seen. The $B6C3F_1$ mouse and the F344 rat predominate in chronic toxicity studies, and thus, so have an extensive data base associated with their response to a wide range of toxic substances as a function of dose and endpoint.

The diets used are the NIH-31 (an autoclavable form of the standard NIH-07 used in most toxicology studies), which is fed to all genotypes as well as special diets (Masoro and Emory-Morse 911A) fed only to diet-comparison cohorts of B6 mice and F344 rats (Table 1). All restrictions start from 14-16 weeks of age, except for one diet/genotype started at 6-8 weeks. The target level for CR is 60% of *ad libitum* consumption. The restricted diets are fortified with vitamin equivalent to what would be received by the animals on an *ad libitum* diet. All animals are singly housed and maintained in SPF barrier conditions. Over the nine-year experimental period, the study is expected to contain over 100,000 animals, up to 22,000 being maintained at any one time.

Presently, animals are being distributed mainly to 14 university laboratories, 10 laboratories at NCTR, and 4 laboratories at NIH, as well as others, as resources permit. The combined expertise of these research units is immense, involving over 50 investigators at the doctoral level and over 200 endpoints being evaluated. In order to maximize the use of the biological material being generated in this project, NCTR will publish, in the near future, a detailed description of the study design and listing of the biological material available for study in our tissue and serum banks.

## FINDINGS

### Body Weight and Survival

Based upon changes observed in their body weights, animals on study are indeed being restricted. An example of this is given in Table 2, which describes the body weight differences at selected ages C57B16 females. For the other groups, the final weight as a percentage of *ad libitum* weight varies with genotype, species and sex, from 30 to 40%. In all genotypes, there is an increase in age-specific survival, consistent with other studies using CR. The magnitude of effect also varies with genotype, sex, and strain; however, the effect is given in Table 3, which gives the survival at selected ages for females of the short-lived mouse strain, DBA/2NNia.

**Table 2.** Mean body weights for female C57BL/6 mice on NIH-31

| Age (wks) | Weight *Ad Lib* (gms) | Weight Restricted (gms) |
|---|---|---|
| 14 | 22.8 | 22.7 |
| 30 | 25.2 | 22.8 |
| 50 | 28.1 | 21.1 |
| 90 | 28.8 | 22.5 |

Body weights are means taken from a cohort of 20 *ad libitum* and restricted animals.

**Table 3.** Survival of DBA/2NNIA female mice on NIH-31.

| Age (wks) | Survival *Ad Lib* (%) | Survival Restricted (%) |
|---|---|---|
| 14 | 100 | 100 |
| 30 | 90 | 93 |
| 50 | 75 | 91 |
| 90 | 65 | 85 |
| 120 | 12 | 45 |

Survival is approximate value derived from a cohort of 100 *ad libitum* and restricted females.

## Intermediary Metabolic Alterations

Intermediary or energy metabolism is central to the metabolic processes which occur in a target cell to metabolize an agent delivered to it, or interact with its active metabolite. Cells, depending on type, activity state (proliferating, quiescent, actively secreting, etc.), and hormonal control, can display widely different metabolism. Liver is a good model of target cell metabolism, although it also exports the results of its activity to the rest of the body.

Gluconeogenesis appears to be significantly enhanced by CR. Gluconeogenic and accessary enzymes, such as, glutamate dehydrogenase (GDH) and glucose-6-phosphatase, etc., are either unchanged or significantly increased, while those related to glycolysis, such as, lactic dehydrogenase (LDH), pyruvate kinase (PK) (the $V_{max}$), sorbitol dehydrogenase (SDH), and alcohol dehydrogenase (ADH) are decreased with CR (Table 4) (12). Decreased also were the enzymes associated with lipid metabolism, such as, malic enzyme and glycerokinase (Table 5). Limited lipid metabolism suggests a decreased potential for fatty acid epoxide formation. A number of studies have measured the mutagenic potential of fatty acid epoxides, and found them to be potent mutagens in the Ames assay system.

