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The effects of dietary restriction on oxidative stress in rodents

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Abstract

Oxidative stress is observed during aging and in numerous age-related diseases. Dietary restriction (DR) is a regimen that protects against disease and extends lifespan in multiple species. However, it is unknown how DR mediates its protective effects. One prominent and consistent effect of DR in a number of systems is the ability to reduce oxidative stress and damage. The purpose of this review is to comprehensively examine the hypothesis that dietary restriction reduces oxidative stress in rodents by decreasing reactive oxygen species (ROS) production and increasing antioxidant enzyme activity, leading to an overall reduction of oxidative damage to macromolecules. The literature reveals that the effects of DR on oxidative stress are complex and likely influenced by a variety of factors, including sex, species, tissue examined, types of ROS and antioxidant enzymes examined, and duration of DR. Here we present a comprehensive review of the existing literature on the effect of DR on mitochondrial ROS generation, antioxidant enzymes and oxidative damage. In a majority of studies, dietary restriction had little effect on mitochondrial ROS production or antioxidant activity. On the other hand, DR decreased oxidative damage in the majority of cases. Although the effects of DR on endogenous antioxidants are mixed, we find that glutathione levels are the most likely antioxidant to be increased by dietary restriction, which supports the emerging redox-stress hypothesis of aging.

Keywords

Dietary restriction; calorie restriction; oxidative stress; reactive oxygen species; antioxidant enzymes; oxidative damage; aging

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Introduction

Denham Harman conceived the first iteration of the free radical theory of aging in 1956 [1]. Harman based his theory on the presumption that lifespan is dependent on metabolic rate – a hypothesis that has since fallen out of favor [2]. He proposed that toxic by-products of metabolism, notably the hydroxyl radical and protonated superoxide, can damage proteins and nucleic acids and lead to cancer and aging. The observations that mitochondria produce the majority of oxygen radicals in the cell and that reactive oxygen species include non-radicals like hydrogen peroxide led to the development of the oxidative stress theory of aging [3]. Oxidative stress results from an imbalance in the rate of reactive oxygen species (ROS) production and detoxification. There is strong support for the oxidative stress theory of aging in invertebrate models, especially *Drosophila*, although data in rodent models is inconclusive as to whether and how oxidative stress affects the aging process [4]. Even more recently, a spinoff of the oxidative stress theory of aging, the redox-stress hypothesis, implicates redox-sensitive signaling pathways in the aging process. The redox stress hypothesis is supported by an oxidizing shift in the cellular redox balance during aging [5–8], which is regulated by the glutathione and thioredoxin systems [9–12]. This redox imbalance can lead to altered protein function and gene transcription [13].

Oxidative stress has conclusively been shown to be associated with aging and age-related diseases, including cancer [14, 15], neurodegeneration [16, 17], cardiovascular disease [18, 19], and diabetes [20]. A number of studies suggest that dietary restriction can protect against these oxidative stress related diseases, including cancer [21], neurodegeneration [22] and cardiovascular disease [23–25]. DR also prevents a number of age-related pathologies, including loss of myenteric neurons [26], hearing loss [27], cataracts [28], insulin resistance [29, 30], and skeletal muscle loss [31, 32]. Although DR is generally thought to reduce oxidative stress, these data are mixed and have not been comprehensively reviewed. Three mechanisms may be responsible for the antioxidant effects of dietary restriction. Specifically, DR could reduce reactive oxygen species production, increase antioxidant enzyme activity or increase the turnover of oxidized macromolecules. These mechanisms are interrelated and often result in confounding results. For example, DR might lead to decreased expression of antioxidant enzymes, but this could be due to reduced production of reactive oxygen species [33]. Here we will review the effects of DR on ROS production, antioxidant enzyme activity and oxidative damage and discuss potential mechanisms for how these effects are achieved.

The following three hypotheses provide a useful framework to test the role of oxidative stress in aging. First, oxidative damage should increase with age; second, manipulations that delay aging should attenuate the age-related change in oxidative damage; and third, specifically modulating the presumptive age determinants in old animals should reverse functional decline. For the first hypothesis, a number of studies have observed increased reactive oxygen species production and oxidative damage, although increased oxidative stress is not universal, as discussed below. For the second hypothesis, dietary restriction, reducing growth hormone and insulin-like growth factor 1 signaling as well as reducing mammalian target of rapamycin signaling with rapamycin are common dietary, genetic and pharmacological interventions, respectively [34–37]. Dietary restriction is the anti-aging

paradigm most commonly used to test the oxidative stress theory of aging, and will be discussed extensively in this review. The third hypothesis has generated the most damning evidence against the oxidative stress theory of aging. Attempts to modulate oxidative stress using mice deficient in or overexpressing antioxidant enzymes do not generally support the oxidative stress theory of aging [38]. Two exceptions to this generalization are the mitochondrially-targeted catalase overexpressing mouse [39], which is long-lived, and the copper zinc superoxide dismutase deficient mouse, which is short-lived [40]. The purpose of this review is not to make a definitive statement on the role of oxidative stress in aging. Rather, our primary objective is to determine the effect of dietary restriction on oxidative stress. As an aside, we will evaluate the effects of DR on oxidative stress during aging.

