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The Naked Mole-Rat Response to Oxidative Stress: Just Deal with It

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Abstract

Significance: The oxidative stress theory of aging has been the most widely accepted theory of aging providing insights into why we age and die for over 50 years, despite mounting evidence from a multitude of species indicating that there is no direct relationship between reactive oxygen species (ROS) and longevity. Here we explore how different species, including the longest lived rodent, the naked mole-rat, have defied the most predominant aging theory. **Recent Advances:** In the case of extremely long-lived naked mole-rat, levels of ROS production are found to be similar to mice, antioxidant defenses unexceptional, and even under constitutive conditions, naked mole-rats combine a pro-oxidant intracellular milieu with high, steady state levels of oxidative damage. Clearly, naked mole-rats can tolerate this level of oxidative stress and must have mechanisms in place to prevent its translation into potentially lethal diseases. **Critical Issues:** In addition to the naked mole-rat, other species from across the phylogenetic spectrum and even certain mouse strains do not support this theory. Moreover, overexpressing or knocking down antioxidant levels alters levels of oxidative damage and even cancer incidence, but does not modulate lifespan. **Future Directions:** Perhaps, it is not oxidative stress that modulates healthspan and longevity, but other cytoprotective mechanisms that allow animals to deal with high levels of oxidative damage and stress, and nevertheless live long, relatively healthy lifespans. Studying these mechanisms in uniquely long-lived species, like the naked mole-rat, may help us tease out the key contributors to aging and longevity. *Antioxid. Redox Signal.* 19, 1388–1399.

Introduction

AGING IS A multicausal process consistently characterized across the animal kingdom by a decline in genomic integrity, an accumulation of oxidative stress, increases in various system pathologies and, most importantly, an increase in mortality. With a greater emphasis on healthspan, rather than lifespan, and an ever increasing demand for improved quality of life into old age, there is a need for unique models of aging and longevity that may aid in the identification of novel mechanisms attenuating age-related diseases and mortality.

Comparative biology has long utilized a broad spectrum of organisms to study specific questions in many different areas of science, including aging (7). Insights into aging using this approach were already evident in Aristotle's 350 BCE treatise on "Longevity and the Shortness of Life," in which he noted that sterile mules lived longer than either the reproductively "salacious" horse and the ass (6), and thereby provided early support for both the "disposable soma theory of aging" (49) and the "rate of living theory of aging" (68). Many of those

early studies revealed good inverse correlations between the maximum lifespan potential (MLSP) of a species and the metabolic rate (68, 77), leading those authors to conclude that lifetime energy potential was constant. However, in those studies primarily involving readily accessible domesticated animals, neither the phylogeny nor size of the organism was taken into consideration; both of these features have a profound effect upon maximum lifespan. For instance, it is widely agreed that birds live about three times longer than similar sized mammals, although it is doubtful that anecdotal reports of centenarian parrots have much validity (65). Even within just the mammalian taxonomic class, the maximum lifespans of the *Chiropterans* (bats) far exceed those of similar sized nonvolant mammals, with reports that Brandt's bat (*Myotis brandtii*) lives more than forty years even under the harsh conditions encountered in the wild (72).

Until recently, humans were considered the longest living mammals based upon reliable records, with the longest lived human, a French woman (Jeanne Calment), who lived 122 years and 165 days (20). This authenticated mammalian

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longevity record may possibly be exceeded by the much larger bowhead whale. Based on data from an individual recently hunted and found to contain harpoon fragments produced in the 1880's, bowhead whale longevity is now estimated to be between 130 and 245 years old (30). Like the metabolic rate, a mouse to elephant allometric relationship can be drawn such that maximum lifespan varies as a function of body size (22). Based upon several thousand nonvolant, nonaquatic mammals, for every doubling of average species mass, a 16% increase is observed in MLSP. A comparable humming bird to ostrich allometric relationship is also observed that supports similar quarter power scaling (42). These allometric-based data can be further manipulated to determine the longevity quotient (LQ), or the relationship between lifespan as predicted by body size and the actual observed lifespan of a species. Long-lived organisms are defined as those with a $LQ > 2$. Based off of this definition of a long-lived organism, multiple species have independently evolved long lifespan, including humans, echidnas, and naked mole-rats, all which live about fourfold longer than predicted, thus a LQ of 4 (22). In sharp contrast, the most commonly used species in aging research, the C57Bl/6 laboratory strain of mice, has a MLSP of 4 years, living approximately as half as long as predicted based upon its body size (22). Novel mechanisms may be identified with a comparative approach by utilizing unique species with varying longevity.

Animals with exceptional longevity, like the longest lived rodent, the naked mole-rat, provide novel insights into a variety of physiological and biochemical questions related not only to lifespan, but also to age-related diseases, including cancer, inflammation, and neurodegeneration (16). Here we are going to review the validity of the oxidative stress theory of aging and highlight findings from the extraordinarily long-lived naked mole-rat in comparison to other rodents as a prime model for studying healthy aging and longevity.

