Oxidative Stress Resistance and Viability of *C. elegans* in the Presence of Manganese through SKN-1 Protein Expression

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Oxidative stress plays an imperative role in viewing how humans respond to diseases, such as diabetes and cancer, as well as in the process of aging. Through research and experimentation of Caenorhabditis elegans, scientists can study organismal and cellular aging that is analogous to that of humans. *C. elegans* are used as an ideal model of study due to their eukaryotic existence, as well as the relative ease of development and growth protocol. Through *C. elegans*, researchers can examine relevant signaling pathways, such as those that regulate metabolism, nutrition, and stress responses. As the complete genome for *C. elegans* has been identified, researchers known the exact cell differentiation pattern of each cell, therefore allowing for in depth study about the responses of *C. elegans* to different conditions and stresses. The SKN-1 protein in this species initiates development of the digestive tracts and other mesendodermal tissues during the primary stages of *C. elegans* development. By studying the genetic mechanisms that are rooted in *C. elegans* aging, humans have the opportunity to identify new human genes, as well as the pathways associated with both disease and aging in humans.

Overview of Oxidative Stress Responses of Organisms

The response of animals to environmental stresses is controlled by a coordination of many cellular regulatory factors, in providing protection in the form of cellular responses in a three-step process. Firstly, a cellular protein that signals the changes detects the environmental changes. Next, gene expression machinery receives the transduced signal from a sensor, and thirdly, the transduced signal activated a variety of transcription factors. Once these factors are activated, a set of stress-responsive genes is then expressed, which gives rise to cellular protection within the organism. This three-step process is imperative in retaining a level of homeostasis within the cellular components of the animal, so the processes must be highly regulated and coordinated in order to ensure the smooth functioning of the stress response.

In the category of environmental stresses, one prominent class is that of oxidative stresses, as oxidative stresses are known to be one of the sources of numerous human diseases, including diabetes, cancer, atherosclerosis (a chronic inflammatory disease affecting arterial blood vessels), and aging (Johnson, 2002). Oxidative stress responses are based off the principle that organisms need to employ effective cellular strategies to detect and provide a detoxification response to metabolites of molecular oxygen (also known as reactive oxygen species), as these are necessitated by their lives in oxygenated environments. The connection to aging and life span is found through the intensity and overall ability of organisms to provide a cellular response to oxidative stress, in addition to the organism's ability to produce appropriate or inappropriate oxidants.

The Utilization of Caenorhabditis elegans in Oxidative Experimentation

The nematode *Caenorhabditis elegans* is regularly used in scientific experiments dealing with oxidative stress, as there has been plenty of research collected in the past ten years sowing that *C. elegans* are an ideal model organism to study and test for cellular and organism aging. *C. elegans* having pathways that are comparable to those of humans in the regulation of nutrition, metabolism, life span, and stress response (Hertweck, 2005). The worm can be easily experimented with, as it is sensitive to a number of charged and uncharged molecules, such as heavy metals, organic toxins, and human neuroactive drugs, which can all easily penetrate the brain (Nass, 2007).

Strains of *C. elegans* are desirable to scientists, as they are inexpensive to breed and can also be frozen so that they can be used at a later

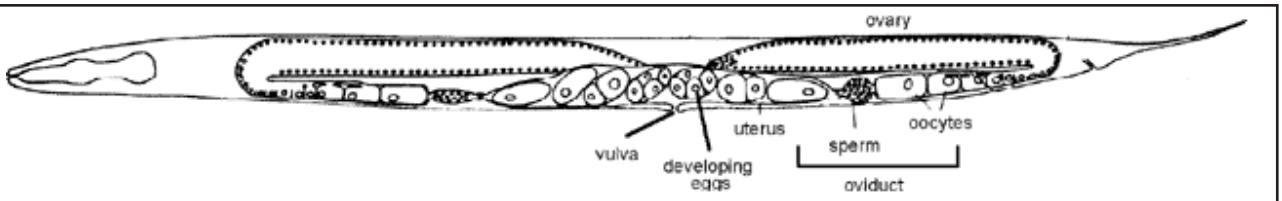

Figure 2. Diagram courtesy of Waksman Student Scholars of Rutgers University.

time. Recently, researchers have been able to map and sequence the entire genome of the *C. elegans*, leading to the implementation of genome-wide investigation and monitoring of gene expression. The monitoring of gene expression allows researchers to better identify the causations of the diseases that can severely render the pathways unable to perform their functions of stress responses. In addition to this, researchers are able to determine new genes and pathways that are associated with the process of aging. As the complete lineage of the *C. elegans* species has been determined, this species can be utilized in studying cellular differentiation, especially in the area of cellular responses.