Dietary restriction is the most well studied aging intervention in rodents, although the effects of DR depend on the extent of restriction, age, sex, species, strain and duration of restriction. Dietary restriction extends lifespan in many, but not all, strains of mice and rats [41, 42]. Protocols for dietary restriction in rodents vary in extent and duration of feeding, with reductions in food from 10–60% and durations from one week to the entire-post weaning lifespan. If a standard protocol exists for long term DR, it would be a gradual reduction in food availability after maturation to 40% relative to animals fed *ad libitum*, and then maintaining this level throughout the study. Some studies use a nutrient supplement for the restricted group, which is considered calorie restriction, while other studies use alternate day feeding or intermittent fasting, in which animals are fed *ad libitum* on some days and fed nothing on others. Although less well studied, alternate day feeding and intermittent fasting produces many, but not all [43–45], of the beneficial effects of DR, including increasing lifespan [41]. The variety of restriction protocols makes comparisons among studies difficult, but in this review we comprehensively surveyed the available literature to search for trends in the effects of dietary restriction on oxidative stress.

Dietary restriction and reactive oxygen species production

For all of our analyses, we included models of DR with differing duration and percent restriction without regard to sex, species or strain. Because mitochondria are a major source of reactive oxygen species (ROS) production in the cell, and because other sources of ROS have been minimally studied with dietary restriction, we only included studies of mitochondrial ROS production. A systematic review of the literature uncovered 157 observations of mitochondrial ROS production using dietary restriction in rodents (Supplemental Table 1) [33, 46–67]. Many reports used isolated mitochondria preparation, saturating concentration of substrates, and ambient oxygen tension (20%), which subjects mitochondria to hyperoxia relative to the *in vivo* environment. Nevertheless, analysis of ROS production in isolated mitochondria is informative as to approximate the maximal production of ROS seen *in vivo* without scavenging. For mitochondrial ROS production, 96% of observations were made in males, with 4% in either female or mixed sex populations, and 63% of observations were in rats. ROS production was more likely to be reduced with dietary restriction in mice (Fisher's exact test, $p < 0.001$). Most studies measured hydrogen peroxide production with the remaining studies measuring superoxide or other, sometimes nonspecific, reactive oxygen species. The distribution of tissues studied reveals that most studies were performed in the liver, heart or skeletal muscle (Table 1). *In*

total, 62% of observed measurements reported no change with DR and 37% reported a decrease in ROS production. Below, we will discuss the effects of DR on ROS production in different tissues and with various electron transport chain substrate and inhibitor combinations.

In Table 2, we have summarized the effects of dietary restriction on ROS production with specific substrate and substrate/inhibitor combinations in all tissues. Substrate combinations like glutamate/malate are not shown due to lack of studies using these substrates. Although there is no difference when comparing complex I linked substrates versus complex II linked substrates without inhibitors, substrate/inhibitor combinations reveal clear differences. When respiring on the complex I linked substrates pyruvate and malate, DR is more likely to reduce ROS production with a complex I inhibitor, and DR is much less likely to reduce ROS production with complex III inhibition. When respiring on the complex II linked substrate succinate, the opposite is true; DR is very likely to reduce ROS production at complex III and much less likely to reduce ROS production at complex I.

In Table 3, we report the overall effects of dietary restriction on ROS production in each tissue. It is interesting to note that the brain showed the most consistent reduction in mitochondrial ROS generation in response to DR. This finding is important due to the role of oxidative stress in neurodegenerative diseases and aging and the protective effects of DR on these conditions [68–73]. Interestingly, the brain was the least responsive to changes in enzymatic antioxidant enzyme activity with DR (discussed below). It may be that because DR is most likely to reduce ROS production in the brain, the need for inducing antioxidant enzymes is reduced. In heart and skeletal muscle, DR was just as likely to have no effect as to decrease ROS production. Interestingly, even though liver is the most commonly studied tissue to date, it is the tissue least likely to show decreased ROS production with dietary restriction.

The effect of DR on antioxidant enzyme activity

We again took an unbiased approach to comprehensively survey the literature on the effects of DR on antioxidant enzyme activity and levels of the redox peptide glutathione in rodents. We included models of DR with differing duration and percent restriction as well as the relatively common model of every other day feeding. There were 372 recorded comparisons (Supplemental Table 2) on the activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) and the levels of glutathione (GSH) [27, 28, 33, 48, 58, 60, 65, 74–104]. Studies of antioxidant enzyme activity are limited by the availability and ease of activity assays. Thus, we found no reports of the effects of DR on methionine sulfide reductase, peroxiredoxin or glutaredoxin activity, protein or mRNA levels. Additionally, isoform specific changes during DR in GPx and to a lesser extent SOD have not been well studied. Only changes in protein or mRNA were found for thioredoxin reductase and the redox protein thioredoxin, which will be discussed separately.