Naked Mole-Rats: A Unique Model of Healthy Aging

Naked mole-rats, (*Heterocephalus glaber*, Rüppell 1842) are the longest-lived rodents known. Although about the size of a mouse, this hairless pink hystricognath rodent (Fig. 1) lives at least eight times as long, with a maximum lifespan of ~32 years in captivity (24). In their natural habitat of the eastern horn of Africa, they are also long-lived with the record for noncaptive animals 17 years [Braude pers. comm. (16)]. In the hot, arid, and semiarid regions where they are naturally found, they live communally in large strictly subterranean colonies of up to 300 animals (11) and work in large packs to excavate foraging burrows and locate underground foods, such as roots, tubers, bulbs, and corms (81).

Cloistered underground, these mole-rats are protected from climatic extremes as well as most predators and pathogens, decreasing their levels of extrinsic mortality (15). Similar to colonies of termites and bees (91), these colonies of eusocial mammals restrict breeding to a single breeding female and one to three breeding males (44). Both breeding males and females in the colony are able to successfully reproduce and raise offspring well into their third decade of life. The actively breeding queen suppresses the reproductive physiology in other members of the colony, and most of the nonbreeders remain in their colony of birth throughout their lives. While long-lived nonbreeders within a colony support the disposable

soma theory of aging, the breeding females do not. Not only do they produce large litters (up to 30 pups per litter), doubling their body mass during pregnancy and dealing with the high metabolic demands of both pregnancy and lactation, but they do not show a menopause or decline in fertility with age and live as long as do nonbreeders in captivity and four times longer than nonbreeders in the wild (19, 82, 88). The latter observation is most likely due to the fact that they have less exposure to dangerous activities, such as foraging and burrow maintenance that may increase the risk of encountering a predator. Similarly, in noncaptive eusocial insects, queens live longer than workers [reviewed in ref. (75)]. Communal existence, like that of bats and herd-dwelling animals, reduces the chance that a particular individual would become fodder for a predator and may certainly contribute to their prolonged lifespan (16).

Naked mole-rats do not have the traditional lifespan curves observed in most rodents; mortality rates do not increase with age. During their long lifespan, naked mole-rats experience very few of the common characteristic changes with age, maintaining body composition, bone density, and metabolic rate, as well as genomic and proteomic integrity well into old age and clearly show a compression of morbidity to the last quartile of life (16). Despite their impeccably maintained healthspan and absence of age-associated diseases over their 30+ years of life, individuals over the age

FIG. 1. Naked mole-rats are small, pink mouse-sized rodents with a maximum lifespan of 32 years, during which they experience very little age-related decline. Here, a very pregnant breeding female, or queen, sleeps with other members of the colony to regulate body heat. The breeding female can successfully reproduce well into her third decade of life. She may double her mass during pregnancy and may carry litters of up to 30 pups. In captivity, breeding females live as long as nonbreeding animals. Photo taken by Raymond Mendez, 1996. Reprinted with permission.

of 25 begin to show the inevitable signs of aging physically, with a decrease in activity and increase in sarcopenic and kyphotic phenotypes (24).

Strikingly, these animals are very resistant to cancer, with no case ever reported in our large colony maintained for over 30 years. This is in sharp contrast to data based upon laboratory rodents. For example, >70% of C57Bl/6 mice die of cancer in the laboratory setting (43). Additionally, unlike cells from other mammals in which transformation by oncogenes rat sarcoma and simian virus 40 large T antigen causes invasive xenograft tumors, transformed naked mole-rat fibroblasts containing this oncogenic cocktail have no tumorigenic effects (55). Not only are naked mole-rats resistant to cancers, but they also appear to resist many of the age-associated diseases. We have found that unlike mice, naked mole-rats show a markedly slower age-related change in diastolic function with approximately a 25% decline over 20 years, whereas the change in diastolic function over a similar proportion of lifespan in C57Bl/6 mice is more than a 50% decline (32). Moreover, a similar pattern of attenuated signs of aging are evident in naked mole-rat brains; their complex social behaviors are maintained with age and the neuron number is also maintained (23). This slow rate of physiological decline and compression of morbidity into the late stages of life is also observed in supercentenarians (>110 years), who show few signs of age-related diseases for at least 80% of their long lifespan (1). This late onset in frailty may reflect that these long-lived species are more likely to die from end organ reserve failure than common age-associated, mid-/late-life onset chronic diseases (28).