C. elegans are a multicellular eukaryote that is the simplest organism with a complete nervous system. Researchers have explored the neural mechanisms dealing with behaviors such as chemotaxis, thermotaxis, mating behavior, etc. It is important to note that although they have a development nervous system, *C. elegans* differ from humans in the fact that their neurons lack the ability to fire action potentials when generating a response. *C. elegans* are also useful in the way in which researchers can easy isolate genes through RNA interference (RNAi), therefore disrupting specific gene function, and allowing researchers to easily deduce what exactly a gene's function is. Worms can ingest genetically transformed bacteria, expressing the double stranded RNA being used, or they can be injected with a solution of double stranded RNA (Baumeister, 2006). These double stranded RNA sequences are complementary to the sequences of the gene that is being disabled, and therefore the function of the genes are disabled.

The Cycle of Life: Stages of Development of C. elegans

C. elegans exists as either a hermaphrodite or a male, with the most common sexual form being the hermaphrodite. Hermaphroditic *C. elegans* lay the eggs and produce sperm, and when self-fertilization occurs, about 300 progeny are produced. Self-fertilization contributes to the homozygosity of alleles, so as long as mutations do not occur, then the individual worms are determined to be genetically identical. After these eggs are fertilized and hatch, they pass through four larval stages titled L1 through L4.

In the L4 stage, hermaphrodites produce all their sperm and then switch over to producing oocytes (see figure above for *C. elegans* sexual anatomy). When self-inseminated, the wild-type hermaphrodite worm produces about 300 eggs, but when inseminated by a male worm, the progeny can exceed 1,000 eggs. The dauer state is the alternative third larval stage of *C. elegans*, which is induced upon overpopulation of the species in a plate or by the absence of food and sustenance on the plate. The dauer larvae do not age and are found to be stress-resistant as well. The average life span of *C. elegans* is about 2-3 weeks, with a generation of approximately 4 days if kept at a temperature of 20°C.

The Use of Manganese in Causing Oxidative Stress

The trace element manganese is essential for good health, yet overexposure or deficiency of the mineral in the body poses dangerous health risks for the individual through oxidative stress. We are exposed to small amount of manganese on a daily basis through water, air and food (ranging from grains and cereals to many fruits and vegetables), but overexposure causes nervous system problems like cognitive disorders, motor problems, etc. As manganese is a component of antioxidants (such as superoxide dismutase (SOD)), it is a chief component of the defense mechanism of the body against reactive oxygen species (ROS) (Aschner, 2000). The ROS superoxide dismutase that is present in the mitochondria contains manganese (MnSOD) and is concentrated to the mitochondrial matrix. When MnSOD comes in contact with hydrogen peroxide is can easily become a highly destructive free radical.

As the *C. elegans* response systems are analogous to those of humans, insight can be gained through this experiment regarding the affect of

hard metals (manganese) on not only *C. elegans*, but also on the response systems (such as oxidative stress responses) of human subjects. Oxidative stress affects humans through degenerative diseases, such as Parkinson's disease and Alzheimer's disease, which have irreversible symptoms and side effects (An, 2005). The *C. elegans* were treated in this experiment with different concentrations of manganese, so that their life viability could be calculated/observed. These varying treatments will help to show a possibly correlation between oxidative stress and increased manganese levels.

Mesendodermal Development in C. elegans through SKN-1

A common mesendodermal tissue area is the basis of production of the endoderm and a mesoderm subset that initiates formation of the heart and blood in vertebrates. In *C. elegans*, the transcription factor SKN-1 induces the mesendodermal development. SKN-1 was identified in a test for the genes that are required maternally in the formation of pharyngeal tissue (Bowerman, 1992). The SKN-1 protein in *C. elegans* induces phase II detoxification gene transcription that is a conserved oxidative stress response. It is necessary to have a SKN-1 response in order to have oxidative stress resistance as well as longevity (An, 2005). Stress causes the SKN-1 protein to accumulate in the intestinal nuclei, therefore activating a protein kinase signaling. Maternally expressed SKN-1 specifies the fate of a single cell in the organism called the EMS blastomere. The EMS blastomere daughter cell E forms the endoderm of the organism, and the MS cell, the sister cell of the EMS, forms the basis of mesodermal derivatives. These derivatives include the posterior portion of the pharynx, as well as a feeding pump that is comparable to the heart. In organisms with mutations of the SKN-1 gene, instead of the anterior pharynx, lineages produce excess hypodermis (Bowerman, 1992).