These early observations did not explain the mechanism through which respiratory quotient (see section-physiologic effects) could increase and decrease. Therefore, we began detailed studies of the affects of CR on the regulation of key enzymes within these metabolic pathways. We demonstrated that pyruvate kinase (PK), a glycolytic enzyme which catalyzes the production of ATP (energy molecule), hormonally influenced (by insulin, glucagon and others) to have an increased affinity for its substrate (increased efficiency) in CR rodents. This allows the glycolytic pathway to operate and produce ATP with input of far lower levels of carbohydrate. Thus, much

**Table 4.** Mean activities of selected enzymes of intermediary metabolism

| Enzyme | *Ad Lib* | Restricted | % Change |
|---|---|---|---|
| **Gluconegenic (and Accessory)** | | | |
| Glutamate Dehydrogenase | 4742 | 6736 | +42 |
| Glucose-6-Phosphatase | 0.22 | 0.33 | +50 |
| Amino Acid Oxidase | 645 | 647 | N.S. |
| **Glycolytic** | | | |
| Alcohol Dehydrogenase | 540 | 294 | -46 |
| Lactate Dehydrogenase | 1457 | 772 | -47 |
| Sorbitol Dehydrogenase | 1639 | 1308 | -20 |
| Pyruvate Kinase (Vmax) | 6621 | 3413 | -48 |

Units are units/liter except glucose-6-phosphatase which is micromole/min/mg microsomal protein. Methods are from Feuers *et al.* (1989) from which table is adapted. Animals used were F-344 rats. N.S. means no significant difference was found by Student's t-test.

**Table 5.** Mean activities of selected enzymes of lipid metabolism

| Enzyme | *Ad Lib* | Restricted | % Change |
|---|---|---|---|
| Fatty Acid Synthetase | 1788 | 1521 | N.S. |
| Glycerol Kinase | 2586 | 1168 | -55 |
| Malic Enzyme | 1363 | 865 | -37 |

Units are units/liter. Methods are from Feuers *et al.* (1989) from which table is adapted. Animals used were F-344 rats. N.S. means no significant difference was found by Student's t-test.

of the available calories are available for conversion to glucose in the liver and export to skeletal muscle to support additional motor activities. The ability of well known and studied hormones to produce these affects is currently under more detailed study. Additionally, we have found that as animal age, PK efficiency deteriorates, but this is offset with CR.

Catalase is an enzyme which is involved in the detoxification of oxygen-free radicals (free radicals are thought to contribute to the aging process by producing cumulative damage to important macromolecules, such as, DNA and proteins) by converting hydrogen peroxide to water. We have found that CR causes the level of this enzyme (and others associated with free radical detoxification) to be increased. The enzyme is rapidly inhibited by its own substrate in *ad libitum* rodents, but in CR animals this inhibition occurs at much slower rates. Therefore, this enzyme becomes much more efficient as well. It appears that the increases in efficiency that have been seen for physiologic measurements occur through specific changes in the regulation of key enzymes in metabolism. The mechanisms for production of efficiency in enzyme activities seem to have commonalties which further study could reveal.

## Microsomal (Drug Metabolizing) Alterations

Studies have focused on the investigation of the effects of long-term caloric restriction on age-related changes in hepatic drug metabolizing enzyme activities in the male

Fischer 344 rat (13, 14, 15). Rats that had been restricted to 60% of their pair-fed control's consumption from 14 weeks post partum exhibited altered enzyme activities when assayed at 9 or 22 months. In general, the drug metabolizing enzyme activities that were most affected were those that are either sex-specific in the rat or those that are partially regulated by corticosteroids, insulin or glucagon.

Caloric restriction appears to evoke changes in hepatic drug metabolizing enzymes isozyme expression that were dependent on age and/or circadian rhythm (Table 6). These effects were associated, in male rats, with a general early partial feminization of hepatic function followed by a delay in the natural, age-dependent feminization, which occurs spontaneously during in senescence. However, such effects were isozyme-specific. Many drug metabolizing enzymes activities remained unchanged by either aging or caloric restriction under the conditions of our investigation, which others were increased by caloric restriction independently of the hepatic feminization process.

Such changes in hepatic drug metabolizing isozyme profiles may result in altered rates of carcinogen activation and detoxication. They may also alter both the rate of superoxide generation in the endoplasmic reticulum and the transformation and elimination of endogenous metabolites. These alterations may, in turn, influence the aging process in the liver and other tissues.

