AN ABSTRACT OF THE THESIS OF

Minhazur R. Sarker for the degree of Honors Baccalaureate of Science in Microbiology presented on August 22nd, 2013. Title: A Comparative Analysis of Rodent Species Shows Oxidative Stress Resilience and HSP70 Expression Levels Correlate with Species Longevity.

Abstract approved:

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Tory Hagen

Researchers of healthy aging have determined trends between a species' body mass and its maximal life span (MLSP). Actual MLSPs are then compared to predicted MLSPs and reported as a longevity quotient (LQ). Long-lived mammals, such as humans and naked mole rats (NMR), have LQs ranging from 5.0 to 10.0, meaning they live 5 to 10 times longer than expected. This LQ similarity leads researchers to believe the NMR may be an ideal model organism for human aging research. Among the proposed theories of aging, the oxidative stress theory, which states that cells have a chronic presence of oxidative stress that increases with age, is one of the most widely accepted. Our work delves deeper into this theory as it pertains to the NMR. We observed that NMR fibroblasts (NMRFs) have a much greater resilience to menadione, an oxidative stressor, than mouse fibroblasts (MFs), which, under the oxidative stress theory of aging, may explain why NMR have delayed onsets of aging phenotypes. We then observed that compared to MFs and rat hepatocytes, NMRFs have a greater basal expression of heat-shock protein 70 (HSP70), a primary role player in homeostatic responses to oxidative stress. This greater HSP70 expression may grant the NMRFs a

stronger initial defense allowing them to maintain viability at greater levels of oxidative stress. Finally, our work investigated the use of geranylgeranyl acetone (GGA) as an HSP70 inducer to slow the insults from oxidative stress. We were not able to show GGA as an efficient HSP70 inducer. Ultimately, our work has discovered some key aspects of the naked mole rat, however, further investigation is needed to determine if naked mole rats may serve as the ideal model species for human healthy aging research.

Key Words: Naked Mole Rats, Geranylgeranyl Acetone, HSP70, Aging, Menadione

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A Comparative Analysis of Rodent Species Shows Oxidative Stress Resilience and HSP70 Expression Levels Correlate with Species Longevity

by

Minhazur R. Sarker

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Minhazur R. Sarker, Author

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Last but not least, I would like to share two quotes that keep me going:

"Don't pay much attention to them, focus on math, science, reading, and writing and at the end of the day, you'll be there" **- Mrs. Flowers (1st Grade Teacher)**

"Nothing's hopeless as long as you believe in it" **- A.S. (July 10th 2011)**

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LIST OF ABBREVIATIONS

Maximal Life Span ---------- MLSP
Longevity Quotient ---------- LQ
Basal Metabolic Rate ---------- BMR
Reactive Oxygen Species ---------- ROS
Heat Shock Protein 70 ---------- HSP70
Heat Shock Factor 1 ---------- HSF1
Geranylgeranyl Acetone ---------- GGA
Nucleotide Binding Domain ---------- NBD
Substrate Binding Domain ---------- SBD
Naked Mole Rat Fibroblasts ---------- NMRFs
Mouse Fibroblasts ---------- MFs

DEDICATION

This thesis is dedicated to my mother and father,

Nahid and Mahfuzur R. Sarker.

Introduction

The process of aging is among the most poorly understood biological processes. Traditionally, aging refers to a gradual deteriorating process that results in decreased physiological function, decreased fertility, and ultimately, increased mortality rate (Buffenstein 2008). Researchers have found that animals with slower aging rates generally show minimal declines in physiology, fertility, and even disease resistance (Finch 1990; Austad 1997; Ricklefs 1998; Buffenstein and Jarvis 2002). In order to measure aging rates, researchers utilize two characteristics: maximal life span (MLSP) and the body mass of the species. The reason behind using these two characteristics is that there exists a general trend between body mass and longevity such that a doubling of a species body mass, on average, results in a 16% increase in longevity (Hulbert, Pamplona et al. 2007; Lewis, Mele et al. 2012). The ratio of actual MLSP to this predicted MLSP is then reported as the longevity quotient (LQ) and used as a relative marker of aging rates. *Homo sapiens* and a few other long-living mammals do not follow the relationship of MLSP to body mass and currently there are no widely-accepted explanations for this discrepancy (Prothero and Jurgens 1987; Austad and Fischer 1991; Buffenstein 2005). Atop this list is the naked mole rat (*Heterocephalus glaber*), which similar to humans, has an LQ ranging from 4 to 10 depending on which equation is used to determine the predicted MLSP (Prothero and Jurgens 1987; Lewis, Mele et al. 2012). Fig. 1 (Buffenstein 2008) diagrams the general trend of MLSP with species body mass in rodents, which shows the naked mole rat MLSP

more than two standard deviations away from the trend line. Fig. 2 (de Magalhães, Costa et al. 2007; Buffenstein 2008) diagrams LQ of rodents based on body mass and again, the naked mole rat defies the general trend. As such, the naked mole rat may be an ideal mammal model for human aging research since, like humans, they are outliers on both MLSP vs. body mass and LQ vs. body mass.

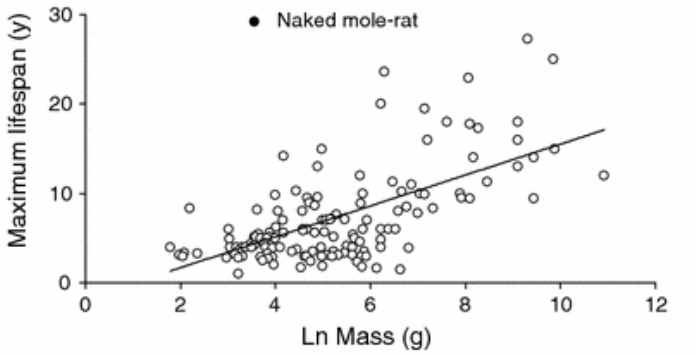
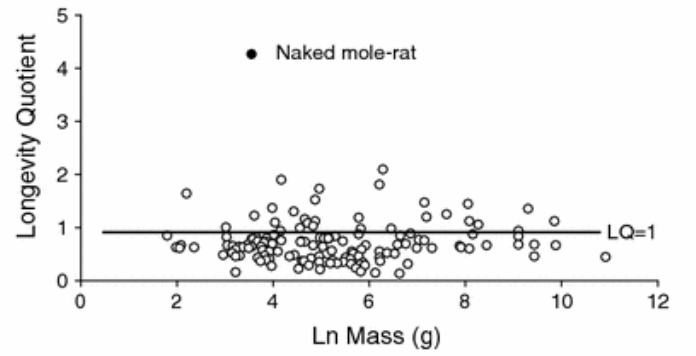


Fig. 1. Naked mole rat lies more than two standard deviations away from predicted maximal lifespan (MLSP). Rodent species display a general trend of MLSP vs. body mass and the naked mole rat is notable exception to this trend. (Buffenstein 2008)



Fig, 2. Naked mole rats live approximately five times longer than expected by mass making it an extreme outlier. Longevity quotients (LQ) of rodents are plotted above, which are ratios of the observed MLSP to the predicted MLSP by body mass. An LQ of one means observed MLSP and predicted MLSP are the same. LQs were calculated using the equation from de Magalhaes et al. (2007) (MLSP = 3.34 $M^{0.193}$ (g)) (Buffenstein 2008)

Naked mole rats hold the record as the longest-living rodent species with an MLSP of 28-30 years. They are eusocial mammals living in colonies underground, much like ants, with one breeding female known as the queen (Buffenstein and Jarvis 2002; Buffenstein 2005; Buffenstein 2008; Azpurua and Seluanov 2013). As a result of living in underground burrow systems, they have become highly adapted to harsh conditions such as low oxygen, high carbon dioxide, and poor ventilation (Jarvis 1981; Sherman, Jarvis et al. 1991;

Lacey, Patton et al. 2000; Gladyshev, Zhang et al. 2011; Delaney, Nagy et al. 2013). These conditions have adapted the naked mole rat to have a low metabolic rate, minimally developed lungs, thermoregulation (poikilothermic), and high circulating hemoglobin and myoglobin concentrations (Jarvis 1981; Sherman, Jarvis et al. 1991; Lacey, Patton et al. 2000; Gladyshev, Zhang et al. 2011; Delaney, Nagy et al. 2013). Previous literature suggests that the naked mole rat is the first known mammal to show negligible senescence over its entire lifespan (Buffenstein 2008). Negligible senescence has three main points: 1) no gradual age-related increase in mortality, 2) no age-related decrease in reproduction rate, and 3) no age-related decline in physiology (Finch 1990; Buffenstein 2008). In addition, naked mole rats have never been documented with any cancers or neoplasms (Buffenstein and Jarvis 2002; Buffenstein 2005; Buffenstein 2008; Seluanov, Hine et al. 2009; Gladyshev, Zhang et al. 2011; Grimes, Lindsey et al. 2012; Azpurua and Seluanov 2013; Delaney, Nagy et al. 2013).

