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Special Issue: Caloric Restriction and Restrictive Diets: Interventions that Target the Biology of Aging: Original Article

Caloric Restriction Study Design Limitations in Rodent and Nonhuman Primate Studies

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Abstract

For a century, we have known that caloric restriction influences aging in many species. However, only recently it was firmly established that the effect is not entirely dependent on the calories provided. Instead, rodent and nonhuman primate models have shown that the rate of aging depends on other variables, including the macronutrient composition of the diet, the amount of time spent in the restricted state, age of onset, the gender and genetic background, and the particular feeding protocol for the control group. The field is further complicated when attempts are made to compare studies across different laboratories, which seemingly contradict each other. Here, we argue that some of the contradictory findings are most likely due to methodological differences. This review focuses on the four methodological differences identified in a recent comparative report from the National Institute on Aging and University of Wisconsin nonhuman primate studies, namely feeding regimen, diet composition, age of onset, and genetics. These factors, that may be influencing the effects of a calorie restriction intervention, are highlighted in the rodent model to draw parallels and elucidate findings reported in a higher species, nonhuman primates.

Keywords: Calorie restriction—Dietary restriction—Monkey—Rodent—Translational

The first research demonstrating that diet influences the rate of development or aging was done in 1915 (1). At nearly the same time in history, dietary intake was found to influence the incidence of cancer (2). These early findings have since been corroborated in many species (for comprehensive review, see Masoro (3); Speakman, Hambly (4); Spindler (5)), most notably by McCay's group in 1935 (recently republished as McCay, Crowell, Maynard (6)). For a time, the effect of diet on aging was thought to be universal, since the effect was observed in subjects as diverse as yeast, spiders, and rats. In short, caloric restriction (CR) came to be thought of as the gold standard for life span extension. However, recent work has led to a re-examination of this hypothesis. In fact, typical control groups, the environment of the subjects, the administration of the restriction as a fixed percentage of ad libitum (AL) intake, and factors coincident to the restriction, may have played a role in obscuring parts of the story. Thus, the generalization that CR is universally successful in slowing the rate of aging has been successfully challenged; making clear that not all CR studies are the same. In this review, we evaluate four specific methodological considerations, which may have contributed to differences in longevity outcomes, reported in two longitudinal studies of CR in monkeys (7).

Primate Study Outcomes

Investigators at the National Institute on Aging (NIA) Intramural Research Program and the University of Wisconsin (UW) have published extensively on their respective longitudinal studies of CR in rhesus monkeys that have been ongoing for over three decades. Their findings have been mostly positive, demonstrating beneficial effects of CR in a long-lived mammal with 93% genetic homology to humans. However, there is one crucial outcome difference between the two studies that has generated significant debate. At UW, CR leads to healthspan and survival extension (8); at NIA,

there is only healthspan extension (9). Any maximal life-span effects of CR have yet to be determined as these studies are still ongoing.

It would be convenient to suggest that one study was right and the other flawed and thus put an end to the debate on whether CR works in primates. However, the story is deeper than that, and data from the rodent world suggests that it has always been this way. A recently published comprehensive comparison of the two nonhuman primate (NHP) studies highlights the differences in study design that likely influenced the divergent outcomes (7). These differences in study design are evident in each of the following areas: (a) feeding regimen, (b) diet composition, (c) age of onset, and (d) genetic differences (Table 1). Here, it should be noted that these methodological considerations are merely the narrow focus of this review and by no means represent an exhaustive list of contributing factors. However, only by examining the unique study design features highlighted in the primate studies and further illustrated in rodent models, we can accurately interpret the outcome and ultimately identify the relevant biological mechanisms.

Terminology

The terms dietary restriction (DR) and CR have often been interchanged to mean the same thing, encompassing a wide variety of nutritional interventions. However, as the field has advanced in the last several decades, a distinction has emerged warranting more precise language. The early studies of McCay and colleagues (10) reported life extension in rats following a reduction in the total number of calories consumed, thus the term CR. The studies did not manipulate the dietary components; it was strictly a reduction in food ration. Several decades later, as rodent diets were manipulated to exert precise control on the contents, experimental restriction took on the more general name of DR to reflect the broader context and varied approaches used to manipulate the nutritional deficit and, technically, it encompasses the term CR. To reduce confusion, CR will be used to describe all study types in this review.