Among the studies on dietary restriction and antioxidant activity, 66% of the measurements were in rats and 83% of measurements were in male animals. On the whole, there were no

differences of the effect of DR on antioxidant activity between species (Chi squared, $p>0.05$). The tissue and enzyme distribution is shown in Table 1; the most studied tissues in order are liver, brain, heart, kidney, skeletal muscle and other. Catalase, SOD and GPx activities each made up about a quarter of the measurements, while GSH levels and GST and GR activities made up the remaining quarter (Table 4). *In total, a majority (53%) of the time no changes were observed in antioxidant activity with dietary restriction, while 38% of the time there was an increase in activity and 8% of the time there was a decrease.*

Below, we discuss the general effects of DR on antioxidant activity and the specific effects in each tissue. We also recorded measurements of antioxidant enzyme mRNA and protein levels which will be discussed separately below. We will summarize the effects of the duration of DR on antioxidant enzymes and glutathione levels as well as the effects of DR on antioxidant activity during aging below.

Table 4 shows the effects of dietary restriction on major antioxidant enzyme activities and glutathione levels, regardless of tissue. These enzymes have been reviewed extensively elsewhere [105–108], so we will only briefly describe their roles in the cell. The antioxidant enzymes work together as part of a complex system to detoxify potentially harmful radicals and ROS. They are generally ubiquitously expressed; however, subcellular compartmentalization is an important part of their ability to regulate cellular protective capacity. A primary line of defense is provided by catalase, a peroxisomal enzyme that catalyzes the reduction of hydrogen peroxide to oxygen and water. Glutathione peroxidase-1, present in both mitochondrial and cytosol, uses glutathione to catalyze the reduction of multiple oxidants, including hydrogen peroxide and fatty acid hydroperoxide. Oxidized glutathione is then reduced by glutathione reductase with the reducing equivalent NADPH. Glutathione S-transferase is involved in detoxification and conjugates glutathione to a number of substrates. Superoxide dismutase is present in the cytosol (CuZnSOD), mitochondria (MnSOD) and extracellular space (EcSOD), and catalyzes the reduction of superoxide anion to oxygen and hydrogen peroxide.

In general, no particular enzyme stands out as being consistently affected by DR. Glutathione levels, which include total glutathione content as well as the ratio of reduced glutathione to oxidized glutathione, depending on the study (when both were available, we included the ratio), were the most likely to change with dietary restriction and increased in the majority of cases. Similarly, glutathione reductase activity was never observed to decrease with DR. These findings support the redox stress theory of aging, for which the glutathione system is a major player. Catalase and glutathione S-transferase activities were the next most likely to change with DR after GSH. In cases where there were changes in these two enzymes, there was a tendency for activity to increase. SOD activity, which includes total SOD activity as well as SOD1 and SOD2 activities, depending on the study, was the least likely to change. Besides being the least likely to change, in cases where SOD activity did change it decreased 39% of the time.

In Table 5, we show the effect of DR on all antioxidant activities and the three most commonly measured enzyme activities (catalase, glutathione peroxidase and superoxide dismutase) in each tissue. There are not enough measurements from other enzymes and

tissues to accurately summarize the effects of DR. These data can be used to draw conclusions about the possible mechanism by which DR protects against disease in each tissue. Regarding liver, it has been the most commonly studied tissue by far (Table 1) and is reflective of general effects of DR on antioxidant activity in that DR had no effect 52% of the time. In the majority of cases, DR increased catalase activity while there was a tendency for SOD activity to be unaffected.

The brain is the second most commonly studied tissue after liver. Measurements of brain antioxidants include studies on whole brain as well as specific brain regions, depending on the experimental design. Brain was the least responsive tissue to the effects of DR on antioxidant activity, with alterations in only 30% of measurements. GPx and SOD activity, in particular, were seldom affected by dietary restriction. Although catalase, GPx and SOD activity have been associated with protection against neurodegenerative conditions [109–113], the observation that DR rarely affects these enzymes suggest that the protective effects of DR are due to other mechanisms, including enhanced neurogenesis, improved neuronal transmission and increased neuronal growth factors [114–116]. Interestingly, although antioxidant enzymes were not likely to increase in the brain with DR, GSH increased in 12 of 14 studies (Supplemental Table 2), suggesting that the beneficial effects of DR in the brain may be due primarily to the glutathione redox system. Glutathione plays a critical role in the brain [117], and disruption of the glutathione system is associated with neurodegeneration and brain aging [118]. The effect of DR on glutathione levels was not as clear in other tissues, suggesting that increased glutathione may be a primary mechanism by which DR protects against neurodegeneration and brain aging.

Heart was the most responsive tissue to dietary restriction, with antioxidant activities changed in 65% of studies. Interestingly, there were a high number of instances where DR decreased antioxidant activity in the heart. Catalase, GPx and SOD activities were reported to decrease at a similar rate. Very interestingly, catalase activity changed with DR in 92% of reports, being increased in 67% of studies. Regardless of the effect, this observation suggests that the heart is highly sensitive to hydrogen peroxide detoxification and has high capacity to regulate catalase activity in response to dietary restriction. Catalase has been shown to be protective in a number of models of cardiac disease [119–122], and DR protects against heart disease and dysfunction [25, 123–125], suggesting that modulation of catalase may be a primary mechanism by which DR protects against cardiac dysfunction.