## DNA Binding Alterations

An example of the ultimate result of some of these changes are their effect on the activation of aflatoxin. Aflatoxin is a human carcinogen which requires metabolic ac-

**Table 6.** Effects of caloric restriction (CR) on hepatic drug metabolizing enzymes in male fischer 344 rats

| | |
|---|---|
| **Enzymes Increased by CR In Middle Aged Male Rats** | |
| Testosterone-5$\alpha$-reductase | [Activity normally higher in females] |
| Corticosterone sulfotransferase | [Activity normally higher in females] |
| Bilirubin UDP-glucuronyltransferase | [Inducble by glucagon and cAMP] |
| **Enzymes Decreased by CR in Middle Aged Male Rats** | |
| Testosterone-16$\alpha$-hydroxylase (P450IIC11) | [Normally higher in males] |
| Glutathione-S-transferase 3:3 | [Normally higher in males] |
| **Enzymes Increased by CR in Aged Male Rats** | |
| Bilirubin UDP-glucuronyltransferase | |
| 4-Nitrophenol hydroxylase (P450IIE1) | |
| Testosterone-16$\alpha$-hydroxylase (P450IIC11) | |
| Testosterone-6$\beta$-hydroxylase (P450IIIA2) | [Normally higher in males] |
| Glutathione-S-transferase 3:3 | |
| $\gamma$-Glutamyltranspeptidase | [Inducible by chronically elevated glucocorticoid] |
| **Enzymes Decreased by CR in Aged Male Rats** | |
| Testosterone-5$\alpha$-reductase | |
| **Enzymes Which Exhibit Differences in Circadian Profiles Due to CR** | |
| Testosterone-5$\alpha$-reductase | |
| Corticosterone sulfotransferase | |
| Bilirubin UDP-glucuronyltransferase | |
| 4-Nitrophenol hydroxylase (P450IIE1) | |

Methods from Leakey *et al.* (1989a, 1989b, 1991).

tivations for its adverse health effects. CR has recently been shown to reduce the incidence of aflatoxin-induced carcinogenesis.

After a single dose administration of labeled aflatoxin $B_1$, hepatic nuclear DNA modification by $AFB_1$ was substantially reduced by caloric restriction. Hepatic $AFB_1$-DNA binding was decreased to less than 50% per mg DNA, and even less per liver (Table 7) (16). Analysis of the individual DNA adducts demonstrated that much lower amounts of the major N-7-guanine adduct and its breakdown product, the formamido-pyrimidine derivative, were present in CR rats compared to *ad libitum* controls (Table 8). Since both phase I and phase II metabolism of $AFB_1$ are effected significantly, the reduction of $AFB_1$-DNA binding by caloric restriction may be attributed partly to the decrease of metabolic activation of $AFB_1$ and the increase of the hepatic $AFB_1$ detoxification activity. In addition to the metabolic changes, CR-induced decreases (50-70%) in hepatic DNA synthesis, as determined by thymidine incorporation may contribute to the reduction in DNA adduction. The potential for proliferation of $AFB_1$-induced genetic lesions was also apparently lower in CR rats since the increase in nuclear DNA synthesis following multiple $AFB_1$ dosing was much less dramatic in the CR group. These results indicate that CR can beneficially modulate chemical carcinogenesis in rats during the initiation stage, and that alterations in both metabolism and DNA modification are mechanistically involved.

## DNA Repair Alterations

As discussed above CR has a major impact on DNA damage. However, the ultimate effect on a cellular system is the amount of DNA damage induced minus the amount of

**Table 7.** Picomoles aflatoxin bound to liver DNA

| Normalization | *Ad Lib* | Restricted | % Change |
|---|---|---|---|
| Bound to Liver (per mg DNA) | 59 | 29 | -51 |
| Bound to Liver (per liver) | 1120 | 420 | -63 |

Three hours after single oral dose of aflatoxin in F-344 rats. Methods are in Pegram *et al.* (1989) from which table was adapted.

**Table 8.** Effect of caloric restriction on concentrations of hydrolysis products of aflatoxin $B_1$-modified hepatic nuclear DNA

| Diet | Concentration (pmol $AFB_1$ derivative/mg DNA)[a] | |
|---|---|---|
| | $AFB_1$-$N^7$-Gua[b] | $AFB_1$-$N^7$-FAP[c] |
| *Ad Libitum* | 44.2 ± 2.3 | 11.7 ± 2.0 |
| Restricted | 19.3 ± 1.9 | 3.4 ± 0.3 |

[a]A single oral dose of [$^3$H]$AFB_1$ (0.1 mg/kg body wt) was administered 3 hr prior to sacrifice. Each value is the mean ± SEM, n = 4.