Table 1. Comparison of Methods: NIA and Wisconsin NHP CR Studies

Study Attribute		NIA	UW
Animal origin/ genetics		Indian & Chinese	Indian
Age of onset			
	Males (age range)	Early- (1 – 5 yrs), Old- (>15 yrs)	Adult- (6 – 14 yrs)
	Females (age range)	Early- (1 – 5 yrs), Adult- (6 – 14 yrs), Old- (>15 yrs)	Adult- (6 – 14 yrs)
Control ration basis		Published standards	Individual baseline intake
Feeding practices			
	Meals per day	2	1
	Overnight access	Yes	No
Feeding regimen		Limited daily	Limited daily
Source of diet		Natural	Semi-purified
	Fat	Soy, corn, fish oils	Corn oil
Nutrient content			
	Sucrose (% of total carbs)	< 7	45

Methodological characteristics of the NIA and Wisconsin longitudinal calorie restriction studies in nonhuman primates (48,49).

Defining the Control Group

The control group often used in CR studies is termed AL, which is Latin for without limit. And so, animals in a true AL condition have unlimited access to food, allowing for self-regulated intake. It is this self-regulation of food intake that calls into question the comparability of an AL control group in CR studies. With unlimited access to food, some strains of rodents will tend to overeat. As a consequence and not surprisingly, true AL fed rodents are more sedentary, obese, and glucose intolerant compared to their CR counterparts (11). For these reasons, their suitability as controls for any model, other than obesity, has been called into question (12). As an alternative, a second control group in which the animals are placed on a mild restriction, conventionally considered to be around 10% CR, could be used. However, this option is less frequently used as having both types of control subjects is too costly to be feasible for most labs. Nevertheless, it is difficult to distinguish either option as the "right" control.

Data from the National Health and Nutrition Examination Survey, 2011–2012, indicate that more than two-thirds of adults are overweight or obese (body mass index [BMI] > 25), and this outcome is on an upward trajectory (13). If it is argued that the control group for a restriction study should be representative of the average constituent in the interested population, then it may be that AL-fed mice best represent the U.S. population. This is a crucial factor when interpreting restriction study outcomes. For example, to determine whether improvements are related to the beneficial effects of CR, or, if they reflect a control group that is compromised due to a poor health status.

Feeding Regimens

The classic CR paradigm has expanded over time and now several feeding regimens are included under the general category of CR. For instance, limited daily feeding (LD) and intermittent fasting (IF) are two forms of CR that are based on the frequency with which meals are provided. Within the categories of LD and IF, there exists a wide variety of feeding paradigms based on experimental manipulation of meal timing, meal volume, and overall caloric intake. These two variants of CR have been successful, at one time or another, at extending life span in mice and other species.

Limited Daily Feeding

Limited daily feeding is a DR feeding regimen in which the subject receives a daily allotment of food that is a fixed percentage (generally 10%–40%) less than a predetermined baseline allotment. It is worth noting that while this procedure provides a daily aliquot of food, it does not control for the portion of the day that the food is available. Thus, the subjects may consume the meal quickly, in which case they would remain without food for the rest of the day (a characteristic that is typical of IF) (14).

Intermittent Fasting

Intermittent fasting is a broad term used to describe any feeding protocol in which periods of access to food are interspersed with periods entirely lacking access to food. IF paradigms include: (a) every other day feeding/fasting, in which 24 hours of access to food is alternated with 24 hours without access and (b) time-restricted

feeding in which there is a period of free access to food in each circadian cycle but only for a defined amount of time (15,16). IF regimen must be employed cautiously because, depending on the length of time fasted, it can lead to gorging behavior in some species, resulting in caloric intake that is unchanged relative to control levels (17).

As can be inferred from the plethora of terms, the initial finding that diet influences aging and that restriction retards it, led to many diverse approaches for exploration. While this has resulted in a deep and rich pool of information, it has also been the cause of some confusion. The actual caloric intake, the ratio of time spent in fasting or fed states, and the influence of macronutrients have the potential to confound CR studies, and as a result, the superficial interpretations of many studies may well be incorrect. A reanalysis of historic studies and a carefully planned set of experiments to explore the roles of the variables described earlier are warranted for further clarity of these issues.