Antioxidant enzymes are also relatively responsive to DR in the kidneys, with 54% of all observations reporting a change in activity. GPx and SOD activity had relatively high levels of reported decreases in activity (15 and 18%, respectively). Strikingly, catalase activity increased in 78% of studies and never decreased, suggesting that, similar to the heart, dietary restriction elicits a response signaling the need for hydrogen peroxide detoxification in the kidney. Dietary restriction has been shown to protect against kidney disease [126–130], and catalase in particular has been implicated in kidney disease [131–136], suggesting catalase modulation may be a primary mechanism by which DR protects against renal disease.

Skeletal muscle is also a responsive tissue to DR in terms of antioxidant activity. Fifty-five percent of observations reported a change in antioxidant activity. Interestingly, 41% of those

changes were a decrease in antioxidant activity. The activity of specific enzymes was highly variable, with no enzyme having consistent changes with dietary restriction. Other tissues that have been studied with DR and antioxidant activity include diaphragm, erythrocytes, eye, inner ear, intestinal mucosa, lens, pancreas, plasma, tendon and testes. There have been 36 observations published on the effects of DR on these tissues. In general, these “other tissues” tended to be less responsive to DR, with 67% of observations having no effect (versus 55% total), and when there were changes in antioxidant activity there were fewer increases (22% increases in “other tissues” versus 36% in all tissues). Whether this reflects a real lack of responsiveness to DR in most tissues or sampling error will need to be determined by more comprehensively studying the effects of DR in these and other tissues.

Effects of DR on antioxidant enzyme protein and mRNA levels

We found 100 recordings of antioxidant protein or mRNA levels with dietary restriction [78, 79, 90, 91, 94, 96, 100, 137–140]. Included in those measurements are mRNA and protein levels of the same enzymes used for activity assays as well as the redox protein thioredoxin and the redox enzyme thioredoxin reductase. Microarray analyses were not included. Thioredoxins are used to reduce oxidized antioxidant enzymes, including methionine sulfoxide reductases and peroxiredoxins. Thioredoxin reductase then reduces oxidized thioredoxin using the reducing equivalent NADPH. This system is a major player in the redox-stress theory of aging. Both thioredoxin levels, which include thioredoxin 1 and 2 (depending on the study), and thioredoxin reductase levels, which include thioredoxin reductase 1 and 2 (depending on the study), tended to not change at the mRNA level with DR (two out of twelve observations) and either increased or stayed the same at the protein level (ten out of twenty observations). Similar to glutathione levels and glutathione reductase activity, there are no reported decreases in thioredoxin or thioredoxin reductase levels with dietary restriction, which again is supportive of the redox stress theory of aging.

When dietary restriction does affect antioxidant activity, does it do so at the transcriptional, translational or post-translational level? Clearly the answer will depend on the DR paradigm, tissue, enzyme and other variables. Notably, measurements of antioxidant enzyme protein and mRNA levels with DR are much less common than activity, making this question difficult to answer. Rarer still are determinations of the effects of DR on antioxidant enzyme post-translational modifications or coregulators, which may be the major regulator of enzyme activity. However, among 70 recorded observations of antioxidant enzyme mRNA levels with dietary restriction, levels did not change 44% of the time and increased 50% of the time [78, 79, 90, 94, 100, 137, 139–141]. For protein, levels did not change 57% of the time and only increased 23% of the time [78, 79, 90, 91, 139]. Although there were only 30 published changes of antioxidant protein levels, this observation suggests the increases in antioxidant activity that do occur with DR may be due to post-translational modifications or coregulators. Consistent with this notion, isocitrate dehydrogenase 2, a generator of the redox-required NADPH, is deacetylated by Sirt3 in the mitochondria in response to dietary restriction [27], and DR is known to alter acetylation of mitochondrial proteins [142, 143]. Post-translational modifications are known to regulate a number of antioxidant enzymes [144–147].

Effect of DR on oxidative damage

A limitation in the study of oxidative modifications is the reliability of reported biomarkers used to measure oxidative damage. Common measurements include carbonyls for proteins, lipid hydroperoxides and aldehydes for lipids, and 8-hydroxy-2-deoxyguanosine for DNA. While bulk measurements of oxidative damage may be indicative of the overall cellular state, it is likely that specific modifications on specific sites of individual proteins, lipids and DNA differentially affect function, similarly to post-translational modifications like phosphorylation and methylation. Indeed, proteomics analyses of carbonylated proteins from young and old animals reveals that oxidative modifications have specific targets [148] and that oxidative modifications do not always reflect a change in protein function. With the relatively limited effect of DR on ROS production and antioxidant enzyme activity, what was the net effect of DR on oxidative damage to macromolecules?