There are three proposed theories to explain the slow aging of naked mole rats. The first theory is the evolutionary theory of aging, which states that living in a protected environment (underground), they have developed selection for physiological function and life-long reproduction (Rose 1991; Austad 1997; Buffenstein and Jarvis 2002). The second theory is the longevity extension theory of social species, which states that living socially results in increased longevity due to overlapping litters, cooperative care, joint foraging, etc (Wilson 2000; Carey and Judge 2001; Buffenstein and Jarvis 2002). The last theory is the environmentally selected life-span theory, which states that living in an unpredictable environment,

nutritionally speaking, results in foraging cooperatively, maintaining an extremely low basal metabolic rate (BMR) and basal energy expenditure, and developing high digestive efficiency to obtain maximal nutrients (Buffenstein 2000; Carey and Judge 2001; Buffenstein and Jarvis 2002). In reality, it is likely that the explanation of naked mole rat longevity is a combination of the three theories proposed. Ultimately, these characteristics of the naked mole rat suggest that they have evolved efficient anti-aging and anti-cancer defenses making this mammal a valuable model organism for aging research (Buffenstein 2005; Buffenstein 2008; Gorbunova, Bozzella et al. 2008; Edrey, Hanes et al. 2011; Delaney, Nagy et al. 2013).

One important anti-aging homeostatic response system that may confer longevity in the naked mole rat is the heat shock protein response. Heat shock proteins (HSPs) are chaperones found in almost all cells that function in overseeing protein quality control. These HSPs decline with age leading to losses in protein stability and ultimately, cell viability (Calderwood, Murshid et al. 2009). HSP70, a specific stress inducible heat shock protein, is among the best-known protectants leading to cellular longevity (Finch 1990; Welch 1992; Morimoto, A et al. 1994; Minowada and Welch 1995; Fink 1999; Jaattela 1999; Kawai, S. et al. 2000; Ikeyama, Kusumoto et al. 2001) and thus, may be a contributing factor in naked mole rat longevity. HSPs are an example of potential anti-aging defenses that may confer longevity in rodent species.

MLSP appears to be taxon specific such that species in similar taxa have similar LQs (de Magalhães, Costa et al. 2007; Buffenstein 2008). However, it would

appear that the naked mole rat observes an LQ that is far higher than relatives in the same taxa such as mice or rats. For example, mouse LQs range from 0.7 to 1.4 and rat LQs range from 0.6 to 1.1 where as naked mole rat LQs range from 5.0 to 10.0 (Prothero and Jurgens 1987; Austad and Fischer 1991; Buffenstein 2005). Currently, the three proposed theories mentioned above attempting to explain the unusual naked mole rat LQ relate to behavioral and environmental aspects rather than on the cellular or the biochemical level. Our work hopes to better understand the discrepancy from a cellular perspective with three aims:

Aim 1: Determine the capacity of naked mole rat fibroblasts and mouse fibroblasts to respond and maintain cellular viability when treated with menadione, a toxicological stressor. We hypothesize that the naked mole rat fibroblasts will display a much greater capacity to respond and remain viable under menadione treatment.

Aim 2: Assess the basal level and robustness of heat shock protein 70 (HSP70) response systems, a homeostatic response system, in various rodent cell types such as naked mole rat fibroblasts, mouse fibroblasts, and hepatocytes from F344 young and old rat. We hypothesize that the naked mole rat will possess a greater basal level and a more robust HSP70 response system than the other rodent cell types.

Aim 3: Determine the efficacy of geranylgeranyl acetone, a pharmaceutical drug, to act as an HSP70 inducer to counteract previously seen age-related declines in HSP70. We hypothesize that geranylgeranyl acetone treatment will induce HSP70 expression levels in the rodent cell types.

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Chapter One:

Naked mole rat fibroblasts are more resilient to menadione, an oxidative stressor, than mouse fibroblasts

1.1 Introduction

To date, there have been hundreds of proposed theories of aging and one of the most studied and widely accepted theories is the oxidative stress theory of aging proposed by Denham Harman in 1956 (Finch 1990; Salmon, Richardson et al. 2010). The oxidative stress theory of aging states that cells have a chronic presence of oxidative stress from an imbalance of pro-oxidants and anti-oxidants leading to lipid, DNA, and protein damage and that this rate of oxidative stress increases with age (Sohal and Weindruch 1996; Beckman and Ames 1998; Grune and Davies 2001; Sohal 2002; Stadtman 2006; Salmon, Richardson et al. 2010). It has also been noted that increases in free radicals and ROS have been seen with an increase in metabolic activity (Commoner, Townsend et al. 1954; Bokov, Chaudhuri et al. 2004). In Fig. 3 (Halliwell 1997; Beckman and Ames 1998; Davies 2000; Bokov, Chaudhuri et al. 2004), we show a simplified biochemical representation of the pathways involved in the oxidative stress theory of aging. This figure shows

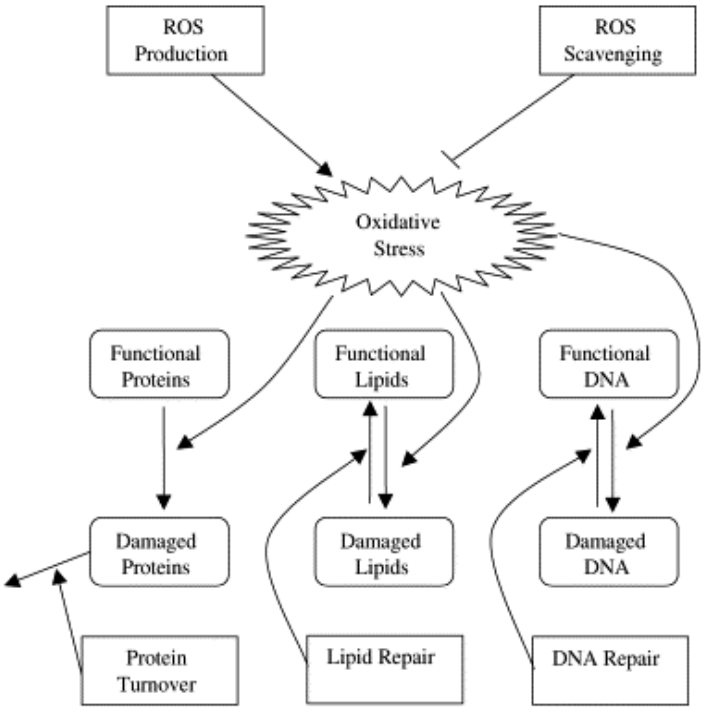

Fig. 3. Summary of the biochemical pathways in the oxidative stress theory of aging. There are two basic pathways: ones that affect net ROS in the cells and the ones that repair or turnover damaged structures. (Bokov, Chaudhuri et al. 2004)

pathways that result in accumulation of ROS as well as the pathways that repair or turn over damaged structures. Within the cell, oxidatively damaged lipids and DNA are repairable whilst damaged proteins must be degraded and newly synthesized (Bokov, Chaudhuri et al. 2004).

If we accept the oxidative stress theory of aging, then three predictions must be met: 1) oxidative damage should increase with age, 2) lifespan extensions should decrease oxidative damage as part of the mechanism, and 3) reducing oxidative damage or ROS production should increase life span (Sohal and Weindruch 1996; Van Voorhies and Ward 1999; Feng, Bussiere et al. 2001; Sohal 2002; Bokov, Chaudhuri et al. 2004). Previous research has shown the presence of an age-related increase in oxidative damage to lipids, proteins, and DNA, thus, supporting the first prediction (Warner 1994; Bohr and Michael Anson 1995; Sohal and Weindruch 1996; Bokov, Chaudhuri et al. 2004). One of the only paradigms to increase maximal lifespan, caloric restriction (Masoro 1988; Weindruch and Walford 1988; Bokov, Chaudhuri et al. 2004; Salmon, Richardson et al. 2010), has shown to decrease the rate of metabolism and subsequent accumulation of oxidative damage, supporting prediction two (Masoro 1988; Sohal and Weindruch 1996; Weindruch and Sohal 1997; Sohal 2002; Bokov, Chaudhuri et al. 2004). Unfortunately, the evidence for the third prediction is a little less clear and further experimentation is necessary. Research has shown that treatment, whether genetic or pharmacological, may show a respective increase or decrease in cell viability, however, it is hard to determine whether the manipulation accumulates with age (Bokov, Chaudhuri et al. 2004). Overall, with the first two predictions being true,

the oxidative stress theory of aging is widely accepted as being at least a partial explanation for the effects of aging.