[b]8,9-Dihydro-8-($N^7$-guanyl)-9-hydroxyaflatoxin $B_1$.

[c]8,9-Dihydro-8-(2,6-diamino-4-oxo-3,4- dihydropyrimid-5-yl formamido)-9-hydroxyaflatoxin $B_1$.

DNA repairs.

DNA repair, as measured by unscheduled DNA synthesis (UDS) after insult by a chemical agent, methylmethanesulfonate (MMS) or ultraviolet light (UV), appears to be enhanced at least 50% by CR (Table 9) (17). These results are consistent with the results of Licastro (18) who found the prevention of an age-related decline in UDS in splenocytes, and those of Waraarchakul and Richardson (19) who found a similar effect after UV exposure in liver and kidney-derived cells. $O^6$ methylguanine acceptor protein activity is increased, by approximately 70% (Table 9). This result contrasts with those of Woodhead, *et al.* (20) who found no change with CR. Although there are a number of possible explanations for this difference, one of the most interesting is related to the extreme temperature dependence of DNA repair. When measured by a continuous monitoring system developed at NCTR, average body temperature is lower with CR (21.) There is a normal diurnal variation in body temperature, that gets accentuated with CR. This core body temperature change has a number of possible mechanisms, and is correlated with metabolic alterations induced by CR, such as, a

**Table 9.** DNA repair

| Agent | *Ad Libitum* | Restricted | % Change |
|---|---|---|---|
| **Unscheduled DNA Synthesis (UDS)** | | | |
| Control | 4704 | 4743 | N.S. |
| MMS | 6603 | 7625 | +52 |
| UV | 9745 | 13044 | +65 |
| **$O^6$-Methylguanine-Acceptor Protein Activity (MGAP)** | | | |
| Control | 0.37 | 0.64 | +73 |

Skin cells were taken from Brown-Norway X F-344 male rats (UDS) or Brown-Norway males (MGAP). Numbers are counts per million cells. Methods are in Lipman *et al.* (1989) from which table was adapted.

**Table 10.** Circadian rhythm, molecular parameters and body temperature

| HALO | Parameter | Ad Libitum | Restricted | Change |
|---|---|---|---|---|
| 1 | TEMP | 36.4 | 34.0 | -2.4 |
| | MGAP | 0.2 | 0.2 | N.S. |
| | C-MYC | 1.0 | 0.4 | -60% |
| 12 | TEMP | 37.2 | 35.6 | -1.6 |
| | MGAP | 0.28 | 0.35 | +25 |
| | C-MYC | 1.8 | 1.35 | -25 |
| 20 | TEMP | 37.5 | 36.7 | -0.8 |
| | MGAP | 0.34 | 0.46 | +35 |
| | C-MYC | 1.6 | 0.4 | -75 |

HALO is hours after lights on (12/12 cycle). Temperature is in degrees Celsius, O6-methylguanine acceptor protein (MGAP) is in femtomoles per microgram DNA, c-*myc* expression is as percentage of a total experimental mean. Methods for MGAP measurement is in Lipman *et al.* (1989), methods for c-*myc* expression is in Nakamura *et al.* (1989). This table was adapted from those studies. $B6C3F_1$ mice were used.

change in respiratory quotient. When the diurnal variation in body temperature is compared to the change in DNA repair (Table 10), it is evident that time of day and repair are highly correlated and temperature may plan a role. Consequently, the time of day (and body temperature) is critical to comparing CR and *ad libitum* animals, a consideration that was not evaluated in the Woodhead, *et al.* study.

The mechanism by which CR enhances DNA repair is completely unknown, as is the mechanism responsible for the correlation to body temperature. Interestingly, it has been previously reported that hyperthermia results in a lowered DNA repair capacity (22), suggesting that repair may be inversely proportional to temperature. It is our belief that the ability to stimulate DNA repair by nutritional modulation has some exciting possibilities in mitigating the negative effects associated with exposure to genotoxic agents.