The Oxidative Damage Theory of Aging

The extended healthspan and notable lifespan of the naked mole-rat is surprising given that even at a very early age, these animals exhibit high levels of a variety of markers of oxidative stress to lipids [isoprostanes, malondialdehyde; (2)], proteins [protein carbonyls and cysteine oxidation; (4,71)], and deoxyribonucleic acid (DNA) [8-oxo-2'-deoxyguanosine (8-OHdG); (4)] in multiple tissues. Oxidative damage, or the damage caused by electron exchange with reactive oxygen species (ROS), an inevitable byproduct of aerobic respiration, is a natural product of normal cell respiration. During electron transfer in the electron transport chain, electrons may leak out of mitochondrial complexes, leading to superoxide formation. At physiologically normal concentrations, these free radicals or ROS most likely have necessary signaling functions within the cell (56, 85), although when these levels reach supraphysiological concentrations, excess ROS becomes dangerous causing promiscuous damage to DNA, lipids, and proteins in the cell. Additionally, the ROS level increase can result from dysfunctional mitochondria, perpetuating high levels of ROS and oxidative damage (31).

Oxidative damage or stress can be measured in multiple tissues within an organism by looking at several different markers. Lipid oxidation is measured by isoprostanes that are formed from ROS peroxidation of fatty acids. ROS damage to protein usually results in modified residues found on carbonyl groups. These carbonyl groups are then rendered resistant to normal degradation. DNA damage from free radicals potentially has the greatest risk for further damage to the cell and organism. Highly reactive free

radicals react with DNA by the formation of double bond adducts, and one of the primary products of this reaction is 8-OHdG. If not repaired, high levels of DNA damage resulting from oxidative stress may result in mutations that contribute to the formation of cancers and other diseases, many associated with aging.

First, hypothesized in 1956, as the "Free Radical Theory of aging," Denham Harman posited that ROS damage to macromolecules within the cell would lead to DNA damage and loss of proteomic integrity, eventually leading to mutations eventually causing age-related diseases, including cancer and neurodegeneration, and a potentially shorter lifespan, particularly in tissues with high rates of aerobic respiration like the heart, brain, and muscle (34). Part of this original theory also speculated that these effects could be countered by the addition of ROS-neutralizing compounds to the cell, thus decreasing the likelihood of accumulated damage. The original Free Radical theory has evolved over the many decades since its first proposition in to the Oxidative Stress Theory of Aging and has also grown to include mitochondrial-based free radical theories of aging. If this theory were true, we would expect an increase in ROS levels would result in a shorter lifespan and higher incidences of neurodegeneration, cancer, and other age-associated diseases. On the other hand, low levels of ROS or damage could predict or result in a long lifespan with low rates of morbidity (Fig. 2).

The Unexceptional Antioxidant Status of Naked Mole-Rats and Other Long-Lived Species

All animals that employ aerobic metabolism have had to evolve multiple mechanisms to deal with ROS byproducts and oxidative stress, and thereby prevent the initial accrual of damage as well as the repair or removal of compromised molecules from the cell (Fig. 2). Antioxidant defenses neutralize potentially dangerous byproducts of aerobic metabolism preventing the autocatalytic chain reaction that draws electrons from adjacent macromolecules within the cell, and thereby propagates oxidative damage. All organisms contain multiple types of antioxidant defenses, including vitamins A and C, polyphenols, flavanoids, selenium-based compounds, glutathione (GSH), and enzymatic antioxidants (*e.g.*, superoxide dismutases [Sod] and catalases). In response to aerobic metabolism, CuZn superoxide dismutase (Sod1) and Mn superoxide dismutase (Sod2) found both in and outside of the mitochondria, remove superoxide radicals *via* O₂ and hydrogen peroxide (H₂O₂) conversions (59). Both catalase and glutathione peroxidase (Gpx) promote the decomposition of the H₂O₂ generated within the cell to water and oxygen, thus effectively neutralizing and eliminating this ROS from the cell (51). Similarly GSH is a reducing equivalent, able to accept electrons and neutralize ROS as well as eliminate other electrophiles and xenobiotics within the cell and serves as a cosubstrate for glutathione transferases and Gpxs (46).

The ratio of reduced (GSH) to glutathione disulfide (GSSG) is also used as a reliable indicator of the oxidative environment within the cell. Although there are clearly multiple antioxidants within the tissues of an organism and multiple back-up systems to compensate when one or more antioxidants are depleted, nevertheless the antioxidant defense suite is never completely efficient and especially under high levels

FIG. 2. Increased levels of ROS can cause oxidative damage to all macromolecules within the cell that, if left unchecked or unrepaired, can further manifest into mutations in DNA, as well as protein and membrane dysfunction. As a direct consequence thereof, these can contribute to uncontrolled proliferation and an accumulation of damage, and eventually cancer and other age-related diseases. Different protective mechanisms in the cell are able, at least to some extent, to repair or deal with oxidative damage *via* detoxification, molecule turnover (*i.e.*, proteasome, autophagy), or DNA repair. Many of these pathways have components that are largely regulated by Nrf2, a transcription factor that modulates the expression of over 600 cytoprotective molecules. The constitutive upregulation of such pathways may immediately protect against potential stressors and has been hypothesized to contribute to a healthy aging and longevity phenotype, despite existing levels of oxidative damage and antioxidant status. DNA, deoxyribonucleic acid; Nrf2, nuclear factor-erythroid 2-related factor-2; ROS, reactive oxygen species.