While holding the oxidative stress theory of aging to be true, one would expect that long-lived animals, such as the naked mole rat (*Heterocephalus glaber*), would accumulate less oxidative damage (Harman 1956; Buffenstein 2008). This relationship does not prove true in the naked mole rat, which exhibits similar levels of ROS and antioxidant defenses but much higher levels of lipid peroxidation, protein carbonylation, and DNA oxidative damage than mice even from a young age (Andziak, O'Connor et al. 2006; Labinskyy, Csiszar et al. 2006; Lambert, Boysen et al. 2007; Buffenstein 2008; Austad 2009; Perez, Buffenstein et al. 2009; Bhattacharya, Leonard et al. 2011; Edrey, Hanes et al. 2011; Rodriguez, Wywial et al. 2011; Delaney, Nagy et al. 2013). Although the level of oxidized molecules is high, naked mole rats do not show any impairment in function suggesting a greater resiliency to cytotoxic insults (Andziak, O'Connor et al. 2006; Pierce, De Waal et al. 2006; Buffenstein 2008; Perez, Buffenstein et al. 2009). These observations suggest that the naked mole rat may possess identical repair mechanisms to other ordent species but has a more resilient response mechanism. These naked mole rat cells also do not show an age-related increase in ROS production or oxidative damage (Pierce, De Waal et al. 2006; Buffenstein 2008; Perez, Buffenstein et al. 2009). Thus, naked mole rats are currently one of the exceptions to the oxidative stress theory of aging.

Our work presented here delves deeper into the third prediction of the oxidative stress theory of aging by introducing mouse fibroblasts and naked mole

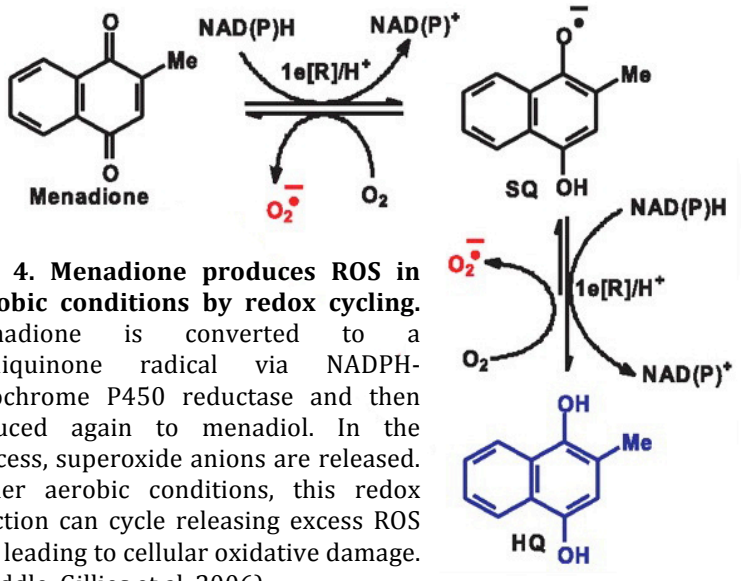

Fig. 4. Menadione produces ROS in aerobic conditions by redox cycling. Menadione is converted to a semiquinone radical via NADPH-cytochrome P450 reductase and then reduced again to menadiol. In the process, superoxide anions are released. Under aerobic conditions, this redox reaction can cycle releasing excess ROS and leading to cellular oxidative damage. (Criddle, Gillies et al. 2006)

rat fibroblasts to an oxidative stressor, menadione. Menadione is a Vitamin K analog (K3) and is capable of redox cycling and arylation, both of which disrupt cellular function (Scott, Atsriku et al. 2005). Lower concentrations of menadione trigger redox-dependent gene expression responses leading to cellular protection (Chuang, Chen et al. 2002; Loor, Kondapalli et al. 2010) while higher concentrations of menadione induce toxic oxidative stress resulting in cell death (Sakagami, Satoh et al. 2000; Loor, Kondapalli et al. 2010). Fig. 4 (Criddle, Gillies et al. 2006) shows the redox cycling mechanism of menadione. First, menadione undergoes a single electron reduction by NADPH-cytochrome P450 reductase yielding a semiquinone radical, which is then further reduced to menadiol (Castro, Mariani et al. 2008). However, under aerobic conditions, the semiquinone radical can participate in redox cycling producing excess amounts of ROS like superoxide anions (O_2^-) (Castro, Mariani et al. 2008). Previous experiments on freshly isolated rat hepatocytes show that menadione treatment results in a loss of cell viability (Toxopeus, van Holsteijn et al. 1993).

We hypothesized that naked mole rat fibroblasts, when compared to mouse fibroblasts, would show a much greater capacity to respond and remain viable in the presence of menadione. To determine this capacity and assess cell viability, we used a lactate dehydrogenase activity assay. With increased oxidative stress, a primary symptom is lipid peroxidation, which decreases membrane integrity, and results in the release of cellular enzymes into the media. LDH is a very stable enzyme that is released into the media and thus, we can use LDH activity as a marker for cell viability. Our results show that naked mole rat fibroblasts are much more resistant to the oxidative stressor, menadione, than mouse fibroblasts.

1.2 Materials and Methods

Cell Culture – Mouse fibroblasts (MFs) and naked mole rat fibroblasts (NMR) were cultured in HyClone DMEM/Low Glucose media containing 10% fetal bovine serum, and incubated at 35°C, which is the optimal temperature for these fibroblasts. Additionally, fresh media was introduced every other day.

Menadione Drug Course Experiments – Menadione was obtained from Sigma Aldrich and placed onto cells using a 0.1% dimethylformamide (DMF) vehicle. MFs and NMR fibroblasts were seeded onto 10cm plates and allowed to grow until approximately 75% confluence. Plates were then treated with vehicle control (0.1% DMF), and various concentrations of menadione. After 24 hours, the media (supernatant) from each plate was collected. These samples were then centrifuged

at 2000rpm for 2 minutes and 1mL of the supernatant was aliquotted and stored in -20°C until utilized for assays.

LDH Assay – Menadione drug course samples were assayed using a Lactate Dehydrogenase Activity Assay Kit from Sigma Aldrich per manufacturer's instructions. Samples were not diluted prior to the assay. A Spectramax190 with the program, SoftMax Pro 5.2, was used to read the 96-well plate. Data was exported and diagramed using GraphPad Prism 6. Finally, data was analyzed with a 2-way ANOVA via GraphPad Prism 6.

1.3 Results

To determine the cells' capacity to respond to the toxicological stressor, menadione, we utilized a Lactate Dehydrogenase (LDH) Assay as a marker for cell viability. Naked mole rat fibroblasts (NMRFs) and mouse fibroblasts (MFs) were treated for 24 hours with various concentrations of menadione in 0.1% DMF solution. The sample supernatants were then assayed for LDH activity (Fig. 5A). These 24-hour menadione concentration courses were repeated for three trials (n=3). Our results show that initially, NMRFs maintain a slightly higher level of LDH than the MFs. However, at concentrations above 18μM, MFs show a significant decrease in cell viability while NMRFs show little change in cell viability. Fig. 5B shows the LDH activity of MFs and NMRFs at 30μM menadione as a percent of the full kill sample. This 30μM treatment killed approximately 74.5% of the MFs while only killing 8.0% of the NMRFs (Fig. 5B). We determined the MF menadione lethal

concentration 50 (LC_{50}) to be approximately 21.3μM. Additionally, a 100μM menadione treatment shows that both cell types observe similar LDH levels during a 100% full kill experiment. We analyzed our data using a 2-way analysis of variance to confirm that the results we obtained are statistically significant (p = 0.0087). These results suggest that the NMRFs may have a stronger oxidative stress response system or have a greater basal oxidative stress resiliency than the MFs.