The SKN-1 protein is primarily present within the ASI chemosensory neurons, but is also located in the intestine through the digestive system of the organism. ASI neurons have localization of SKN-1 in the nuclei, which then promotes and induces phase II gene expression (noting that phase II deals with cellular metabolism). In the intestine, SKN-1 is activated by the oxidative stress, causing the protein to then accumulate within minutes in nuclei and instigate gene expression that is then mediated through posttranscriptional regulation of SKN-1 (An, 2005). With the onset of oxidative stress, the life span of the SKN-1 protein is shortened by 25-30%, therefore demonstrating the intensity of the stress defense mechanism.

SKN-1 Responses to Oxidative Stress

SKN-1 functions during postembryonic stages are responsible for oxidative stress resistance and longevity. The oxidative stress response initiated by SKN-1 appears to be conserved among *C. elegans*, vertebrates, and single-celled eukaryotes. This trend in lineage implies that this is an ancient pathway in which the role of SKN-1 in initiating mesendodermal development (especially of the digestive system) may have evolved from the detoxification mechanism (An, 2003).

SKN-1 is still unique from any other known protein as its DNA binding mechanisms differs in that it binds through a basic region to DNA with high affinity as a monomer. SKN-1 works by binding to DNA using a unique mechanism that may be distantly related to that of basic leucine-zipper proteins that work with the main oxidative stress response in vertebrates and yeast. Similarly, SKN-1 workings in *C. elegans* consist of a mechanism comparable to that of the leucine-zipper proteins, but instead it has a distinct mode of target gene recognition. A principle phase II detoxification gene is regulated by SKN-1 during the postembryonic stages. It is regulated by constitutive and stress-inducible mechanisms in the ASI chemosensory neurons and the intestine. Under normal conditions, SKN-1 is present in the ASI nuclei. Under stressed conditions, SKN-1 accumulates in the intestinal nuclei. It is important to note that SKN-1 mutants are highly sensitive to any type of oxidative stress, and due to this, they have a shortened lifespan.

The C. elegans Experiment- Part I

For the first part of the research, I compared the viability of two different worm strains, SKN-1 and GCS-1 (worms that have the SKN-1 and GCS-1 markers, respectively), under the oxidative stress response caused by various manganese concentrations. I prepared over 50 agar plates with a bacterial growth medium (either NA22 or OP50 bacterial strands) so that the *C. elegans* could be well-nourished through the process. By using both 8P and

NGM plates, the worm strands were cultured and then treated, respectively. I propagated the strands that were frozen at -80°C, so I had to defrost them, consequently bringing them out of their dormant dauer state (a state where the dauer larvae do not age and are stress-resistant). Once the 8P plates appeared to be fully populated, I began the process of synchronization in order to get all the worms in the same growth stage. By washing the plates and then bleaching the worms, the adult worms lysed and if they were carrying eggs as hermaphroditic worms, the eggs remained in solution after numerous repetitions of centrifuging and washing.

For the duration of this project, I treated the *C. elegans* worm strands with solutions of manganese chloride ($MnCl_2$-$4H_2O$) and salt (NaCl) at different concentrations (2 M, 1 M, 0.7 M, 0.4 M, 0.2 M, 0.06 M, 0.002 M, 0.00002 M, and 0 M). By doing this, on both the SKN-1 and GCS-1 strands after synchronization, I studied the effects of the manganese solution upon the larvae that are all in the same stage of development. I found the LD50 (lethal dose to kill 50%) to be 452.6 mM for GCS-1 and 123.2 mM for SKN-1, which both contains errors, as the SKN should be more resistant to the manganese compared to GCS. I believe I have isolated the few sources of error in the parameters of: not having enough alive bacterial medium used (caused by not shaking the tube at consistent points during the spreading process), as well as problems in the beginning with counting the large populations of moving worms.