of oxidative stress, oxidative damage still occurs (8). Nevertheless, based upon the basic principles of the oxidative stress theory of aging that accrued oxidative stress induces age-related changes that ultimately determine the lifespan of an organism, one would surmise that constitutive levels of antioxidants and, in particular, the predominant enzymatic antioxidants, would positively correlate with species lifespan. Therefore, we have compared the status of the antioxidant enzymes from several species.

Superoxide dismutases

Superoxide dismutases (Sods) exist in two commonly studied forms, Sod1 and Sod2. Sod1 is located in the intermembrane space of the mitochondria and is primarily responsible for scavenging free superoxide radicals in the cytoplasm, whereas Sod2 also scavenges free superoxide radicals, however, in the mitochondrial matrix. Mice experimentally manipulated to lack Sod1 (a complete knockout) have increased levels of oxidative damage and a higher incidence of liver neoplasia, as well as a 30% shorter average and maximum lifespan compared to wild-type mice (25, 64, 70). Although Sod2 knockout mice show increased signs of oxidative stress, these mice are not viable past neonatal stages (54). Mice heterozygous for Sod2 show an increase in oxidative damage well into adulthood; however, there is no difference in the lifespan compared to wild-type mice (89). On

the opposite end of the spectrum, in keeping with predictions based upon the oxidative stress theory of aging, one would expect that overexpression of Sod1 and/or Sod2 would result in an extension of lifespan. However, this is not observed for either transgenic, even though their fibroblasts have been reported to be more resistant to toxins (60, 70).

Studies in other species have revealed equivocal results. Several studies report that Sod1 and Sod2 levels are negatively correlated with longevity in multiple species, including insects, amphibians, birds, rodents, and nonhuman primates (9, 67, 69), and that this pattern is evident in a variety of tissues, including liver, brain, and heart. Other studies report a positive correlation among seven vertebrate species (66) and two other large comparative vertebrate studies reveal no relationship whatsoever (57, 86) and studies involving fewer bird species reveal no difference in Sod levels between short-lived quail and long-lived parrots (62). In naked mole-rats, Sod1 activity is 1.35-fold higher than levels observed in physiologically age matched mice and age does not have an effect on its activity in either species (3). Naked mole-rats also have slightly higher activity levels of Sod2 in young and adult animals compared to age-matched mice. However, naked mole-rats do not experience the age-related increase in Sod2 activity observed in mice by 18 months of age. It is unlikely that this relatively low difference in Sod1 or Sod2 activity between naked mole-rats and mice is responsible for the profound disparity in longevity.

Catalase activity

The antioxidant enzyme catalase catalyzes the decomposition of H_2O_2 to water and is primarily expressed in peroxisomes of all aerobic organisms. Embryonic fibroblasts of transgenic mice that overexpress catalase are more resistant to H_2O_2 (60). Moreover, mice that overexpress catalase in their peroxisomes and nucleus have slight increases in median lifespan, but no change in maximum lifespan, whereas those mice overexpressing catalase in the mitochondria reportedly may have a 20% increase in median and maximum lifespan (80).

Comparative studies in other mammals as well as birds reveal a negative correlation between catalase activity/levels and maximum lifespan (67). We have previously reported that catalase activity did not correlate with maximum lifespan in bats and rodents with the highest levels evident in both short-lived rats (MLSP 4 years) and long-lived vampire bats (MLSP 34 years) (18). Catalase activity in mice and naked mole-rats was also found to be similar, although old individual naked mole-rats maintain levels of catalase activity, while that of mice declines with age (3). Concurring with these findings of no correlation between catalase levels and longevity is a recent study using five species of birds with a seven-fold range in longevity and no indications that catalase correlates either positively or negatively with longevity (62).

Glutathione peroxidase

It is only in assessments of Gpx, do our data involving seven rodent species reveal a strong negative correlation with species longevity (Fig. 3). Cellular glutathione peroxidase (cGpx) levels do not appear to correlate with body mass; the decreased levels of cGpx in longer lived species also appear to be linked to phylogeny as well as maximum lifespan. Hystricognaths (guinea pigs and mole-rat species) have overall

lower cGpx levels compared to mice and rats. It is possible that the extremely high levels of cGpx observed in laboratory mice may not be reflective of wild-derived counterparts, but rather may have been unintentionally selected for during inbreeding in captivity. This strong correlation between MLSP and cGpx confirms many of the data found in other species (mammals and birds), that there is a negative correlation by cGpx levels and maximum lifespan (9, 67). However, a recent study comparing three long-lived parrot species with short-lived quail reported a significant positive correlation between cGpx and longevity (62).