For studies of oxidative damage to macromolecules, we found 315 reported comparisons with dietary restriction in rodents [27, 33, 48, 49, 52–54, 57, 60–67, 75–77, 85–87, 91, 97–99, 103, 104, 137, 149–182]. Seventy-two percent of these observations were made in rats; the remaining observations were made in mice. There was no difference between species in overall effects of dietary restriction on oxidative damage (Chi squared, $p > 0.05$). Males, again, were studied at a strikingly disproportionate amount (94%). Regarding the distribution of tissues studied, liver was the most well studied, followed by brain, skeletal muscle, heart, other tissues and kidney (Table 1). A significant proportion (21%) of measurements was done on mitochondrial fractions, which will be discussed separately below. Lipid, protein and DNA oxidation was well represented, with RNA oxidation being rarely studied (Table 6). *In total, a majority of observations (53%) indicated reduced oxidative damage with dietary restriction with 44% of observations indicating no effect.*

The oxidized product as well as the methods for quantifying these products varied among studies we reviewed. For DNA oxidation, the most common measurement was 8-oxo-deoxyguanosine. Common readouts for lipid oxidation include 4-hydroxynonenal, malondialdehyde and F2-isoprostanes. The most frequent measurement of protein oxidation was carbonyls, and various tyrosine modifications (nitrotyrosine, dityrosine and o-tyrosine) were relatively common. Overall, DNA oxidation was most likely to be decreased with dietary restriction (Table 6). Consistent with reduction in DNA oxidation, DR also reduces or prevents induced and endogenous DNA mutations [27, 183–185]. For lipid and protein oxidation, dietary restriction was about as likely to have no effect on oxidative damage as to decrease oxidative damage.

Mitochondria are major generators of reactive oxygen species and may play a role in the effects of dietary restriction [186]. Did DR have significant impact on oxidative damage in mitochondria? Of 67 observations of oxidative damage in mitochondria with DR, only 46% reported decreased oxidative damage. Forty-three percent of studies reported no change, and 10% of studies reported an *increase* in oxidative damage with dietary restriction. *Thus, DR did not have a particularly noteworthy effect on oxidative damage in mitochondria, consistent with the modest effects of DR on mitochondrial ROS production discussed previously.*

On the whole, oxidative damage was not affected preferentially by DR in any specific tissue (Table 7). Interestingly, oxidative damage was the only parameter measured that did not find tissuespecific differences between the distributions of effects, suggesting the effects of DR on oxidative damage are universal among all tissues. Oxidative damage decreased more than half the time with DR in all tissues except skeletal muscle. Did DR have effects on specific macromolecules in each tissue (Table 8)? In the brain, interestingly, lipid oxidation was least likely to be affected by DR, while in the heart, lipids were the most likely to be affected by dietary restriction. In the liver, protein oxidation was rarely decreased by DR, and in skeletal muscle, oxidative damage to each macromolecule was decreased half the time with dietary restriction. Interestingly, lipid oxidation *increased* in skeletal muscle in 21% of studies. Protein oxidation, on the other hand, increased in heart and liver in 13% of studies.

Effects of dietary restriction on oxidative stress during aging

Although this review was not intended to comprehensively review the effects of oxidative stress during aging, a large number of studies examined here were on old animals, and the results in this section are likely reflective of all age-reported changes in oxidative stress. For example, we found a similar degree of effect for DNA oxidation compared with a recent comprehensive analysis of all age-related changes in DNA oxidation [187]. Related to the study of DR on old animals, the duration of DR may influence whether oxidative stress is reduced. Based on Table 9, duration of dietary restriction had a marked effect on whether oxidative stress was attenuated. For example, ROS production was more likely to be decreased with DR if the duration of restriction was more than twenty months. Similarly, longer durations of DR were more likely to increase antioxidant activity and reduce oxidative damage. Potentially, these effects may be due to the fact that studies of longer durations of dietary restriction are done on older animals, and DR may have independent interactions with the aging process. Note that in some studies, older animals were restricted for short durations, so duration of DR *is not necessarily* reflective of the age of animals tested. Below we will discuss the effects of aging and dietary restriction on mitochondrial ROS production, antioxidant enzyme activity and oxidative damage.

Protection by DR against age-related changes includes partial and full reversal of phenotype. Of 21 studies on ROS production during aging, there was no effect of aging 52% of the time. Of the remaining 48% of aging studies showing an aging effect, 60% of those studies demonstrated that DR prevents age-related changes in ROS production and 40% of studies showed no effect of DR. Thus, when ROS production does increase with age, DR tends to prevent this change. A number of studies were on the effects of DR on antioxidant enzyme activity during aging. Among 177 observations in those studies, in 55% of the measurements there were no age-related changes in antioxidant activity, while in 32% of the cases DR prevented the age-related change and in 13% of the cases DR did not prevent the age-related change. Among the cases which did not observe any effect of age, DR independently affected enzyme activity 38% of the time, and in the vast majority of those cases DR increased enzyme activity.