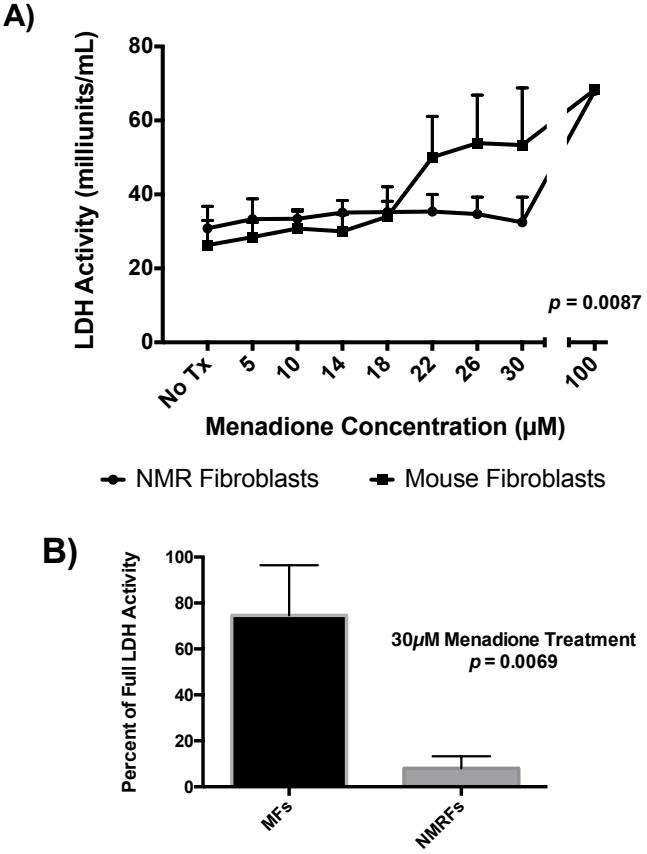

Fig. 5. Naked mole rat fibroblasts are more resilient to menadione than mouse fibroblasts *in vitro.* Naked mole rat fibroblasts (NMRFs) and mouse fibroblasts (MFs) were treated for 24 hours with menadione in 0.1% DMF solution at the indicated concentrations in (A) (100μM correlated to a full kill). Cell supernatants were then assayed for lactate dehydrogenase (LDH) activity as a marker for cell viability. A greater LDH level correlates to less resiliency to the menadione treatment. The chart shows the LDH assay results (mean + SD) across three separate experiments. (p = 0.0087) LDH activity values indicate that a 30μM menadione treatment killed 74.6% of the MFs while only killing 8.0% of the NMRFs (B), suggesting a much greater menadione resiliency in naked mole rat fibroblasts. (p = 0.0069)

1.4 Discussion

In this chapter, we utilized an LDH assay to determine cell viability of naked mole rat fibroblasts (NMRFs) and mouse fibroblasts (MFs) in the presence of an oxidative stressor, menadione. The third prediction of the oxidative stress theory of aging states that a reduction in ROS production should increase life span and here

we show that an increase in ROS decreased cellular viability. From our results, we conclude that NMRFs have a much greater capacity to respond to oxidative stress than MFs. Even with only three replicate experiments, a clear trend is present. The 100μM full kill treatment shows us that at a 100% cell viability loss, both NMRFs and MFs observe the same total LDH activity. Furthermore, since the full kill shows a significant increase in LDH activity for the NMRFs, the finding validates the use of an LDH assay as a marker of cell viability for our comparison.

As stated in the oxidative stress theory of aging, there is an age-related increase in reactive oxygen species (ROS) within cells (Warner 1994; Bohr and Michael Anson 1995; Sohal and Weindruch 1996; Bokov, Chaudhuri et al. 2004). NMR cells may be able mitigate the negative effects that would normally result from this age-related increase in ROS through two mechanisms. The first mechanism hinges on the idea that there is a transitioning concentration at which menadione stops triggering redox-dependent genes and starts causing decreases in cell viability. NMR cells may have a higher transitioning concentration and as a result, are more resilient to increasing concentrations of menadione. A second mechanism favors the idea that NMR cells possess stronger stress response systems and that they are able to induce them for cellular protection when the need is present (ex. menadione-induced ROS production). The greater capacity to withstand oxidative stress by NMRFs may explain why although naked mole rats have greater levels of lipid peroxidation, protein carbonylation, and DNA oxidative damage than mice, they show no negative impact on function (Andziak, O'Connor et

al. 2006; Pierce, De Waal et al. 2006; Buffenstein 2008; Perez, Buffenstein et al. 2009).

Further investigation is needed in order to determine the mechanism that confers the greater capacity in NMRFs to withstand oxidative stress. These experiments can first identify the mechanism utilized by NMRFs, knockdown that mechanism, co-treat the knockdown with menadione, and show a decrease in oxidative stress capacity. This knockdown experiment would confirm that the identified mechanism was resulting in the greater capacity to respond to oxidative stress. Additionally, further experiments can compare the capacity to respond to oxidative stress between cells isolated from young and old animals to determine whether this high NMRF oxidative stress capacity diminishes with age.

1.5 Conclusion

We report that naked mole rat fibroblasts are more resilient than mouse fibroblasts to an oxidative stressor, menadione. This finding is consistent with the naked mole rats ability to maintain a higher level of lipid peroxidation, protein carbonylation, and DNA oxidative damage than mice even from a young age without any impact on cellular function. Further investigation is necessary to determine how the naked mole rat is able to withstand extensive oxidative stress and whether these findings can lead to implications for human healthy aging.

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Chapter Two:

Naked mole rat fibroblasts maintain greater HSP70 levels, both basal and post-heat shock, relative to other rodent species within the same taxa

2.1 Introduction

We have previously shown that naked mole rat fibroblasts are more resistant to a toxicological stressor, menadione, than mouse fibroblasts. Menadione causes stress by inducing the formation of reactive oxygen species (ROS) within the cell via redox cycling (Castro, Mariani et al. 2008). These ROS damage lipids, DNA, and proteins, leading to a significant decrease in cell viability. Naturally, cells have response mechanisms for coping with an oxidative insult. There are a vast number of homeostatic and stress response systems, but one of the most prominent is the heat shock protein (HSP) response system.

Heat shock proteins are a family of highly conserved chaperone proteins found in almost all cells and organisms (Li and Srivastava 2004; Bozaykut, Sozen et al. 2013; Kamal and Omran 2013). HSPs are abundant in cells making up approximately 1-2% of the total protein in unstressed cells and upregulated to 4-6% in response to stress (Garrido, Gurbuxani et al. 2001; Bozaykut, Sozen et al. 2013). These chaperone proteins serve many functions throughout the life cycle of other proteins, as they are involved in protein folding, trafficking, remodeling, and degradation (Calderwood, Murshid et al. 2009; Patury, Miyata et al. 2009; Bozaykut, Sozen et al. 2013; Kamal and Omran 2013). Fig. 6 depicts a simplified HSP response pathway highlighting the key steps and the activation of heat shock factor 1 (HSF1), the key transcription factor for HSPs. Typical stress response inducers include reactive oxygen species, heavy metals, heat-shock, drugs, and inflammation (Lindquist and Craig 1988; Garrido, Gurbuxani et al. 2001; Calderwood, Murshid et al. 2009; Bozaykut, Sozen et al. 2013). These stressors

result in protein damage, which attracts the induced heat shock proteins to repair the damaged proteins by refolding them or, if the damage is far too extensive, target them for degradation (Freeman, Michels et al. 2000; Soti, Nagy et al. 2005; Calderwood, Murshid et al. 2009; Bozaykut, Sozen et al. 2013; Kamal and Omran 2013). Fig. 7 (Calderwood, Murshid et al. 2009) summarizes the four possible fates of damaged proteins in aging cells.

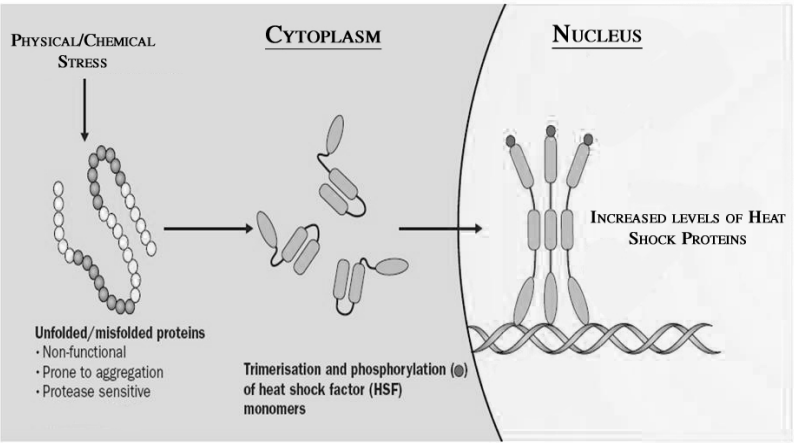

Fig. 6. Summary of a typical heat shock protein (HSP) response pathway. Physical and chemical stressors result in protein, lipid, and DNA damage, which trigger the activation of heat shock factor 1 (HSF1). HSF1, a transcription factor, then relocates to the nucleus to activate HSP genes. Newly synthesized HSPs will then repair cellular damage or target components for degradation.