## Cellular Replication and Gene Expression Alterations

Cellular replication and the expression of genes associated with cellular proliferation are a prerequisite for tumor formation. Studies measuring cell replication during CR are surprisingly absent, especially since it has been assumed, based on the work of Tannenbaum (23), that promotion is the stage most affected by CR. It is almost dogma that cellular proliferation is an important part of promotion and that CR effects proliferation and hence promotion. Weindruch and Walford (4) have demonstrated a greatly decreased number of cells in the immunological system with CR. However, this small number may not mean that the turnover is less, rather it may simply indicate that the setpoint, the difference between turnover birth and death may be different. Prabhu, *et al.* (24) measured oral labial mucosal cells and during CR, and found no difference as a result of diet. Preliminary observations in our lab show little change in the proliferative rate of the cells in the liver or bone marrow with CR. This, combined with the results described above for aflatoxin, which indicated that initiation was greatly effected by CR, indicate that, although it has been assumed that CR effects tumorigenesis by altering cellular proliferation, little current information supports this assumption.

Although proliferation may not be effected, the induction of changes consistent with tumorigenesis may be. One such biomarker is an oncogene, c-myc. c-Myc, like other oncogenes, is associated with cellular proliferation control. This is indicated by the inhibition of expression seen in c-myc with CR (Table 10). c-Myc oncogene expression appears to be temperature dependent (Table 10) (25).

Such a decrease in expression may also be related to the ability to withstand viral challenge referred to above. Similar decreases may occur in the expression of the viral genomes, which is necessary for their action, although we have no data on this parameter.

## Physiological and Behavioral Alteractions

Little is known above the primary mechanisms by which caloric restriction interacts with environmental factors that relate to disease, longevity, and toxicity (26). To study these endpoints, behavioral measures, such as, food and water consumption, the number of feeding and drinking episodes, gross motor activity and physiological

measures, such as oxygen consumption, carbon dioxide production, respiratory quotient (RQ), and body temperature, were continuously and concurrently measured over a 10-day interval in Fischer 344 rats and B6C3F$_1$ mice that were fed *ad libitum* or fed a caloric restricted diet (60% of *ad libitum*) (21, 27). Average daily averages for behavioral and physiological variables in rats and mice are given in Table 11-14, respectively (21, 28).

Three different feeding protocols (daytime feeding (daily), nighttime feeding (daily) and alternate day feeding) were used to determine if the timing of various rhythms was synchronized to the photoperiod cycle, the feeding cycle, or a combination of these environmental factors. The results of this study indicate that: 1) calorically restricted animals eat fewer meals but consume more food per meal and spend more time eating than *ad libitum* animals, suggesting that the timing and duration of meals can alter physiological performance; 2) caloric restriction significantly lowers average body temperature and increases the amplitude of the body temperature rhythm; and 3) caloric restriction increases motor activity with no increase in metabolic rate, indicating greater metabolic efficiency. The fact that restricted animals express periods of circadian torpor (low body temperature and metabolism), concurrently with low RQ, suggest that rapid changes in metabolic pathways, from carbohydrates to fatty acid metabolism, may trigger a more energy efficient form of metabolic regulation (21, 27).

Nighttime feeding was found to synchronize physiological performance between *ad libitum* and caloric restricted animals better than daytime feeding. Restricted rats and mice responded differently to daytime feeding (photoperiod cycle 180° out of phase with feeding cue). Rats expressed a bimodal circadian pattern with absorptive behavior (food consumption) synchronized to the feeding cue and spontaneous behavior synchronized primarily to the photoperiod rhythm. In mice, however, the timing of nearly all of the variables measured was shifted to synchronize with the presentation of food (28).

Three different biological rhythms (48, 24 and 12 hour) were identified in mice adapted to alternate day restricted feeding. For physiological variables related to energy metabolism, the 48 hour rhythm was the most significant component, indicating that they were synchronized to the feeding cue, whereas for behavioral parameters, such as, motor activity, the 24 and 12 hour components were more significant than the 48 hour rhythm, indicating that they were synchronized primarily to the photoperiod rhythm.

Moreover, as mentioned above, core body temperature and oxygen were highly corrlated to *c-myc* protooncogene expression (16) and the rate of DNA repair (24), suggesting that biological mechanisms which modulate energy expenditure may also regualte aging and disease processes at the molecular level.

These results provide useful chronobiological markers and baseline data to be used in pharmacology and therapeutics. For example, clinical patients, who are on a restricted dietary regimen, should be given drugs that effect metabolism, or thermoregulation, according to a schedule that is synchronized to the feeding cycle (hours after feeding), and therapeutic regimens that relate to behavioral performance should be synchronized to the photoperiod cycle (hours after light on) (28).