Differing Responses to Feeding Regimens

As noted in Anson and colleagues (18), the ability of the various CR paradigms to improve healthspan and extend life span provides opportunities for discerning the mechanisms underlying the modulation of aging rate by CR. For example, a cell's circadian clock components regulate metabolic efficiency, changing in response to dark and light cycles, food intake, and other stimuli from the body and the environment. A design that alters the time of day at which an animal is fed allows us to determine if circadian rhythm and its proponents play a role in the mechanism of CR.

Diet Composition

CR study outcomes are influenced, not only by feeding frequency, timing, and volume but also by the amount of protein, carbohydrate, and fat that are combined to create the diet (19). The composition of the diet affects both the nutrient content and palatability, the latter likely playing a much larger role in rodent studies. In fact, unpalatable food may result in a self-imposed restriction in an animal study and ultimately compromise the integrity of the intervention. Here, we describe a few of the variations in macronutrient composition of rodent diets, which were also apparent in the NHP studies and may affect physiological processes and ultimately healthspan and life-span outcomes (20–22).

Natural Versus Purified Rodent Diets

In general, diets given to laboratory animals are composed of either natural or purified ingredients. A natural diet is one that is formulated using agricultural products and byproducts, such as whole grains or high-protein meals (soybean meal or fishmeal) (23). Conversely, a purified diet is formulated with a more refined and restricted set of ingredients, such as sugar and starch (carbohydrates) or casein and soy (protein). Natural diet ingredients vary from batch to batch because the nutritional content fluctuates depending on the harvest location and growing season. On the other hand, purified diets are identical from batch to batch. This consistency can be achieved because the ingredients are refined and, each ingredient then, contains a single nutrient or nutrient class. For this reason, purified diets limit the chance that an uncontrolled variable could affect experimental results more so than natural diets. However, natural diets are generally a more complete source of nutrition compared to purified diets.

Macronutrient Source

Carbohydrates

Carbohydrates generally provide the largest percentage of macronutrients in the diet and provide most of the energy. Early work aimed at addressing dietary components for long-term studies showed a 10% reduction to life span in rats consuming a sucrose-based diet compared to cornstarch (24). Not surprisingly, another early report showed a reduction in life span when mice consumed 20% of their calories as glucose (25). In fact, physiological outcomes will vary depending on which sugar is primarily consumed and these outcomes can persist even under CR conditions (26).

Protein

Soy is the main protein source in most natural ingredient diets and is one of the richest sources of isoflavones, a polyphenolic compound classified as a phytoestrogen, plant derived compounds with estrogenic activity. The earliest standardized purified diet, AIN-76A was manufactured with casein protein, the most abundant protein in milk and phytoestrogen-free. Reformulations of the AIN-76A diet, now labeled as AIN-93, increased the soy component and are considered a better choice for long-term studies (27).

Despite the now widespread use of soy in rodent diets, it is not universal so it is important to recognize the physiological and behavioral effects of this phytoestrogen and the potential to influence study outcomes. The physiological effects of soy isoflavones tend to be beneficial and include protection from renal disease (28) and prostate cancer (29), and reductions in adipose tissue and anxiety (30). In combination with a high fat diet, soy protein isolate reduced plasma insulin and markers of inflammation as compared to casein (31). In another study, the replacement of casein with soy protein resulted in reduced nephropathy and increased median life span in Fischer 344 rats (32). In fact, some evidence suggests that, in rodents and insects, alterations in the protein content of a diet affect the lifespan benefits of CR (19). Taken together, these results indicate that the source and content of dietary protein can influence experimental outcomes, particularly when the aim is to investigate the impact of CR on healthspan and life span.

Fat

Dietary fat sources can also impact health outcomes, particularly in longitudinal studies. In fact, the omega-6 polyunsaturated fatty acid, linoleic acid, is reported to have pro-inflammatory effects, and an increased omega-6: Omega-3 ratio has been associated with increased risk for cardiovascular disease, rheumatoid arthritis, osteoarthritis, periodontal disease, and cancer (33,34). However, high-fat diets high in plant based oils, like flaxseed or safflower, were associated with increased bone strength and density, lower body weight, and extended life span (34,35).