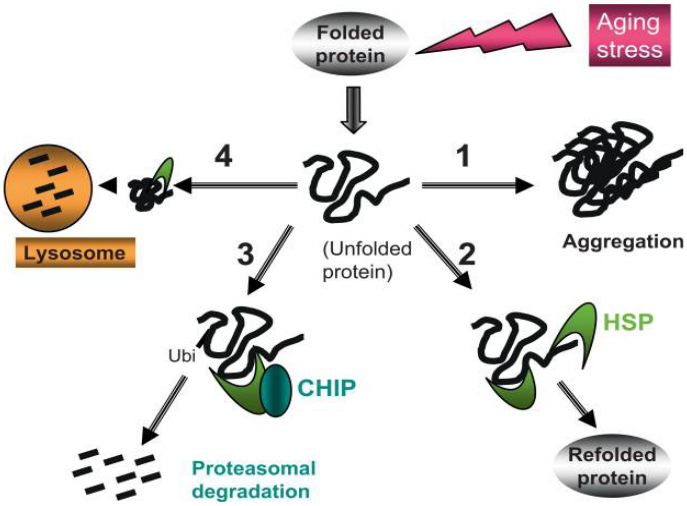

Fig. 7. Denatured proteins from aging stress have four distinct fates. Unfolded proteins can follow one of four fates: 1) in absence of repair, they can aggregate and disrupt cellular signaling, 2) be repaired by HSPs, 3) be targeted by HSPs for proteasomal degradation, and 4) be targeted by HSPs for lysosomal degradation. (Calderwood, Murshid et al. 2009)

Studies have shown that an age-related decline in certain heat shock proteins occurs in neuronal tissues, liver, and skeletal and cardiac muscle (Gagliano, Grizzi et al. 2007; Kayani, Morton et al. 2008; Winklhofer, Tatzelt et al. 2008; Calderwood, Murshid et al. 2009). Over time, cells lose the

ability to activate HSP transcriptional pathways, leading to many physiological disorders (Calderwood, Murshid et al. 2009). For example, in neuronal tissues the decline in HSP synthesis has been shown to lead to a number of neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease (Yasuda, Shichinohe et al. 2004; Winklhofer, Tatzelt et al. 2008; Calderwood, Murshid et al. 2009). These neurodegenerative disorders result from proteins denaturation and formation of aggregates. In turn, this disrupts in cellular signaling and function. As a result of this age-related decline, heat shock protein responses are important therapeutic targets for delaying the onset of aging phenotypes and/or treating degenerative disorders (Haigis and Yankner 2010).

HSP70 is a 70kDa stress-inducible protein and is a dominant role-player of protein homeostasis (Patury, Miyata et al. 2009). Additionally, it is among the best known protectants for cell survival and longevity (Finch 1990; Welch 1992; Morimoto, A et al. 1994; Minowada and Welch 1995; Fink 1999; Jaattela 1999; Kawai, S. et al. 2000; Ikeyama, Kusumoto et al. 2001). Many organisms express different forms of HSP70 highlighting the complexity of the HSP70's functions within the cell (Yasuda, Shichinohe et al. 2004; Patury, Miyata et al. 2009). Constitutive HSP70 levels have been used as a marker for cellular stress, where a greater basal level indicates a greater capacity to respond to cellular stress (Bratton, Jardine et al. 1997; Bozaykut, Sozen et al. 2013).

We speculate that naked mole fibroblasts have a higher capacity to respond to menadione than the mouse fibroblasts as a result of greater HSP70 basal levels and more robust HSP70 stress response systems. A greater basal level will grant

the naked mole rat a stronger initial defense and a more robust response will grant the naked mole rat a greater ability to respond to increasing concentrations of menadione. To test our speculation, we chose to utilize a comparative analysis of rodent heat shock protein systems. Since naked mole rats and mice have longevity quotients of 0.7 to 1.4 and 5.0 to 10.0 (Prothero and Jurgens 1987; Austad and Fischer 1991; Buffenstein 2005), respectively, a comparison of these two rodents shows the differences between heat shock protein systems in long-lived and short-lived rodents. Also since rats have a longevity quotient of 0.6 to 1.1 (Prothero and Jurgens 1987; Austad and Fischer 1991; Buffenstein 2005), a second comparison was made between NMRFs and isolated F344 rat hepatocytes, from both young and old rats, in order to observe the differences in heat shock protein systems with age.

We hypothesized that the naked mole rat fibroblasts would maintain a higher basal HSP70 level as well as possess a more robust HSP70 response system than the mouse fibroblasts. We also hypothesized that the hepatocytes from the old F344 rat will display a lower basal HSP70 expression and a weaker, potentially minimal, robustness of HSP70 response than the hepatocytes from the young F344 rat. Our results show that the naked mole rat fibroblasts maintain a much higher basal level but somewhat similar robustness of response as the mouse fibroblasts and that the hepatocytes from the old rat maintain a significantly lower basal HSP70 expression with a nonexistent response.

2.2 Materials and Methods

Cell Culture – Mouse fibroblasts (MFs) and naked mole rat fibroblasts (NMR) were cultured in HyClone DMEM/Low Glucose media containing 10% fetal bovine serum, and incubated at 35°C, which is the optimal temperature for these fibroblasts. Fresh media was introduced every other day. Fischer 344 rat hepatocytes (young = 6-8 months and old = 24-28 months) were isolated as needed for experiments and cultured in Williams' E Media containing 5% fetal bovine serum, and incubated at 37°C (Shenvi, Dixon et al. 2008). Fresh media was administered daily to these primary cells.

Heat Shock Experiments – Cells of each organism were seeded onto 10cm plates: 1 control and 1 experimental. When the fibroblasts were approximately 75% confluent, the treatments were administered. Primary rat hepatocytes were seeded at 75% and then treatments were administered. The control plates were allowed to grow continuously at the normal 35-37°C while the experimental group plates were heat shocked by placing the plates in a 41°C incubator for 1 hour (incubator did not have CO_2). After a 3-hour recovery period, the plates were scraped and the cell pellet was resuspended in TNSEV (50 mM Tris, 1% NP40, 100mM NaCl, 2mM EDTA, 1mM sodium orthovanadate) lysis buffer with appropriate phosphatase and protease inhibitors. The cell lysates were stored at -80°C until used for SDS PAGE/Western blot.

SDS PAGE/Western Blot – Sample concentrations were determined via a Bio-Rad Protein Assay based on the Bradford protein assay. Assay readings were conducted in a Beckman Coulter DU 800 Spectrophotometer. After assaying, samples were prepared with 2x Laemmli Loading Buffer and loaded onto a Bio-Rad Ready Gel 7.5% Tris-HCl gel. Gel boxes were operated at approximately 120V and proteins were transferred to PVDF membrane via a semi-dry transfer protocol. Membranes were blocked in 3% bovine serum albumin solution for 2 hours. Anti-HSP70 antibody [3A3] (Abcam, Cambridge, MA) incubations were performed for 1 hour and then the membrane was washed for three ten-minute cycles. Anti-mouse IgG HRP-linked antibody (Cell Signaling, Beverly, MA) incubations were performed for 30 minutes and then the membrane was washed for three ten-minute cycles. After the incubations and washes, the membrane was treated for 5 minutes with SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific. Finally, blots were imaged on a Bio-Rad ChemiDoc MP Imaging System with Image Lab 4.0 and proteins levels were quantified using ImageJ (NIH). Data and values were collected and analyzed using GraphPad Prism 6.

2.3 Results

To characterize heat shock profile systems in rodent species, we utilized cell culture experiments followed by Western blot analysis to quantify HSP70 levels. Various rodent cell types were treated with a 41°C heat shock for 1-hour followed by a 3-hour recovery. This 3-hour recovery period is critical in order to allow the

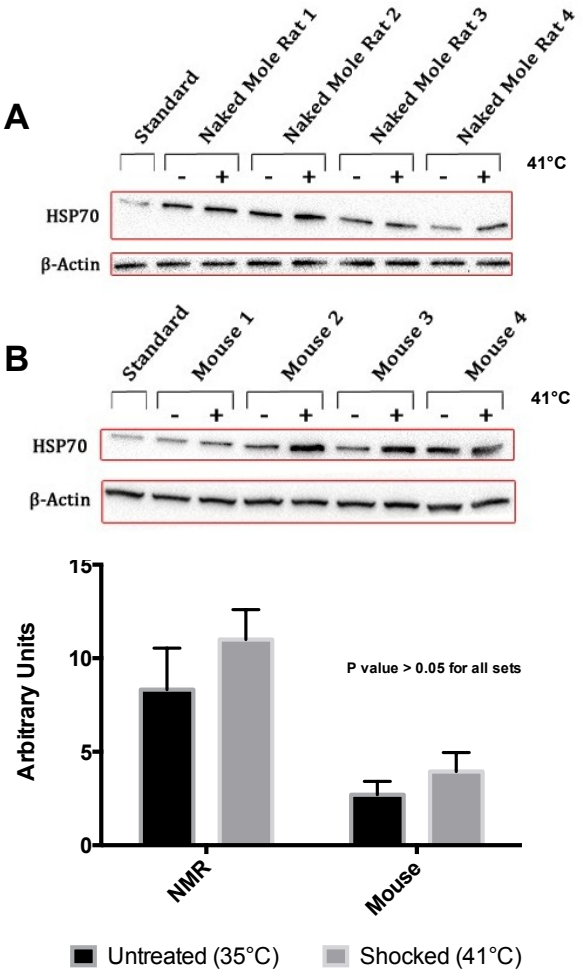

Fig. 8. NMRF HSP70 response systems are far more robust than MFs suggesting a correlation with species longevity. HSP70 levels were assessed by Western blot using protein samples from naked mole rat fibroblasts (NMRFs) (A) and mouse fibroblasts (MFs) (B) treated with a 41°C heat shock for 1-hour followed by a 3-hour recovery. All samples were analyzed against a standard HepG2 protein sample to insure consistency across blots. NMRFs express a greater basal HSP70 level than MFs and maintain a higher HSP70 expression post heat shock. NMRFs maintain a 3.5-fold greater basal HSP70 level than the MFs. This difference decreases to 3-fold post heat shock implying the robustness of HSP70 response systems in MFs may be greater than that of the NMRFs. NMRFs HSP70 levels, when compared to the MFs, suggest that HSP systems may correlate to species longevity in rodents.

cells to sense the heat shock, trigger HSP70 transcriptional activators, and translate new HSP70. To insure consistency across blots and species, we utilized a single standard (HepG2 protein sample) and calculated all values as a relative measure against the standard.