**Table 11.** Fischer 344 male rats-results of T-test analysis-means and standard errors for various physiological measures

| | *Ad libitum* Group Mean + S.E. | LF Restricted Group Fed During Light Period Mean + S.E. | Percent of *Ad Lib* | DF Restricted Group Fed During Dark Period Mean + S.E. | Percent of *Ad Lib* | Significance Levels for Various Comparisons | | |
|---|---|---|---|---|---|---|---|---|
| Average body temperature/day (group average) | 37.40 ± .12°C | 36.58 ± .19°C | 97.81 | 36.485 ± .24°C | 97.55 | A** | A** | |
| Average body temperature/day (group range) | 37.65-37.00°C (.65°C) | 37.25-34.99°C (2.26°C) | 347.69 | 37.17-36.22°C (.95°C) | 146.15 | NA | NA | NA |
| Average body temperature/hr (24 hr range) | 38.02-36.81°C (1.21°C) | 37.38-35.82°C (1.56°C) | 128.92 | 37.74-35.92°C (1.81°C) | 149.59 | A* | B** | C* |
| Average total activity/day | .1705 ± .0053 mv sec.$^{-1}$ | .1959 ± .0072 mv sec.$^{-1}$ | 111.92 | .2059 ± .0068 mv sec.$^{-1}$ | 120.76 | A* | B** | |
| Average oxygen consumption/day (per rat) | 463.34 ± 21.84 *ml* hr$^{-1}$ | 308.83 ± 8.31 *ml* hr$^{-1}$ | 66.65 | — | — | A*** | | |
| Average $CO_2$ production/day (per rat) | 417.65 ± 25.98 *ml* hr$^{-1}$ | 279.91 ± 6.87 *ml* hr$^{-1}$ | 67.04 | — | — | A*** | | |
| Average oxygen consumption/day (gm$^{-1}$ LBM) | 1.354 ± .064 *ml* gm$^{-1}$hr$^{-1}$ | 1.373 ± .037 *ml* gm$^{-1}$hr$^{-1}$ | 101.40 | — | — | | | |
| Average respiratory quotient/hr (24 hr range) | .911-.883 (.028) | 1.005-0.883 (.172) | 614.28 | — | — | A*** | | |

A = *Ad libitum* × LF restricted comparison (significant effect), B = *Ad libitum* × DF restricted comparison (significant effect), C = LF restricted × DF restricted comparison (significant effect). * = P< .05, ** = P< .01, *** = P< .001. LBM = Lean body mass. NA = Not applicable.