Considering the evidence presented here regarding the potential effects of the various macronutrients assembled to make a diet, it appears that the story can get complicated very quickly. And, there are additional study design elements we have yet to consider.

Age of Onset

The point within the life cycle in which CR is initiated can affect study outcomes across species and is therefore a critical methodological consideration. In the laboratory, mice are weaned at 3–4 weeks of age and considered mature by approximately 6 weeks, with an optimal age of reproduction between 2 and 10 months (36). Typically, longitudinal

rodent CR studies are initiated just after weaning and maintained throughout the life span, thus affecting development throughout adulthood. However, adult- and old-onset studies introduce CR later in life. Adult-onset studies generally refer to instances in which CR is initiated after 6 months, but before 20 months of age, while old-onset studies are initiated when animals are greater than 20 months of age. Here, we provide examples of how the age at which CR is initiated affects life span and healthspan outcomes in murine models of aging.

Early-Onset CR (Weaning—6 Months)

In rodent models, there is contradictory evidence regarding the benefits of CR initiated during early life. For example, Weindruch and colleagues (11) found that 55% or 65% CR started at or before weaning increased mean and maximum life span of long-lived female F1 hybrid mice (B6C3F1) but did not improve all healthspan measures. Among mice with lymphoma, longevity was increased by 8–12 months, though early-onset CR did not reduce overall tumor incidence for restricted versus control mice. Conversely, Cameron and colleagues (37) found that 12 months of early-onset CR provided healthspan and life-span benefits to C57BL/6 mice. Here, short-term CR improved fasting insulin levels, glucose tolerance, and body mass in males; health improvements which were maintained even after an AL diet had resumed for several months. In females, CR provided life-span benefits but these only persisted if CR was maintained. In contrast to the Weindruch and colleagues' (11) female F1 hybrid mice, in C57BL/6 mice, long-term CR reduced tumor incidence in females and postponed tumor onset in males (37).

Adult-Onset CR (6–20 Months)

Consistent with early-onset studies, healthspan and life-span outcomes vary across rodent models of adult-onset CR. Adult B10C3F1 and C57BL/6J mice on a CR diet showed a 10% to 20% increase in maximum life span and had reduced incidence of lymphoma (38). After only 2 months, adult-onset CR resulted in life-span benefits for B6C3F1 mice, whose remaining time to death increased by 42% compared to controls (39). Conversely, 33% CR initiated in 18-month-old rats did not significantly impact median life span compared to controls (40). With such widespread differences, it is difficult to ascertain whether these study outcome variations are due to genetics, sex, age, or some other variable, or perhaps a combination of them all (41).

Old-Onset CR (>20 Months)

Studies of CR initiated in older mice (>20 months of age) demonstrate less life-span benefits compared to CR initiated earlier in life. For example, Forster and colleagues (42) reported that 40% CR in 24-month-old mice increased mortality in three strains commonly used in research (ie, C57BL/6, DBA/2, and B6D2F$_1$), with the increase in mortality being most pronounced in the DBA/2 strain. Conversely, old-onset CR rapidly reversed aging associated cardiomyopathy and, after 2 months of CR, the aging hearts of old mice were indistinguishable from those of young mice (43). Old-onset CR also ameliorated the age-dependent loss of cognitive and motor skills (44).

Genetics

The numerous transgenic and knockout strains available in murine species have provided the opportunity to explore how genetic factors modulate the longevity- and health-promoting effects of CR. The great variability observed in survival rates between strains of mice demonstrates that the effects of CR are not universal (45). For example, long-term CR resulted in an increase in median and maximum life span in C57BL/6 and B6D2F1 mice but had no effect on DBA/2 mice (42). Moreover, when 41 recombinant inbred mouse strains were given a 40% CR diet, it was reported to decrease life span in more strains than it benefited (46). Supporting the assertion that life span and healthspan effects of CR are notably associated with genetic determinants. The recent publication by Mitchell and colleagues (45) highlights the complicated relationship between genetics and CR response with additional complexity when sex and level of restriction are considered.

Transferring Design Considerations to NHP Studies

It is apparent that, even in rodent studies that employ inbred, genetically homozygous animals, the effects of CR on healthspan and life span are on a continuum. Even the slightest variation in study design can profoundly alter experimental outcomes (47), demonstrating that the effects of CR are not universal and absolute. This methodological limitation, present in all life-span studies, is exemplified in the longitudinal studies in NHPs at the NIA and UW, each designed to test the CR intervention in a long-lived species with aging characteristics similar to humans.