Naked mole rat fibroblast (NMRF) HSP70 response systems are far stronger than that of mouse fibroblasts (MFs) (Fig. 8). At a basal level, NMRFs maintain almost a 3.5-fold greater level of HSP70 than the MFs. Post heat shock, NMRFs and MFs increase their HSP70 levels by 32.2% and 45.9%, respectively, which brings the difference down to 3-fold. This result suggests that the MFs have a more robust HSP70 response than the NMRFs, but even post heat shock, the NMRFs maintain a much higher level of HSP70.

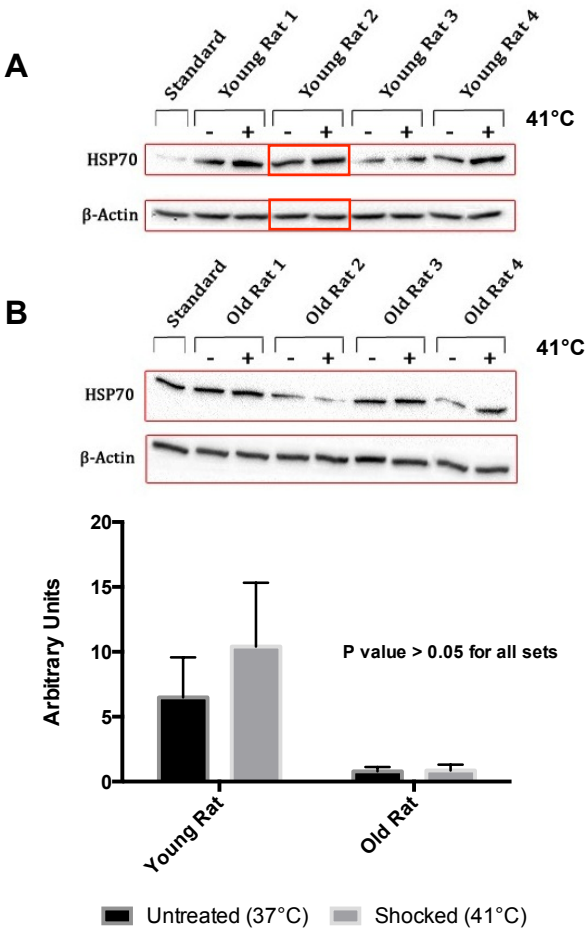

Fig. 9. HSP70 basal level and robustness of response decrease with age in F344 rat hepatocytes. HSP70 levels were assessed by Western blot using protein samples from F344 rat hepatocytes, both young (A) and old (B), treated with a 41°C heat shock for 1-hour followed by a 3-hour recovery. All samples were analyzed against a standard HepG2 protein sample to insure consistency across blots. Young rat hepatocytes express an approximately 8-fold higher basal level of HSP70 than the old rat hepatocytes, which increases to approximately 12-fold post heat shock. Old rat hepatocyte HSP70 levels, when compared to the young rat hepatocytes, confirm that HSP systems decline with age both in the basal level as well as the robustness of response.

F344 rat hepatocyte HSP70 response systems decrease with age in both basal level and robustness of response (Fig. 9). At a basal level, hepatocytes from young rats maintain an 8-fold greater level of HSP70 than hepatocytes from old rats. This difference increases to 12-fold post heat shock as a result of the hepatocytes from young rats increasing their HSP70 by 60.2% while the hepatocytes from old rats increase their HSP70 by only 10.1%. This result confirms the age-related decline of heat shock protein systems seen in the literature.

For all species, n = 4 and an unpaired t-test suggests the results are not significant ($p > 0.05$), however, a clear trend is present in the data.

2.4 Discussion

We observed a significant difference in HSP70 basal levels between naked mole rat fibroblasts (NMRFs) and mouse fibroblasts (MFs). NMRFs maintain a 3.5-fold higher basal level of HSP70 than MFs, which decreases to 3-fold post heat shock. This reduction indicates that although NMRFs maintain a higher basal expression of HSP70, the robustness of shock related induction of HSP70 in both cell types is similar and maybe even more robust in MFs. Since HSP responses are vital role players in response to oxidative stress (Calderwood, Murshid et al. 2009; Bozaykut, Sozen et al. 2013), this result may explain why NMR cells display a greater capacity to respond to oxidative stress as seen in our previous work. To confirm this correlation, we could develop HSP70 knockdown NMRFs and MFs, treat them with menadione, and show that this specific knockdown results in a loss of NMRFs ability to respond to oxidative stress.

Our results also confirmed an age-related decline in HSP70 response systems in isolated F344 rat hepatocytes. Hepatocytes from old rats show an 8-fold lower HSP70 basal expression with an almost non-existent induction post-heat shock when compared to hepatocytes from young rats. This age-related decline has previously been seen in multiple regions and especially in liver cells (Gagliano, Grizzi et al. 2007). The widely accepted oxidative stress theory of aging states that over time, reactive oxygen species (ROS) increase and as a result, cellular components (lipids, DNA, and proteins) become highly damaged leading to decreases in cellular viability (Harman 1956). The age-related decline in HSP70 response systems would result in decreased repair and turnover mechanisms,

which would allow ROS damaged molecules to accumulate in cells. As such, age-related declines in HSP70 are consistent with the oxidative stress theory of aging.

Although statistical analysis with Student's t-test produced *p*-values > 0.05 for all samples, a clear trend is present such that more replicate experiments could result in statistical significance. Additionally, the gel box utilized for the naked mole rat fibroblast samples and the old rat hepatocyte samples may have caused the Bio-Rad Ready Gel 7.5% Tris-HCl gel to distort and thus caused the bands to appear angled rather than linear as seen in Fig. 8 and Fig. 9. This distortion does not affect our data since molecular weight marker measurements confirmed the HSP70 bands were approximately 70kDa. On the blot image for the young rat samples, the indicated boxed region shows a sample that has been cropped and placed in. This sample was originally run incorrectly and the bands placed in its spot are the correct samples. This latter cropped in sample was also run under the same protocol and with the same standard sample as a relative measure, and thus, the subsequent data analysis is accurate.

2.5 Conclusion

Our results observed that naked mole rat fibroblasts maintain a higher basal HSP70 expression than mouse fibroblasts and that there exists an age-related decline in HSP70 response systems in F344 rat hepatocytes. These differences in HSP70 expression levels are of concern since HSP70 is a vital role-player against oxidative stress in aging cells. Delaying the onset of the age-related decline in

HSP70 expression or finding a means of pharmacologically or genetically inducing HSP70 levels may prove quite worthwhile for the future of aging research.

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Chapter Three:

Geranylgeranyl acetone (GGA) is not an efficient HSP70 inducer in rodent species

3.1 Introduction

From the heat shock profiling experiments, our longevity comparative analysis results showed that naked mole rat fibroblasts maintain a much higher level of basal HSP70 than the mouse fibroblasts and this trend remains even post-heat shock. Our age-related rodent comparison of F344 young and old rat hepatocytes confirmed previous studies suggesting that HSP70 response systems decline with age. Fig. 10 shows a quick summary of our results from the comparative analysis experiments.

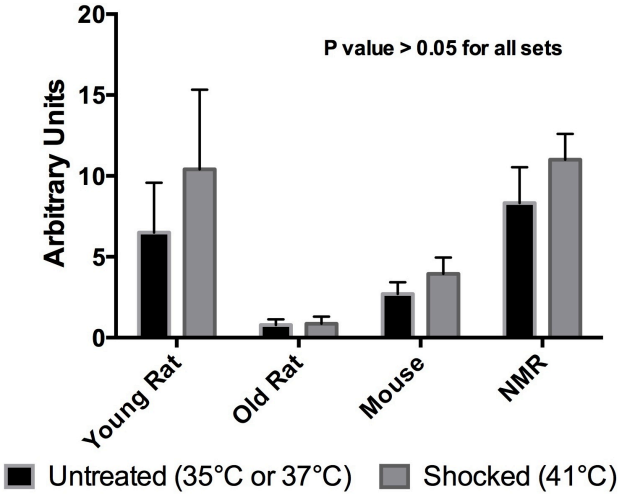

Fig. 10. NMRFs HSP70 response systems are far more robust than MFs and rat hepatocytes show an age-related decline in HSP70 response systems. A quick summary of our results from chapter two highlighting that NMRFs maintain a higher basal level of HSP70 and have a more robust response system. Additionally, we confirmed age-related declines in HSP70 response systems using F344 young and old rat hepatocytes.