Indeed, the most striking difference among species was that observed between naked mole-rats and mice, with cGpx levels 70-fold lower in the naked mole-rat and more in keeping with knockout levels in mice. Since cGpx is also often measured as an indication of the antioxidant status of an organism, these data would support the premise that naked mole-rats have poor antioxidant defenses against oxidative stress and that this may contribute to the high levels of oxidative damage in naked mole-rats. Gpx's exist in several different isoforms, many of which are unique to certain tissue types (79). Gpx4 is the isoform that offers cytoprotection by detoxifying complex lipid peroxides in cellular membranes and is found in multiple compartments of the cell. Mice heterozygous for Gpx4 are more sensitive to oxidative stress, but surprisingly defy predictions based upon the oxidative stress theory of aging and show an increase in median lifespan and delay in cancer incidence, although mean and maximum lifespan are not different compared to wild-type mice (74). Clearly, cGpx is not playing a critical role in the longevity of the naked mole-rat and these extremely low levels, may even contribute to the pronounced cancer resistance observed in this species.

This strikingly low cGpx activity in naked mole-rats may be due to several factors. Quantitatively, the levels of cGpx are

FIG. 3. cGpx levels in liver lysates significantly correlate with maximum lifespan. cGpx is lowest in the naked mole-rat compared to all other rodent species measured. The highest levels were found in the traditional laboratory rodents. The extremely low levels of cGpx in the naked mole-rat are reportedly associated with low selenium levels and a divergence in its genetic sequence. cGpx, cellular glutathione peroxidase.

similar to those in selenium-deficient mice and cGpx-knockout mice, and both of those models are more susceptible to oxidative stress compared to wild-type mice (3, 21, 29, 38, 90). Naked mole-rats, despite having evolved to live in hypoxic underground deep burrows, are nevertheless very tolerant of the relatively hyperoxic captive environments, thriving in captivity and attaining their extreme longevity under these potentially hostile conditions. Like selenium-deficient mice, they have lower levels of selenium and thyroid hormone concentrations (58) and these low selenium levels are attributed to GPx1 sequence differences (47).

Our findings concerning antioxidant status concur with studies in multiple tissues in species representative of the major phylogenetic groups (yeasts, worms, insects, amphibians, birds, reptiles, and mammals); little to no correlation occurs between antioxidant status (*i.e.*, catalase, Sod, and Gpx) and lifespan. Moreover, studies involving genetic manipulation of antioxidant expression of antioxidants Sod1, Sod2, and catalase reveal no lifespan extension (70). Conversely, when these genes are knocked-out of mice, lifespan is unaffected, although these animals often show an increase in oxidative damage in multiple tissues. In keeping with predictions of the oxidative stress theory of aging, higher levels of oxidative damage lead to an increase in the rate of progression and severity in age-related phenotypes, like muscle loss and concomitant pathology and even result in increased cancer incidence. Clearly, they would have a pronounced impact upon healthspan, leading to an expansion of the period of morbidity. This would indicate that in healthy organisms housed under optimal husbandry, free of pathogens, unhealthy diets, and other harmful environmental conditions that antioxidant status is not a critical component of aging and lifespan. It is possible, however, that under more natural conditions of fluctuating temperatures, poor food choices, and exposure to pathogens, compromises in health status induced antioxidant defenses may present as protective anti-aging actions, and support the basic tenets of the oxidative stress theory of aging by contributing to prolonged lifespan and delayed age-related pathology.

The Role of GSH in Naked Mole-Rats

Not only is GSH also considered an antioxidant, but it has multiple other functions. Together with its disulfide form, GSSG, it is the most abundant redox couple in the cell, signaling changes in the oxidant status. GSH is also a good marker of overall antioxidant health and maintains other antioxidant molecules in their functional reduced forms. GSH scavenges ROS in the cell and reacts directly with electrophiles as well. It also serves as a cosubstrate, necessary for the function of GPXs and glutathione-S-transferases (GSTs) [reviewed in ref. (92)].

GSH is synthesized from cysteine, glutamate, and glycine. Importantly, GSH serves as a storage and transport molecule for cysteine within the cell, and is thus involved in cell metabolism as well as regulation of cellular proliferation, apoptosis, immune response, and mitochondrial function and maintenance. GSH levels are largely regulated by GSH synthetase and glutamyl cysteine synthetase (glutathione cysteine ligase), the rate-limiting step in GSH synthesis.

Studies from many different animal models show a negative correlation between multiple components of GSH

pathways and maximum lifespan using multiple tissues. Long-lived strains of *Drosophila* show an inverse relationship between GSH and long-lifespan (61). Similarly long-lived dwarf mice have lower GSH levels in their liver tissue compared to their normal littermates as do naked mole-rats (13). This inverse pattern was also observed among different mammalian and bird species (57).