Although the two studies were implemented independently (NIA in 1987 and UW in 1989), and without the intent to be replicative, it was natural to make comparisons and expect that the outcomes might answer the question about translation of CR to humans. In 2009, UW reported that healthspan and age-related survival effects were improved in monkeys that had been on CR for 20 years (8). Yet, rather than confirming this finding and solidifying the beneficial effects of CR in monkeys, the NIA reported only a marginal increase in healthspan with no increase in survival (9). These apparently disparate findings created a media stir and some backlash within the CR field. The initial response was to declare one study correct and the other flawed, with no universal agreement regarding which study fell into either category. However, if considered in the context of the rodent literature, it may be more appropriate to view each study as answering different questions.

A recently published comprehensive comparison of the two monkey studies highlights the differences in study design that likely influenced the divergent outcomes (7). These differences in study design are evident in each of the following areas: (a) feeding regimen, (b) age of onset, (c) diet composition, and (d) genetic differences (Table 1).

Regarding feeding regimen, neither the NIA nor UW study fits neatly into one category or another. And, control animals were not free-fed and thus, were not AL. However, a version of LD feeding regimen with an element of time-restricted feeding was employed at both sites. All UW monkeys received one allotment in the morning and had no access to food overnight. Whereas, all NIA monkeys received two meals daily, with a limited amount of time to consume the morning portion and the evening portion remaining available overnight.

As is apparent in rodent studies, the age at which the CR is initiated impacts the healthspan and life-span effects of CR. The UW monkeys were all adults when CR was initiated. Whereas, NIA tested CR in young, adult, and old monkeys to determine the effect of age of onset. The smaller group sizes at NIA complicate the interpretation of the results but provide a more comprehensive data set.

Within both NHP studies, the control groups were considered healthy and the macronutrients in the diet were consistent between groups. Thus, within each study the diet composition was not a variable. However, diet composition between the two studies was considerably different. UW's diet was composed of purified ingredients, with lactalbumin as the primary protein source; corn starch, dextrin, and sucrose were the carbohydrate sources. NIA's natural ingredient diet included soybean and fishmeal for protein, and wheat, corn, and sucrose as the carbohydrate source. A primary difference here existed in the quantity of sucrose: at 45% of the carbohydrates at UW but only 6.8% at NIA.

As reported in mice, all strains do not respond to CR alike. From differing effects on body weight to healthspan and life-span effects, no two strains are equal. Rhesus monkeys are far more genetically diverse than mice and greater than 93% genetically like humans. Just as a group of humans of the same sex, age, and family background vary, so do monkeys. Thus, another major point of difference between the two NHP studies is the source of animals for each cohort. UW's study included animals from one facility, all Indian origin; thus, the more homogenous study population. Conversely, the NIA animals were obtained from multiple facilities and were of mixed origin (Chinese and Indian). Because of this, it is difficult to determine the contribution of country of origin and consequential genetic variation on the effects of CR.

As outlined here, the methodological differences between the NIA and UW studies are overtly small yet latently substantial. They preclude any straightforward combination of results to answer one overarching question. Instead, each study should be interpreted independently, with study variables as the basis of the analysis. In uncovering disparate survival outcomes, together these two studies are more informative about the effect of CR and the many influences on survival, than had they come to the same conclusions.

Conclusion

In summary, our initial belief that the rate of aging is directly proportional to caloric intake (with obvious limits at the higher and lower ends of the spectrum) has now been shown to be incorrect. DR works through a variety of mechanisms, as evidenced by the fact that its pro longevity and pro health effects vary based on several modifiable study design factors including: the diet composition, age of onset, feeding regimens, and genetics and sex of the organism. The fact that nutrition influences aging in many animal models is nevertheless valuable, and given our incomplete understanding of aging itself, it continues to provide an avenue of investigation that is not even close to reaching its full potential. A complete understanding of the mechanisms behind CR is imperative in ameliorating the aging process in humans.

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Conflict of Interest

The authors declare that there is no potential conflict of interest.

Author Contributions

All authors contributed to writing and revising the paper.

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