**Table 12.** Fischer 344 male rats-results of T-test analysis-means and standard errors for various behavioral measures

| | *Ad libitum* Group Mean + S.E. | LF Restricted Group Fed During Light Period Mean + S.E. | Percent of *Ad Lib* | DF Restricted Group Fed During Dark Period Mean + S.E. | Percent of *Ad Lib* | Significance Levels for Various Comparisons | | |
|---|---|---|---|---|---|---|---|---|
| Total food consumption/day | 16.324 ± 0.745 gm. | 10.554 ± 0.064 gm. | 64.65 | 10.165 ± 0.046 gm. | 62.27 | A*** | B*** | C*** |
| Food Consumption (gm$^{-1}$ LBM) | 0.048 ± 0.002 gm. | 0.047 ± 0.0003 gm. | 97.92 | 0.045 ± 0.0002 gm. | 93.75 | | | |
| Total water consumption/day | 16.156 ± 0.910 gm. | 14.170 ± 0.712 gm. | 87.71 | 14.796 ± 0.246 gm. | 91.58 | | | |
| Water consumption (gm$^{-1}$ LBM) | 0.047 ± 0.003 gm. | 0.063 ± 0.0031 gm. | 134.04 | 0.066 ± 0.001 gm. | 140.42 | A*** | B*** | |
| Average food consumption/episode | 1.503 ± .089 gm. | 3.900 ± 0.269 gm. | 259.48 | 2.216 ± 0.291 gm. | 147.44 | A*** | B* | C*** |
| Average water consumption/episode | 1.693 ± 0.236 gm. | 1.564 ± 0.122 gm. | 92.38 | 1.433 ± 0.069 gm. | 84.64 | | | |
| Number feeding episodes/day | 11.221 ± 0.631 | 3.197 ± 0.526 | 28.49 | 5.650 ± 0.321 | 50.35 | A*** | B*** | C*** |
| Number drinking episodes/day | 11.649 ± 1.476 | 9.880 ± 0.893 | 84.81 | 10.992 ± 0.585 | 94.00 | | | |
| Average time active/feeding episode | 8.636 ± 0.384 min. | 25.993 ± 1.570 min. | 300.98 | 15.967 ± 0.854 min. | 184.89 | A*** | B*** | C*** |
| Average time active/drinking episode | 4.896 ± 0.201 min. | 4.518 ± 0.164 min. | 92.28 | 5.786 ± 0.697 min. | 118.18 | | | |
| Total time feeding/day | 100.571 ± 4.159 min. | 72.706 ± 3.291 min. | 72.29 | 88.967 ± 4.370 min. | 88.46 | A*** | | C** |
| Total time feeding/day (gm$^{-1}$ food) | 6.161 ± 0.144 min. | 6.888 ± 0.317 min. | 111.80 | 8.752 ± 0.619 min. | 142.05 | | B** | C** |
| Total time drinking/day | 60.883 ± 7.817 min. | 46.931 ± 4.285 min. | 77.08 | 66.494 ± 6.297 min. | 109.22 | | | C* |
| Total time drinking/day (gm$^{-1}$ water) | 3.768 ± 0.438 min. | 3.312 ± 0.717 min. | 87.90 | 4.490 ± 0.550 min. | 119.16 | | | C* |
| Total food consum/total water consum × 100 | 101.040 ± 0.827% | 74.481 ± 0.388% | 73.71 | 68.701 ± 1.252% | 67.99 | A*** | B*** | |

A = *Ad libitum* × LF restricted comparison (significant effect), B = *Ad libitum* × DF restricted comparison (significant effect), C = LF restricted × DF restricted comparison (significant effect). * = P< .05, ** = P< .01, *** = P< 0.001. LBM = Lean body mass.

**Table 13.** $B_6C_3F_1$ male mice-results of T-test analysis-means and standard errors for various behavioral measures

| Measurement | *Ad Libitum* Group Mean ± SE | LF Restricted Group Mean ± SE | Percent of *Ad Lib* | DF Restricted Group Mean + SE | Percent of *Ad Lib* | Significance Levels for Various Comparisons AL vs LF | AL vs DF | LF vs DF |
|---|---|---|---|---|---|---|---|---|
| Total food consumption/day (g) | 5.21 ± 0.10 | 3.35 ± 0.02 | 64.3 | 3.41 ± 0.08 | 65.5 | A*** | B*** | — |
| Food consumption ($g^{-1}$ LBM) (g) | 0.193 ± 0.004 | 0.153 ± 0.001 | 79.3 | 0.132 ± 0.001 | 68.4 | A* | B** | — |
| Total water consumption/day (g) | 3.64 ± 0.38 | 5.15 ± 1.07 | 141.5 | 3.72 ± 0.07 | 102.2 | — | — | — |
| Water consumption ($g^{-1}$ LBM) (g) | 0.135 ± 0.014 | 0.235 ± 0.149 | 174.1 | 0.170 ± 0.003 | 125.9 | A* | — | — |
| Average food consumption/episode (g) | 0.32 ± 0.01 | 1.20 ± 0.09 | 375.0 | 0.81 ± 0.02 | 253.1 | A*** | B*** | C* |
| Average water consumption/episode (g) | 0.33 ± 0.03 | 0.57 ± 0.13 | 172.7 | 0.41 ± 0.01 | 124.2 | A*** | — | — |
| Number feeding episodes/day | 16.51 ± 0.69 | 2.80 ± 0.23 | 17.0 | 4.23 ± 0.37 | 25.6 | A*** | B*** | C* |
| Number drinking episodes/day | 11.13 ± 1.28 | 9.03 ± 0.44 | 81.1 | 8.99 ± 0.67 | 80.8 | — | — | — |
| Average time active/feeding episode (min) | 15.94 ± 3.01 | 58.85 ± 4.18 | 369.2 | 59.87 ± 11.34 | 375.6 | A*** | B*** | — |
| Average time active/drinking episode (min) | 7.49 ± 1.90 | 9.40 ± 0.66 | 125.5 | 8.01 ± 1.34 | 106.9 | A* | — | — |
| Total time feeding/day (min) | 246.88 ± 29.68 | 164.78 ± 2.81 | 66.7 | 253.21 ± 37.77 | 102.6 | A* | — | C* |
| Total time feeding/day ($g^{-1}$ food) (min) | 47.42 ± 2.40 | 49.20 ± 0.71 | 103.8 | 74.31 ± 11.09 | 156.7 | — | B*** | C* |
| Total time drinking/day (min) | 83.33 ± 12.79 | 84.80 ± 2.72 | 101.8 | 72.03 ± 5.29 | 86.4 | — | — | — |
| Total time drinking/day ($g^{-1}$ water) (min) | 22.92 ± 3.49 | 18.81 ± 2.84 | 82.1 | 20.00 ± 2.98 | 87.3 | — | B*** | — |
| Total food consumption/total water consumption × 100 (%) | 143.21 ± 9.52 | 65.07 ± 21.20 | 45.4 | 91.68 ± 14.00 | 64.0 | A** | B* | — |