We have previously shown that naked mole-rats have lower levels of GSH in liver, similar GSSG levels, as well as a lower GSH/GSSG ratio compared to shorter lived age-matched mice, indicative of a more pro-oxidative milieu (4). These data, together with similar observations in birds and other mammals, support the counterintuitive notion of pro-oxidative cellular environments being associated with long lifespan. We observe similar results when we compare GSH, GSSG, and GSH/GSSG ratios in liver tissue of young animals from multiple rodent species (Fig. 4). Although there are slight differences in levels between the different species, there does not appear to be a clear relationship between GSH, GSSG, or the GSH/GSSG ratio in the rodent species observed here. Shorter lived guinea pigs appear to have the lowest GSH and

FIG. 4. GSH and GSSG levels are not indicative of longevity. The ratio of GSH/GSSG levels in the cell can be indicative of an oxidative environment within the cell. GSH and GSSG levels, as well as GSH/GSSG ratios in liver tissue do not appear to correlate with maximum longevity or body size of five different rodents. A lower GSH/GSSG ratio in the naked mole-rat is indicative of a pro-oxidative environment compared to shorter lived species, like the mouse and guinea pig. GSH, glutathione; GSSG, glutathione disulfide.

GSSG levels compared to similarly sized blind and Damaraland mole-rats, in contrast to what is observed in smaller rodent species, including the mouse and naked mole-rat.

As previously mentioned, GSH is necessary for the activity of GSTs, which catalyze the conjugation of GSH with a variety of toxic molecules in the cell, most often for drug metabolism and toxin elimination. There are many different isoforms of GSTs, found in different compartments of the cell, including the cytosol, nucleus, and mitochondria (36). Additionally, different tissues express different quantities of GST isoforms, and these molecules are often upregulated in response to oxidative damage or xenobiotic response. Unlike antioxidants in the cell, certain GSTs have been shown to be more intimately associated with longevity, and potentially have a positive relationship with long lifespan. Long-lived mutant *Caenorhabditis elegans* show an upregulation of GST enzymes, and overexpression of GSTs increase worm median lifespan. Long-lived dwarf mice also reveal an increase in GSTs (14), and caloric restriction has been shown to enhance not only detoxification, but GST levels as well.

When we measured GST in liver tissue of different rodent species (Fig. 5), naked mole-rats have the highest levels compared to other shorter lived mouse species. However, other larger long-lived rodents, like the Damaraland and blind mole-rat, have much lower GST levels compared to the shorter lived guinea pig; thus, high cytosolic GST levels in naked mole-rats may reflect a cytoprotective mechanism unique to that species. Alternatively, the guinea pig, known to differ from other rodents in their dietary requirements and to be stressed by poor lighting conditions and confined laboratory housing (87) may nevertheless be encountering stressful situations that lead to the induction of high levels of GST.

High Constitutive Levels of Oxidative Damage in the Naked Mole-Rat

High levels of oxidative damage in naked mole-rats are not a unique quirky trait; long-lived vampire bats and many

longer lived birds have higher levels of oxidative damage to DNA than do shorter lived mammals measured under identical conditions (3, 18, 27, 33, 37). High levels of oxidative damage in both mole-rats and bats have been attributed to their high intracellular iron content wreaking havoc with redox status (16, 27). In the case of the naked mole-rat, high levels of damage at a young age may also reflect that atmospheric conditions above ground represent hyperoxic stress to a natural underground dweller.

Naked mole-rats, having led a strictly subterranean existence for more than 25 million years, have evolved a suite of traits to tolerate the hypoxic and hypercapnic conditions commonly encountered below ground (24) and in early life, do not have in place the appropriate defenses and existing defenses to fend off oxidative stress. Not surprisingly then, isoprostanes in urine are highest in very young individuals and 2–10-fold higher in naked mole-rats than in mice (2). High levels of isoprostanes have been similarly observed in skeletal muscle, heart tissues, livers, kidneys, and brains of naked mole-rats (summarized in Table 1).

These high levels of lipid peroxidation are at variance with their membrane peroxidation index (41) and most likely reflect that at a young age, the defenses against ROS are overwhelmed and a higher steady state level of oxidative stress is attained. Naked mole-rat membrane phospholipids, like that of larger species, contain proportionately less easily oxidized n-3 polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA), but rather have more n-6 PUFAs (such as arachidonic acid) as well as a greater proportion of monounsaturated fatty acids that are more resistant to oxidation. These peroxidation resistant membrane lipids ought to impede the self-propagating autocatalytic process of oxidative damage to adjacent macromolecules and may indeed limit the degree of oxidative damage incurred. The disproportionately high amounts of n-6 PUFA in naked mole-rat membranes may contribute to the very high levels of F_2 isoprostanes. F_2 isoprostanes are routinely used as overall markers of lipid peroxidation, but in essence are only markers of oxidized arachidonic acid and not DHA, the predominant phospholipid in mouse membranes.