Further evidence against a role for antioxidant enzymes in the lifespan-extending effects of DR comes from an important study on mice deficient in the transcription factor Nuclear

factor (erythroid-derived 2)-like 2 (Nrf2^{-/-}) [188], which regulates the expression of hundreds of antioxidant and detoxification genes [189]. When compared to control mice on DR, Nrf2^{-/-} mice that are dietary restricted did have increased incidences of induced cancer, implicating the Nrf2 pathway in the anti-cancer effects of DR. However, the lifespan-extending effects of DR are still maintained in Nrf2^{-/-} mice. Although the regulation of antioxidant enzymes is complex, this study strongly suggests the anti-aging effects of DR in mice are not solely due to upregulation of antioxidant proteins.

Unlike the effects of DR on ROS production and antioxidant enzyme activity during aging – which showed mitigating effects of DR in 29% and 32% of reported cases, respectively – in sixty-six percent of studies on old animals (n=100) DR prevents the age-related change in oxidative damage. Interestingly, there was a significantly different distribution of effects comparing studies of aging versus all studies for oxidative damage, unlike antioxidant activity or ROS production (Chi-squared or Fisher's exact test). This suggests that DR has an independent interaction with aging in terms of oxidative damage. Moreover, DR reduces oxidative damage in 73% of aging studies (versus 53% of all studies on oxidative damage and dietary restriction). In 21% of studies on aging that included DR there was no increase in oxidative damage. Thus, oxidative damage tends to increase with age, and DR prevents this increase in the majority of cases.

The marked effects of DR on oxidative damage during aging may be due not to changes in ROS production and antioxidant enzyme activity but instead the effects of DR on protein turnover. This can be achieved through increasing synthesis or degradation of macromolecules. DR prevents the age-related decline in protein synthesis [190, 191] and increases macromolecular breakdown by autophagic [192, 193] and proteasomal degradation [194–203]. DR also increases DNA repair activity [91, 204–207], which may reduce DNA oxidation, and reduces DNA mutations [27, 183–185]. Furthermore, animals fed DR have increased oxidized DNA in the urine of animals fed DR, which suggests increased removal of damaged DNA [28]. Thus, the reduction in oxidative damage with DR may be attributed to increased turnover of oxidized macromolecules.

Concluding remarks

Recent evidence from rodent studies calls into question the oxidative stress theory of aging. In this review, we analyzed the effects of dietary restriction, the gold standard for anti-aging interventions, on oxidative stress. Our analysis is limited in that we did not take into account strain or sex, due to the diversity of strains tested and the lack of studies done in females. Statistical analysis was included only when there were sufficient numbers of observations. Furthermore, our analysis did not take into account the extent of effect of DR on the various oxidative parameters because data was not uniformly expressed and raw data was not always available for analysis. Similarly, our analysis does not take into account the relative ages across aging studies, thus relying on the expertise of others to determine how old an animal need to be for an aging study. However, this review is the first to take a comprehensive and unbiased approach to analyzing this subject matter and avoids the pitfalls of many reviews that emphasize only positive results.

Studies of the effects of dietary restriction on oxidative stress in rodents disproportionately use male animals. Because there are differences in how sexes respond to DR in regards to oxidative stress and other parameters [66, 99, 208], it is crucial that female animals are included in future studies of dietary restriction. We also found that for all three parameters – ROS production, antioxidant enzyme activity and oxidative damage – studies that made more than ten measurements were less likely to find differences between DR and *ad libitum* control animals (Chi-squared or Fisher's exact test, $p < 0.05$). This observation reinforces the importance of integrative, comprehensive analysis in DR and aging studies. In addition, there have been only a few studies on non-mitochondrial ROS production, which comes from a variety of sources, including peroxisomal oxidases, NADPH oxidases and xanthine oxidase, and mediates essential cellular functions [209]. More research on oxidative stress and dietary restriction is also necessary in tissues other than liver, brain, heart, kidney and skeletal muscle and on antioxidant enzymes like the glutaredoxins and peroxiredoxins. Furthermore, research in this area should move toward analysis of the mechanisms by which DR does affect oxidative stress, including macromolecule synthesis and degradation, post-translational modifications to antioxidant enzymes and related signaling pathways, redox-sensitive epigenetic regulation of gene transcription and further understanding of the effects of oxidation to the multitude of lipid signaling intermediates.

Our review of the literature on the effects of dietary restriction on oxidative stress reveals that DR tends not to affect mitochondrial reactive oxygen species production or antioxidant enzyme activity. Despite this observation, DR rarely induces a pro-oxidant environment by increasing ROS production or decreasing antioxidant enzymes. Thus, in total, DR likely produces a more reductive environment overall. In contrast to the modest effects of DR on ROS production and antioxidant enzyme activity, DR often prevents oxidative damage to macromolecules, especially in studies of old animals. Increased degradation of oxidized macromolecules may explain the enhanced effects of DR on oxidative damage.