This notable age-related decline in HSP70 is of major concern to researchers of healthy aging since HSP70 is one of the best-known factors protecting cells (Finch 1990; Welch 1992; Jacquier-Sarlin, Fuller et al. 1994; Morimoto, A et al. 1994; Minowada and Welch 1995; Fink 1999; Jaattela 1999; Kawai, S. et al. 2000; Ikeyama, Kusumoto et al. 2001). A significant decrease in HSP70 levels, as seen in the hepatocytes from F344 old rats, results in an accumulation of protein aggregates that disrupt simple cellular pathways and functions and thus, HSP70 is a prime target for therapeutic drug targeting. Unfortunately, most HSP inducers are cellular toxins (Wiegant, Spieker et al. 1988; Kamal and Omran 2013). Structurally

speaking, HSP70 has a substrate binding domain and a nucleotide-binding domain, which are locations of interest for therapies. Both domains have been utilized in attempts to find a way to induce HSP70 *in vitro* (Patury, Miyata et al. 2009).

There have been a few novel treatments involving the nucleotide-binding site, however, none have been commercially licensed:

1) Nucleotide mimetics: compounds that resemble ATP and bind the nucleotide binding site and affect the activity of HSP70 and/or affinity of HSP70 for substrates (unfolded proteins) (Patury, Miyata et al. 2009; Powers, Clarke et al. 2009; Williamson, Borgognoni et al. 2009)
2) (−)-epigallocatechin gallate (EGCG): an antioxidant that binds ER-resident HSP70 and triggers apoptotic activity making it a potential cancer therapy drug (Ermakova, Kang et al. 2006; Patury, Miyata et al. 2009)
3) MKT-077: a cationic, rhodacyanine dye that binds mitochondrial HSP70, potentially disrupts p53-mtHSP70 interactions and activates apoptotic signaling making it another potential anti-cancer drug (Wadhwa, Sugihara et al. 2000; Kaul, Reddel et al. 2001; Patury, Miyata et al. 2009)
4) Sulfoglycolipids: a glycolipid that binds to testis-specific HSP70 to decrease V_{max}, hinting at a potential noncompetitive mechanism (Boulanger, Faulds et al. 1995; Patury, Miyata et al. 2009)

Additionally, there have also been a few novel treatments involving the substrate-binding site:

1) Substrate mimetics: short antibacterial peptides have shown to bind *E. coli* DnaK and effectively manipulated chaperone function (Otvos, O et al. 2000; Patury, Miyata et al. 2009)
2) Acyl benzamides: compounds that bind and competitively inhibit *E. coli* DnaK and show anti-HSP70 functions suggesting a potential anti-micorbial and anti-cancer therapy (Patury, Miyata et al. 2009)
3) Geranylgeranyl acetone (GGA): a pharmacological inducer of heat shock proteins that induces cellular stress responses without damaging the cell (Sinn, Chu et al. 2007; Patury, Miyata et al. 2009)

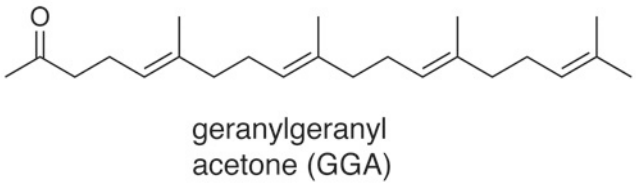

Fig. 11. Structure of Geranylgeranyl Acetone (GGA). GGA has been commercially licensed as an antiulcer drug in Japan and has been shown to be efficient HSP70 inducer via induction of HSF1.

The majority of treatments and drugs have focused on anti-cancer therapy rather than anti-aging. Of the seven example treatments above, only geranylgeranyl acetone (GGA) shows promise as an anti-aging therapy. Fig. 12 shows the chemical structure of GGA, which is known for its uses as an anti gastric ulcer drug in Japan for the past two decades (Hirakawa, Rokutan et al. 1996; Kawai, S. et al. 2000; Ikeyama, Kusumoto et al. 2001; Yasuda, Shichinohe et al. 2004; Namba, Tanaka et al. 2011; Kamal and Omran 2013), and thus, we know that GGA is not detrimental to cells in the human body. Many research groups have shown GGA to induce HSP70 in various cell types such as hippocampal neurons of rats and

gastric mucosa of guinea pigs and rats (Hirakawa, Rokutan et al. 1996; Kawai, S. et al. 2000; Ikeyama, Kusumoto et al. 2001; Yasuda, Shichinohe et al. 2004; Namba, Tanaka et al. 2011). As an efficient HSP70 inducer, GGA could provide a protective effect against various cellular stressors such as reactive oxygen species, free radicals, heavy metals, and heat-shock (Tanaka, Namba et al. 2007; Ogawa, Shimizu et al. 2008; Suemasu, Tanaka et al. 2009; Namba, Tanaka et al. 2011; Kamal and Omran 2013). Mechanisms of GGA action are only beginning to emerge but it has been reported that GGA acts via activating the transcriptional activator, heat shock factor 1, and not by directly activating HSP70 (Ikeyama, Kusumoto et al. 2001; Hoogstra-Berends, Meijering et al. 2012). Currently, the viability of GGA as an anti-aging therapy is still being explored.

We chose to utilize GGA to see if we could induce HSP70 levels in mouse fibroblasts that match those in naked mole rat fibroblasts. After confirming GGA as an HSP70 inducer, we hoped to co-treat these cells with GGA and menadione, an oxidative stressor, to determine if pharmacologically induced HSP70 offers protective effects and mitigates the loss in mouse cell viability seen in our previous work. Additionally, we chose to utilize GGA on hepatocytes from F344 old rats to see if we could rejuvenate declining HSP70 response systems back to the level found in hepatocytes from F344 young rats.

We hypothesized that GGA will function as an efficient HSP70 inducer in both experiments and will also offer protective effects against menadione in the mouse fibroblasts. Unfortunately, our results were not able to confirm GGA as an

efficient HSP70 inducer as shown in the literature and our subsequent experimental approaches were placed on hold.

3.2 Materials and Methods

Cell Culture – Mouse fibroblasts (MFs) and naked mole rat fibroblasts (NMR) were cultured in HyClone DMEM/Low Glucose media containing 10% fetal bovine serum, and incubated at 35°C, which is the optimal temperature for these fibroblasts. Fresh media was introduced every other day. Fischer 344 rat hepatocytes (young = 6-8 months and old = 24-28 months) were isolated as needed for experiments and cultured in Williams' Media E containing 5% fetal bovine serum, and incubated at 37°C (Shenvi, Dixon et al. 2008). Fresh media was administered daily to these primary cells.

GGA Time Course Experiments – GGA was obtained from Sigma Aldrich and was placed onto cells using a 0.1% dimethylformamide (DMF) vehicle. Cells of each organism were seeded onto 6-well plates and treated at a concentration of 100μM GGA. Each time course had a vehicle control (0.1% DMF), and various time points to follow the course of the drug treatment. Samples were harvested by scraping the plates and the cell pellets were resuspended in TNSEV (50 mM Tris, 1% NP40, 100mM NaCl, 2mM EDTA, 1mM sodium orthovanadate) lysis buffer with appropriate phosphatase and protease inhibitors. The cell lysates were stored at -80°C until used for SDS-PAGE/Western blot.

SDS PAGE/Western Blot – Sample concentrations were determined via a Bio-Rad Protein Assay based on the Bradford protein assay. Assay readings were conducted in a Beckman Coulter DU 800 Spectrophotometer. After assaying, samples were prepared with 2x Laemmli Loading Buffer and loaded onto a Bio-Rad Ready Gel 7.5% Tris-HCl gel. Gel boxes were operated at approximately 120V and proteins were transferred to PVDF membrane via a semi-dry transfer protocol. Membranes were blocked in 3% bovine serum albumin solution for 2 hours. Anti-HSP70 antibody [3A3] (Abcam, Cambridge, MA) incubations were performed for 1 hour and then the membrane was washed for three ten-minute cycles. Anti-mouse IgG HRP-linked antibody (Cell Signaling, Beverly, MA) incubations were performed for 30 minutes and then the membrane was washed for three ten-minute cycles. After the incubations and washes, the membrane was treated for 5 minutes with SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific. Finally, blots were imaged on a Bio-Rad ChemiDoc MP Imaging System with Image Lab 4.0 and proteins levels were quantified using ImageJ (NIH). Data and values were collected and analyzed using GraphPad Prism 6.