Naked mole-rats share this trait of membranes with an exceptionally low peroxidation index relative to body size with both echidnas (40) and humans (73). Moreover, all three species also have similar longevity quotients ($LQ \approx 4$). Similarly, even within a single species, longer lived strains have membranes with a lower peroxidation index (41) and while dietary restriction does not influence mass-specific basal metabolic rate, membrane fatty acid composition varies proportionally with the degree of dietary restriction (26). Collectively, these findings led to the formation of the “membrane pacemaker theory of aging (42),” which has found strong support from studies comparing the long-lived queen and worker bees, as well as several comparative studies in both birds and mammals (39).

High levels of oxidative damage to proteins may affect multiple cellular processes, and thereby contribute significantly to the characteristic age-related decline in functionality and impaired repair of DNA and protein damage with concomitant impacts upon genomic and protein integrity (63). Assessments of protein carbonylation reveal that some proteins bear the brunt of carbonylation and others appear

FIG. 5. Cytosolic GST levels do not correlate with species longevity, lifestyle, or body size. Nevertheless, levels are highest in the longest living rodent, the naked mole-rat. Overall, it appears the naked mole-rat may potentially be utilizing high, constitutive levels of GST as a mechanism to protect against constantly high levels of oxidative damage within the cell. GST, glutathione-S-transferase.

TABLE 1. NAKED MOLE-RATS, WHEN COMPARED TO SHORTER LIVED MICE, SHOW HIGHER LEVELS OF OXIDATIVE DAMAGE TO EVERY MACROMOLECULE EXAMINED TO DATE AND IN A WIDE VARIETY OF TISSUES AND URINE

Oxidative damage	Tissue	Naked mole-rats vs. mice
Isoprostanes	Brain, Heart, Liver, Kidney, Muscle, Urine	↑↑↑↑↑↑↑↑↑↑
Malondialdehyde	Liver	↑↑
Protein carbonyls	Heart, Kidney	↑
Cysteine oxidation	Liver	↑
8-oxo-2'-deoxyguanosine	Liver, Kidney, Urine	Urine: ↓↓↓ Other tissues: ↑↑↑↑↑↑↑↑
Antioxidant levels		
CuZnSOD	Liver	No difference
MnSOD	Liver	↑
Catalase	Liver	No difference
Glutathione peroxidase	Liver	↓↓↓↓↓↓↓↓↓↓
Thioredoxin	Liver	↓
Glutathione	Liver	↓
Glutathione reductase	Liver	↓

Arrows indicate the fold-difference between species. Moreover, antioxidant levels, for the most part, are lower in the longer lived species with only MnSOD showing a slight increase in naked mole-rat liver tissue. Most striking are the incredibly low levels of glutathione peroxidase observed in the naked mole-rat, although these are due Gpx sequence differences and concomitant low levels of selenium.

Gpx, glutathione peroxidase; SOD, superoxide dismutase.

impervious to the high levels of oxidative damage (4). Moreover, analyses of damaged proteins in the subcellular fractions have revealed pronounced species differences among short-lived mice and long-lived naked mole-rats; levels of protein carbonylation were markedly lower in the nuclear and endoplasmic reticular fractions of naked mole-rats when compared to mice, suggesting that these regions responsible for the maintenance of genomic and protein integrity are better protected against damage accrual (10). Protein oxidation in the nuclear compartment may obstruct transcriptional regulation by damaging key enzymes (*e.g.*, polymerases, ribosomal subunits) impairing signaling pathways and even modifying interactions between DNA and histones (12, 50). Moreover, damage to DNA repair enzymes can also have deleterious effects on genomic integrity, leading to the accrual and propagation of mutations and increasing the long-term risk of cancers. The low level of carbonylated proteins in the naked mole-rat nuclear lysates may, therefore, indirectly contribute to their pronounced resistance to cancer.

Surprisingly, it appeared that the observed high protein carbonyls in whole liver lysates were due to the threefold higher levels of protein damage in the mitochondria. The high levels of accrued oxidative damage in the mitochondrial fraction may be indicative of high levels of ROS production, an unexceptional mitochondrial antioxidant defense as well as modest mitochondrial proteolytic activity. Most intracellular carbonylated proteins are eliminated from the cell by the ubiquitin proteasome system (UPS). Low levels of carbonyls in nuclear and endoplasmic reticulum fractions most likely reflects the markedly higher proteasome-specific chymotrypsin-like and trypsin-like proteolytic activity in naked mole-rat liver whole tissue and subcellular fractionated lysates as well as the greater proportion of highly efficient immunoproteasome/hybrid proteasomes and in the naked mole-rat liver tissue (76).