C. elegans Lifespan Experiment- Part II

For this part of the experiment, I focused on the viability of the worms at the different concentrations over their lifespan to see which concentrations tended to keep the worms alive the longest. I populated the SKN-1 GFP worm strain to fill the large 8P plates, and spread them onto small agar NGM plates with 500 µL of OP50 bacteria on them. After completing a dose response on the new vial of the SKN-1 worms, I initiated the treatments with the manganese chloride, with the concentrations of 0 mM, 0.02 mM, 2mM, 60 mM, 200 mM, 400 mM, 700 mM, 1 M, 2 M, 3 M, and 4 M of the manganese solution. I then put ten worms from each treatment onto a small FUDR plate, producing triplicates of each concentration and making sure the plates were correctly labeled. I scored the worms every day to check the viability/lifespan under the given oxidative stress of the manganese and recorded these results. Every third day of counting, I would transfer the worms to new small plates with OP50 bacteria.

The method of transferring the worms between plates was to use a platinum pick to transfer the living worms to new plates or to remove the dead worms and eggs from the existing plates. Burning the pick after removal of waste ensures that the new plate is not contaminated.

In my first trial, these small agar plates were also mixed with 1mL FUDR per 2L agar solution bottle. The low level of FUDR is important, as the eggs laid by adults in the presence of FUDR do not hatch. This makes the culture grow in a synchronous fashion so that it is not complicated by the presence of eggs and younger worms. In the later trials, there was no FUDR available in the lab, so in lieu of this, I had to pick the worms onto new plates with every counting.

Results of Lifespan Treatments Experiment:

The lifespan of each concentration was calculated by the averaging the number of days that it took for the worm population of each plate to reach zero from the triplicate experiments (therefore, all worms were dead). By using a platinum wire every other day to pick out the eggs and larvae from the plates, I made sure that the lifespan counts were based off of the original plated worms and not their progeny.

Concentration	Lifespan (days)
0 mM	16
0.02 mM	14.667
2 mM	13.667
60 mM	12.333
200 mM	13.667
400 mM	12.667
700 mM	13
1M	11.333
2M	11
3M	11.333
4M	9

Table 1: Effects of the concentration of manganese upon the viability of C. elegans

These results were in accordance with my initial hypothesis that the greater the manganese concentrations, the greater the amount of oxidative stress, and therefore, the lower the amount of lifespan days. As can be inferred from Table 1, the control concentration of 0 mM corresponded to an

average lifespan of 16 days, which was the longest recorded. This is assumed to be due to the plate having the least amount of oxidative stress caused upon these *C. elegans,* as there was no Mn within this control plate to affect the worms. As the concentrations increased, especially in the last four concentrations of 1 M, 2 M, 3 M, and 4 M, the lifespan was greatly reduced and the worms tended to die very early on in the experiments. In the lower concentrations besides the control, the lifespan determinations were somewhat similar, which was expected by the relatively close concentrations of manganese administered.

Conclusion

As oxidative stress comes as a response to environmental stresses caused by both chemical and physical factors, as well as it playing a major role in many chronic human diseases, it is important to study the effects of oxidative stress in other eukaryotes, such as *C. elegans*. Through my experiments, I was able to collect evidence that supporting the hypothesis that an increase in manganese leads to a direct increase in oxidative stress, which was shown by the decrease in lifespan of the distressed worms. In order to increase the statistical validity of the experiment, the next step would be to perform additional lifespan counts for more plates under the given concentrations.

By identifying the roles of SKN-1 and other proteins and transcription factors, scientists can identify similarities with human life systems, such as the immune or nervous systems. The process of aging and of lengthening life spans can be applied to models formed by the studies of *C. elegans*, as it is known that their system responses are analogous to that of human subjects. The next move in applying the *C. elegans* research to humans would be translating the study to a higher-level organism, such as mice, so that the effects and findings can have a strengthened correlation to the systems of humans. By studying the responses of *C. elegans* and higher-level organisms to oxidative stressors (ROS species and antioxidants), there is the potential to moderate the symptoms of many human diseases, including cancer (ovarian, colon, etc), atherosclerosis, diabetes, inflammations, infertility, as well as aging diseases and processes in the future.