3.3 Results

To determine the efficacy of geranylgeranylacetone (GGA) as an HSP70 inducer, rodent cells were treated with 100μM GGA in 0.1% DMF at various time intervals as shown in Fig. 13 and then the samples were analyzed by Western blot. GGA did

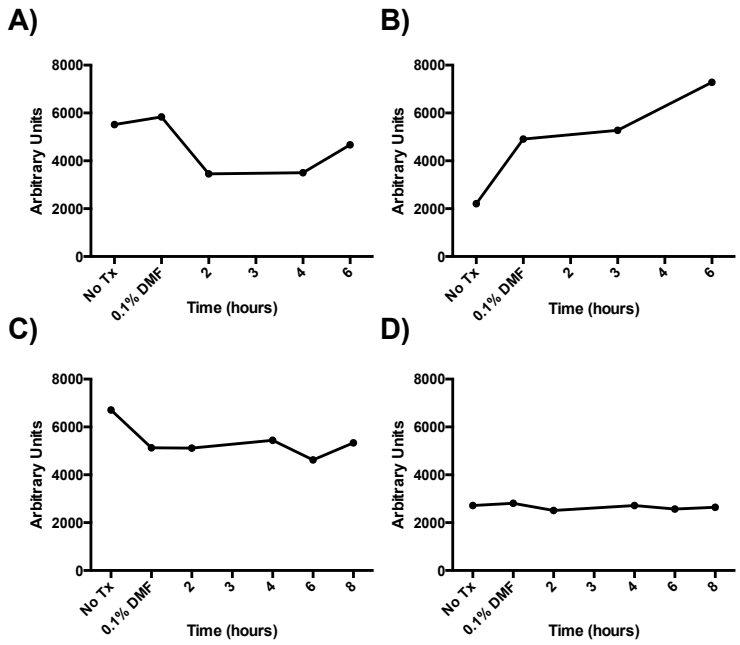

Fig. 12. Geranylgeranylacetone is not an efficient inducer of HSP70 in rodent cells. HSP70 levels were assessed by Western blot using protein samples from naked mole rat fibroblasts (A), mouse fibroblasts (B), and F344 hepatocytes from young rats (C) and old rats (D) treated with 100μM Geranylgeranylacetone (GGA) in 0.1% DMF for the indicated time intervals. GGA did not efficiently induce HSP70 in any of the rodent cell types tested and furthermore, in the naked mole rat fibroblasts, GGA initially knocked down the expressed level of HSP70.

not significantly induce HSP70 in any of the rodent cell types (Fig. 13). The hepatocytes from F344 young rats show a slight decrease in HSP70 compared to the DMF control and subsequent GGA treatment resulted in no significant difference. The hepatocytes from F344 old rats show no response to the GGA treatment or the DMF control. Of all the cell types, only mouse fibroblasts exhibited an HSP70 induction with GGA treatment, however, the induction was not significant when compared to the DMF control. Additionally, naked mole rat fibroblasts show a significant decline in HSP70 levels with a 2h GGA treatment, which returns to normal levels at 6h post treatment. Overall, GGA is not an efficient HSP70 inducer in the rodent cell types tested.

3.4 Discussion

We were unable to confirm geranylgeranyl acetone (GGA) as an efficient HSP70 inducer in naked mole rate fibroblasts (NMRFs), mouse fibroblasts (MFs), or hepatocytes from F344 young and old rats. Previous research has shown GGA to be an efficient HSP70 inducer (Kawai, S. et al. 2000; Yasuda, Shichinohe et al. 2004) and we wished to utilize GGA for two reasons: 1) induce MF HSP70 levels to that seen in NMRFs and then introduce an oxidative insult (menadione) to determine if induced HSP70 results in oxidative protection, and 2) induce HSP70 expression in hepatocytes from old rats to that seen in hepatocytes from young rats. Unfortunately, as of not being able to confirm GGA as an HSP70 inducer, our further work was placed on hold.

Hepatocytes from both young and old F344 rats show no response to GGA since HSP70 expression levels remain fairly consistent throughout the entire time course (Fig. 13). This led us to conclude that perhaps GGA is not an efficient HSP70 inducer in hepatocytes. MFs show a slight HSP70 induction with GGA, however, the induction relative to the no-treatment and DMF 0.1% (vehicle) control was fairly insignificant. Once again, we concluded that GGA was not an efficient HSP70 inducer. NMRFs show an interesting response such that GGA treatment knocked down HSP70 expression. This surprising result may suggest that NMRFs possess a maximized HSP70 response system and any outside intervention may only serve to decrease the efficiency rather than increase it further.

We performed many experiments with GGA changing concentrations, time intervals, vehicles (DMSO, DMF, or ethanol), and even alterations in the media (data not shown). All of our attempts produced similar results, that is no HSP70

induction. After speculating that our GGA may be defective, we ordered fresh GGA and performed a round of experiments with no successful induction. All of our results, collectively, lead us to conclude that GGA is not an HSP70 inducer in rodent cells. Upon further investigation, we speculate that GGA may never have adequately reached our cells. The structure of GGA confers hydrophobic characteristics, which may have been an issue during our drug treatments. Additionally, in the literature GGA has shown HSP70 induction during gastric ulcer treatments, which leads us to believe a gastric acidic pH activation may be crucial. As it stands currently, we believe that GGA may not be a future anti-aging remedy as we had previously hypothesized.

3.5 Conclusion

Our results from the geranylgeranyl acetone (GGA) time course failed to confirm GGA as an efficient HSP70 inducer and as such, we did not complete the proposed experiments. Looking forward, we hope to determine the efficacy of other pharmacological drugs as HSP70 inducers as well as further study GGA to ensure that it truly does not have a future as an anti-aging remedy. Shortly after GGA experiments had begun, we started testing the efficacy of a few other drugs: acetaminophen, lipoic acid, and tunicamycin, which all need further investigation to fully characterize their ability to induce HSP70 (data not shown). Finding a compound to reverse age-related declines in HSP70 and potentially chemically induce oxidative stress protectants would lead to overall healthier aging.

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Conclusion

The work presented in this thesis has delved deeper into understanding why a long-lived rodent species, the naked mole rat, exceeds the LQ for rodents in general. We observed a greater capacity in naked mole rat fibroblasts (NMRFs) than mouse fibroblasts (MFs) to respond to an oxidative stressor, menadione. We then proposed that this greater capacity is a result of stronger homeostatic response systems such as heat shock proteins (HSPs). We focused on HSP70 and observed a much greater basal HSP70 expression in NMRFs than in MFs. We also confirmed an age-related decline in HSP70 expression, both basal and robustness of response, in primary hepatocytes isolated from F344 young and old rats. Since HSP70 is such a vital role-player in maintaining cell longevity and viability, we investigated potential HSP70 inducers as a way to counteract age-related declines and confer an oxidative stress capacity in MFs that matches NMRFs. We focused on a pharmacological drug, geranylgeranyl acetone (GGA), but we were not able to conclude GGA to be an efficient HSP70 inducer in rodent species, especially compared to actual heat shock, which can be viewed as a positive control. This GGA conclusion will need further analysis to determine if the GGA, a hydrophobic compound, ever reached the cells during the treatment process.

Further investigation is needed to confirm that HSP70 response systems are responsible for the greater capacity to respond to oxidative stress in NMRFs. One confirmation experiment we propose is by utilizing HSP70 knockdown rodents then co-treating these rodents with menadione and showing a decline in oxidative

stress capacity. Along with further HSP70 experiments, PCR and expression assays following heat shock factor 1, HSF1, will determine if the HSP70 induction we observed is by transcriptional or translational means. These HSF1 experiments may even conclude that GGA confers a slow HSP70 induction and thus, maybe even our 24h time courses were not long enough (data not shown). Although we concluded GGA as an inefficient HSP70 inducer, the future of other potential pharmacological HSP70 inducers such as tunicamycin, acetaminophen, and lipoic acid, warrant further investigation. Additionally, determining the basal expression levels and robustness of response of other HSPs such as HSP60 and HSP90 may prove to be a worthwhile endeavor in painting a complete HSP profile.

Our experimental approach utilized good model systems when comparing naked mole rat fibroblasts with mouse fibroblasts and comparing hepatocytes from F344 young and old rats. Concerns may arise when comparing across these two models (ex. comparing naked mole rat fibroblasts to hepatocytes from young rats) since these are two vastly different cell types and HSP70 and oxidative stress resiliency may not be comparable across cell types. Ideal future experiments would look towards using all fibroblasts or all hepatocytes, however, that was not possible for our preliminary work presented here. Ultimately, we investigated the prospect of using the naked mole rat as a model species within the context of the oxidative stress theory of aging but further work is needed to fully conclude the naked mole rat as a model species for human healthy aging.

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