A major caveat of the aging changes as well as the DR effects is the tendency to not publish negative data. Thus, the moderately beneficial effects of DR on oxidative stress reported here are likely over representative of reality. In addition, these observations were admittedly subject to our own interpretation of the reported data. The protective effects of DR in aging and disease are likely mediated by a number of mechanisms, including prevention of apoptosis in post-mitotic cells [210–212], preservation of stem cell function [213], reducing inflammation [138, 151, 214–216], hormesis [217], and reducing senescent cells [173]. As the redox stress theory of aging points out, oxidative modifications are often more than just damage and can affect function. Thus, alternative roles of oxidative stress in the cell need to be further examined. For example, DR prevents the age-related *decrease* in oxidative modification to histones, which may play a role in the age-related changes in gene transcription [218].

We find evidence consistent with the redox stress theory of aging in our review. Most notably, the glutathione system is commonly altered with dietary restriction. In further support of the effects of DR on redox stress, DR increases sulfhydryl content [60, 156, 157] and modulates the activity of redox-sensitive transcription factors [8]. Furthermore, a recent paper compared two strains of mice, one for which lifespan is extended by DR and one for

which DR has no effect on lifespan, and found that mice from the strain responsive to DR are more likely to upregulate redox-related antioxidants [102], including glutamyl cysteine ligase, the rate-limiting step in GSH biosynthesis. NADPH is required for the final reduction of oxidized glutathione by glutathione reductase and thioredoxin by thioredoxin reductase [219]. Interestingly, one generator of NADPH, NADPH-dependent isocitrate dehydrogenase, increases with age in liver and testes but decreases in kidney, and DR prevented all of these changes [220]. DR also upregulates other NADPH generators, including malic enzyme, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase [221], and transketolase, an enzyme necessary for the production of NADPH via the pentose phosphate pathway [194]. Lastly, in non-human primates, metabolomics analysis reveals that dietary restriction upregulates the pentose phosphate pathway [222].

A strength of the redox stress theory over the oxidative stress theory is that it acknowledges the fact that oxidation is not merely damage but also affects enzymatic activity, signaling pathways and gene transcription. Interestingly, redox stress is associated with a number of post-translational modifications, including SUMOylation, acetylation, phosphorylation and glutathionylation [223–225]. However, there are over 300 post-translational modifications to proteins [226] that independently regulate enzymatic activity, signaling pathways and gene transcription just as elegantly as redox modifications. Furthermore, in some cases, non-oxidative modifications regulate the same exact pathways regulated by redox chemistry. For example, the activity of the redox-regulated transcription factor NF- κ B is regulated by a number of post-translational modifications [227]. In this context, we question the rationale of focusing on redox biology as a major mediator of the aging process. Instead, comprehensive analysis of changes in post-translational and epigenetic modifications during aging will elucidate more completely the processes that accompany the aging process. This line of research is especially relevant because many substrates for post-translational and epigenetic modifications are intermediates in pathways regulated by mitochondria [228]. Consistent with this idea, a recent proteomic analysis revealed strikingly few changes in total protein content during aging in multiple tissues [229], suggesting that alternative methods of regulation, *including but not limited to redox regulation*, are major factors in the declining physiological function that occurs with age. We emphasize that the use of dietary restriction or other anti-aging paradigms are essential to these future studies to elucidate the cause(s) of aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DR   dietary restriction

ROS	reactive oxygen species
PM	pyruvate/malate
S	succinate
AA	antimycin A
R	rotenone
Cat	catalase
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GST	glutathione S-transferase
SOD	superoxide dismutase
NADPH	nicotinamide adenine dinucleotide phosphate

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- DR tends to have no effect but otherwise reduces mitochondrial ROS production
- DR tends to have no effect but otherwise increases endogenous antioxidants
- DR often reduces oxidative damage to macromolecules
- DR tends to increase redox antioxidants like glutathione
- DR during aging is more likely to affect oxidative stress parameters

Table 1
**Percentage of studies per tissue that examined mitochondrial ROS production,
antioxidant enzyme activity and oxidative damage**

Liver, brain, heart, skeletal muscle and kidney are the most studied tissues in analyses of dietary restriction and oxidative stress.

Tissue	Reactive Oxygen Species (n=157)	Antioxidants (n=350)	Damage (n=315)
Brain	10	22	22
Heart	19	14	12
Kidney	4	13	6
Liver	52	37	35
Sk. muscle	15	5	15
Other	0	10	10

Table 2
Effects of specific electron transport chain substrates and inhibitors on mitochondrial reactive oxygen species production with dietary restriction

Complex I substrates PM, the complex II substrate S, the Complex I inhibitor R and the complex III inhibitor AA are common in studies of DR and ROS production. DR tends to reduce ROS production at Complex I when respiring on PM and Complex III when respiring on S. Substrates/inhibitors: pyruvate/malate (PM), succinate (S), antimycin A (AA), rotenone (R).