Proteins that escape degradation by intracellular proteolytic machinery may accumulate and clump into large insoluble aggregates (83). It is commonly thought that these

insoluble aggregates play a pivotal role in the decline in healthspan during aging, particularly neurodegeneration. Naked mole-rats, like dietary restricted mice, show markedly lower levels of insoluble protein carbonyls than observed in physiologically age-matched *ad libitum* fed C57Bl6 mice (84). This reduced tendency to form insoluble aggregates may reflect the rapid and efficient removal of soluble protein carbonyls as well as possibly the presence of high levels of small molecular chaperones that may protect proteins against unfolding and formation of aggregates. In support of this premise are our findings that proteins from naked mole-rats or more resistant to unfolding induced by high concentrations of urea or iron ascorbate as well as in response to high temperatures (71).

A striking difference between naked mole-rats and mice is that levels of oxidative damage do not appear to increase with age in naked mole-rats, but rather they maintain high steady state levels, similar to those observed in old mice (2). Not only do naked mole-rats continue to live with this damage, but transcriptome analyses reveal that gene expression is unchanged with age at least in brain, liver, and kidney tissues over a 20-year period (48). At ages equivalent to that of octogenarians, naked mole-rats maintain youthful levels of gene expression as well as cancer-free good health with few signs of pathology related to the high levels of oxidative damage to proteins or DNA. It is possible that they have acquired a suite of cytoprotective defenses to enable them to tolerate the high level of daily oxidative insults without compromising functionality and/or have acquired highly efficient repair mechanisms to maintain DNA and proteome integrity. Evidence in support of this premise is (i) the observation of high levels of expression of many DNA repair enzymes and proteins involved in the UPS (48, 93); (ii) low levels of ubiquitinated proteins and high levels of proteasome activity that are sustained over more than 20 years of life (71, 76); (iii) maintained membrane composition with age (41); and (iv) unchanged levels of oxidative damage to both lipids (2) as well as proteins (71) with aging. We hypothesize that one such mechanism that may be playing a role in the regulation is nuclear factor-

erythroid 2-related factor-2 (Nrf2)-signaling activity, which we find to be constitutively upregulated in naked mole-rat fibroblasts (53). The Nrf2-signaling pathway has been shown to regulate over 600 cytoprotective molecules, including those involved in xenobiotic metabolism, GSH synthesis, and DNA repair, as well as subunits of the proteasome. Thus, this may be a regulator of many of the potential mechanisms already identified as enhanced in the naked mole-rat (52).

Clearly, naked mole-rats are able to tolerate and deal with this level of damage potentially utilizing a variety of means. Unlike shorter lived mice that tend to die within a year of acquiring such high levels, high levels of oxidative damage do not impact upon naked mole-rat longevity. Protective mechanisms may also contribute to the profound cytotoxin resistance observed in naked mole-rat skin fibroblasts compared to that of shorter lived mouse species (53, 78), a phenomena often associated with long-lived organisms (35, 45). Having a higher set point for oxidative damage and concomitant tolerance of this oxidative damage may underlie the large differences in species longevity. This would limit the role of oxidative stress in leading to diseases, including cancer, myopathy, vascular disease, and neurodegeneration, known to result from unchecked ROS. Through greater tolerance of oxidative stress and the mitigation of the translation of this damage into pathological states, naked mole-rats may have arrived at a tolerable disparate adaptive state of flux.

At best, naked mole-rats provide only equivocal support for the oxidative stress theory of aging. While the pro-oxidative intracellular milieu and unexceptional antioxidant defense may explain the high levels of observed oxidative damage and run counter to that predicted by the oxidative stress theory of aging, the absence of age-related changes in antioxidant status and oxidative damage levels provide some support for this theory. However, clearly many other factors play a more dominant role in the aging process. For example, mechanisms are in place to maintain steady state and eliminate damaged proteins and DNA, and thereby maintain proteostasis and genomic integrity. These cytoprotective mechanisms may contribute to the maintenance of a healthy stem cell population, youthful proteome, and genomic integrity and, thereby, prevent many diseases commonly associated with aging.

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Abbreviations Used

8-OHdG = 8-oxo-2'-deoxyguanosine
cGPx = cellular glutathione peroxidase
DHA = docosahexaenoic acid
DNA = deoxyribonucleic acid
Gpx = glutathione peroxidase
GSH = glutathione
GSSG = glutathione disulfide
GST = glutathione-S-transferase
H₂O₂ = hydrogen peroxide
LQ = longevity quotient
MLSP = maximum lifespan potential
Nrf2 = nuclear factor-erythroid 2-related factor-2
PUFA = polyunsaturated fatty acids
ROS = reactive oxygen species
Sod = superoxide dismutase
Sod1 = CuZn superoxide dismutase
Sod2 = Mn superoxide dismutase
UPS = ubiquitin proteasome system