A = AL × LF restricted comparison (significant effect), B = AL × DF restricted comparison (significant effect), C = LF restricted × DF restricted comparison (significant effect). * = $P < .05$, ** = $P < .01$, *** = $P < .001$, AL = *Ad libitum*. LF = Restricted group fed during light period, DF = Restricted group fed during dark period, LBM = Lean body mass

**Table 14.** $B_6C_3F_1$ male mice-results of T-test analysis-means and standard errors for various physiological measures

| Measurement | *Ad Libitum* Group Mean ± SE | LF Restricted Group Mean ± SE | Percent of *Ad Lib* | DF Restricted Group Mean + SE | Percent of *Ad Lib* | Significance Levels for Various Comparisons AL vs LF | AL vs DF | LF vs DF |
|---|---|---|---|---|---|---|---|---|
| Average body temperature/day (group average) (°C) | 36.78 ± 0.08 | 35.54 ± 0.15 | 96.6 | 35.11 ± 0.18 | 95.5 | A*** | B*** | — |
| Average body temperature/hr (max-min) (24h range) (°C) | 37.98 – 35.81 | 38.15-32.24 | | 37.52-32.93 | | | | |
| | (2.18) | (5.91) | 271.1 | (4.59) | 210.6 | A*** | B*** | — |
| Average activity/day (pulse/hr) | 10.54 ± 2.30 | 18.26 ± 1.71 | 173.2 | 26.50 ± 5.57 | 251.4 | A* | B** | — |
| Average $O_2$ consumption/day (g LBM) ($ml/g^{-1}hr^{-1}$) | 3.34 ± 0.16 | 3.44 ± 0.16 | 103.0 | 3.19 ± 0.06 | 95.5 | — | — | — |
| Average $CO_2$ consumption/day (g LBM) ($ml/g^{-1}hr^{-1}$) | 3.05 ± 0.15 | 3.03 ± 0.17 | 99.3 | 2.83 ± 0.12 | 92.8 | — | — | — |
| Average RQ/day | 0.91 ± 0.01 | 0.88 ± 0.01 | 96.7 | 0.89 ± 0.02 | 97.8 | A*** | B* | — |
| RQ variation/day (max-min) (24-h range) | 0.95 ± 0.86 | 0.99- 0.80 | | 1.01- 0.77 | | | | |
| | (0.10) | (0.20) | 200.0 | (0.24) | 240.0 | A*** | B*** | — |

A = AL × LF restricted comparison (significant effect), B = AL × DF restricted comparison (significant effect), C = LF restricted × DF restricted comparison (significant effect). * = $P < .05$, ** = $P < .01$, *** = $P < .001$. AL = *Ad libitum*, LF = Restricted group fed during light period, DF = Restricted group fed during dark period, LBM = Lean body mass

## CONCLUSION

Caloric restriction is a paradigm which significantly alters many molecular, physiological, and biochemical parameters. Such nutritional modification may be important to dissect out the relationship of nutrition and toxicity and identify appropriate biomarkers of both carcinogenesis and aging. Integrated projects, such as the one described above, are useful in attacking complex integrated phenomena, such as those that underlie the biodynamics of degenerative disease processes.

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