Substrate/Inhibitor	% Decrease	% No change
PM (n=38)	32	68
PM AA (n=8)	13	88
PM R (n=16)	38	63
S (n=38)	39	61
S AA (n=25)	60	36
S R (n=14)	21	79

Table 3
Effects of dietary restriction on mitochondrial ROS production in various tissues

ROS production is commonly measured in liver, heart, skeletal muscle, brain and kidney. Distribution of effects between tissues is statistically significant (Chi-squared, $p < 0.01$).

Tissue	% Decrease	% No change	% Increase
Brain (n=15)	60	40	
Heart (n=30)	50	50	
Kidney (n=7)	57	43	
Liver (n=81)	23	75	1
Sk. muscle (n=24)	46	54	

Table 4
Overall distribution and effects of dietary restriction on antioxidant enzyme activity

Catalase, GPx and SOD activities are commonly measured in studies of DR and oxidative stress. Distribution of effects between enzymes is statistically significant (Chi-squared, $p < 0.01$).

Enzyme	% Total	% Decrease	% Increase	% No change
Catalase (n=87)	23	8	46	46
Glutathione peroxidase (n=93)	25	9	31	60
Glutathione reductase (n=20)	5	0	40	60
Glutathione (n=61)	16	2	62	36
Glutathione S-transferase (n=16)	4	13	38	50
Superoxide dismutase (n=95)	26	14	22	64

Table 5
Effects of dietary restriction on specific antioxidant enzyme activities in each tissue

Liver, brain, heart and kidney are commonly analyzed in studies of DR and oxidative stress. Brain is least responsive to changes in antioxidant activity overall. Catalase often changes in heart and kidney with dietary restriction. Liver reflects the overall changes in antioxidant activity with dietary restriction. Distribution of effects in all enzymes between tissues is statistically significant (Chi-squared, $p < 0.01$).

Tissue	Enzyme	% Increase	% No change	% Decrease
Brain	All enzymes (n=74)	30	68	1
	Cat (n=22)	27	73	0
	GPx (n=22)	14	82	5
	SOD (n=23)	17	83	0
Heart	All enzymes (n=51)	45	35	20
	Cat (n=12)	67	8	25
	GPx (n=15)	40	40	20
	SOD (n=15)	20	53	27
Kidney	All enzymes (n=50)	46	46	8
	Cat (n=9)	78	22	0
	GPx (n=13)	31	54	15
	SOD (n=11)	36	45	18
Liver	All enzymes (n=123)	44	52	4
	Cat (n=31)	53	44	3
	GPx (n=28)	48	52	0
	SOD (n=30)	29	65	6

Table 6
Distribution of macromolecules studied with respect to oxidative damage during dietary restriction

Lipid, protein and DNA oxidation are well characterized with dietary restriction. No significant effect was found in the distribution of effects between macromolecules (Chi-squared, $p>0.05$).

Molecule	% Total	% Decrease	% No change	% Increase
DNA (n=92)	29	58	42	0
Lipid (n=115)	37	49	48	3
Protein (n=101)	32	50	45	6
RNA (n=7)	2	43	57	0

Table 7
Effects of dietary restriction on oxidative damage in specific tissues

Oxidation of macromolecules is more likely to change with dietary restriction than ROS production or antioxidant activity. There is no significant difference between the distributions of effects between tissues (Chisquared $p>0.05$).

Tissue	% Decrease	% No change	% Increase
Brain (n=70)	56	44	0
Heart (n=38)	58	39	3
Kidney (n=18)	56	44	0
Liver (n=109)	50	46	5
Sk. muscle (n=47)	47	47	6

Table 8
Tissue- and macromolecule-specific effects of dietary restriction on oxidative damage

Lipid oxidation is least likely to decrease in brain, while in heart lipid oxidation is the most likely to decline with dietary restriction. Protein oxidation rarely declines in the liver with dietary restriction. Oxidative damage to all macromolecules tends to decline in skeletal muscle.

Brain	Heart			Liver			Sk. muscle		
	Target	Effect	%	Target	Effect	%	Target	Effect	%
DNA (n=13)		Decrease	69		Decrease	53		Decrease	47
		No change	31	DNA (n=15)	No change	47	DNA (n=15)	No change	53
		Increase	–		Increase	–		Increase	–
Lipid (n=27)		Decrease	41		Decrease	75		Decrease	50
		No change	59	Lipid (n=8)	No change	25	Lipid (n=14)	No change	29
		Increase	–		Increase	–		Increase	21
Protein (n=30)		Decrease	63		Decrease	50		Decrease	50
		No change	37	Protein (n=15)	No change	31	Protein (n=12)	No change	50
		Increase	–		Increase	13		Increase	–

Table 9
Duration of dietary restriction affects ROS production, antioxidant enzyme activity and oxidative damage

The longer animals were dietary restricted, the more likely reduced oxidation was observed for each parameter.

Duration (months)	Effect	Reactive oxygen species	Antioxidants	Damage
Less than 10	% Decrease	34	11	45
	% No change	65	52	51
	% Increase	1	37	4
10 to 19	% Decrease	40	3	53
	% No change	60	67	44
	% Increase	–	30	3
20+	% Decrease	56	6	64
	% No change	44	40	34
	% Increase	–	55	2