Effects of Oxidative Stress in intestines of *Caenorhabditis elegans*

by

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Abstract

Stress is a major phenomenon present in lives of all organisms in the planet. Organisms respond differently to various kinds of stress. For example, some common types of stress experienced during a lifetime are salt, heat, or oxidative stress. This experiment attempts to discover the ubiquitin stress response in the intestines of *Caenorhabditis elegans* under oxidative stress. *Caenorhabditis elegans* are used to study stress because proteins that respond to stress such as ubiquitin are highly conserved. Ubiquitin in *C. elegans* and ubiquitin in humans differ only by one amino acid which makes them 98.68% genetically identical. Therefore, carefully studying the ubiquitin response in *C. elegans* gives us a good understanding of the way it works in humans as well. ERT 261 and ERT 264 (intestinal strains) one day adult *Caenorhabditis elegans* were treated with hydrogen peroxide for oxidative stress, sodium chloride for salt stress (positive control), and M9 buffer for an unstressed condition (negative control). Previously, Dr. Lynn Boyd's lab has looked at salt stress in the intestine and both salt and oxidative stress in the gonads. In both the cases, nuclear spheres (referred to as foci) were observed, ubiquitin being a major component of it. Foci are the spherical regions enriched with ubiquitin observed with in the nuclei of cells under stress conditions. In this experiment, foci were observed in the intestinal tissue of *C. elegans* under oxidative stress. In addition to whether or not nuclear spheres formed in the intestine, this experiment used different concentrations of hydrogen peroxide to cause different levels of oxidative stress and thus a relationship between the level of oxidative stress and the percentage of nuclei with spheres observed was developed. At 5mM, 50% of the ERT

261 had nuclear spheres, while at 10mM and 15mM it was 44% and 58%. To take the experiment another step further, recovery period for up to 24 hours was allowed and the percentage of nuclei with foci were observed. A decrease by a 9% in M9, 25% in 500mM sodium chloride, 42% in 5mM, 36% in 10mM, and 42% in 15mM of hydrogen peroxide was observed after the 12 hours recovery period when compared to those after 1 hour of stressing. However, no spheres were observed after 24 hours due to intense auto fluorescence in the intestinal strain of *Caenorhabditis elegans*.

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Definition of Terms

Hydrolysis: A reaction that involves the breaking of a bond in a molecule with the help of water.

Gene: It is the unit of heredity of any living organism.

ATP: An adenosine triphosphate molecule consists of three phosphate groups. ATP is produced by many different enzymes which include ATP synthase, adenosine diphosphate (ADP), or adenosine monophosphate (AMP).

Green Fluorescent Protein: Green fluorescent protein (GFP) is a 238 amino acids long protein which had been primarily isolated from marine jellyfish. When shone under the blue-violet range in the electromagnetic spectrum, it exhibits bright green fluorescence which is particularly important in gene expression in a region of interest in a study organism.

Proteasome: Protein complex responsible for breaking down damaged or unwanted proteins and disassembling ubiquitinated substrates at the final step of degradation by ubiquitination.

Degradation: Process followed by ubiquitin molecules to attach themselves to a target substrate to be broken down by a proteasome.

E1: In the three step pathway of protein degradation by ubiquitination, E1 is the very first enzyme that selects and activates ubiquitin as well as identifies the proteins that require degradation.

E2: E2, also known as the ubiquitin conjugating enzyme (UBC), is the second enzyme in the three step pathway of degradation by ubiquitination. Its main job is to attach ubiquitin molecules to targeted substrates in order to degrade them.

E3: E3, also known as ubiquitin ligase, is the third enzyme in the final step of degradation by ubiquitination. Its job is to recruit ubiquitin rich E2s and recognize protein substrates for the ubiquitin to be attached to the substrate and be degraded by a proteasome.

OP50: Particular strain of *Escherichia coli* normally consumed by *C. elegans* with no experimental conditions applied.

ERT 261: A strain of *Caenorhabditis elegans* that have a green fluorescent protein (GFP) tag on its ubiquitin so that it will fluoresce under the fluorescent microscope.

ERT 264: A strain of *Caenorhabditis elegans* that have a green fluorescent protein (GFP) tag on its ubiquitin that is mutated and is unable to form poly ubiquitin chains due to the di-glycine being replaced by di-alanine.

Introduction

Stress is a major phenomenon that organisms go through regularly. Organisms respond differently depending on the type of stressor. Stress responses develop from within the cells of organisms in order to maintain the homeostasis of the body even at the most extreme situations. Particularly in humans, stress can affect the physical and physiological functioning, mental and psychological well-being, as well as social life and relationships. Hence, it is extremely important to address the negative effects different stressors have on organisms, study and understand the pattern in which each work, and find new ways to minimize the chances of acquiring health problems caused by them.

This particular experiment was designed to study the effects of oxidative stress in the intestine of *C. elegans* and compare the results to that of salt stress in the intestine which had been studied in the Boyd Lab in the past. *Caenorhabditis elegans* is a species of nematodes widely used in laboratory experiments designed to study ubiquitin and stress response. Past and current researchers in Dr. Lynn Boyd's lab have looked at different tissues of the body of *C. elegans* such as the gonads and the intestines and in both cases, GFP::Ub foci (spherical regions enriched with ubiquitin) were observed in the oval-shaped nuclei of cells. This is the first time that the intestines have been studied under oxidative stress.

The intestines are an important organ in *C. elegans* that hold roughly one-third of the total somatic mass and carry out vital role in the digestion and absorption of nutrients[1]. The intestines hold the highest number of peroxisomes which is one of the only two places where the oxidation of fatty acid takes place in *C. elegans*[1]. The oxidation of fatty acid is important because nematodes heavily rely on lipids for energy

metabolism and storage[1]. Therefore, the intestines serve as the major site for such biochemical activities to take place and allow the organism to function properly[1].

Our bodies produce free radicals (molecules comprised of oxygen with an unpaired electron or electrons) which highly react with vital components of cells, including proteins, by snatching their electrons in order to stabilize their own selves[2]. As a result of the stabilization, it destabilizes the DNA and other components of the cell triggering an overall domino-effect reaction[2]. Antioxidants on the other hand are molecules residing inside cells that lend an electron to the problem causing free radicals and still manage to stay stable themselves[2]. An imbalance between the manufacture of antioxidants and free radicals is referred to as oxidative stress[2]. Oxidative stress is a root cause for Alzheimer's, Parkinson's, cancer, and cardiovascular diseases[2].

To understand how stress works, it is vital first to understand the role of the regulatory protein known as ubiquitin, and the different pathways it follows. The function of ubiquitin is to attach itself to a protein and signal another protein complex known as a proteasome to come and degrade that particular protein[3]. Under normal conditions, it takes a very long time to break a peptide bond by the process of hydrolysis without the help of a catalyst. Even though the stability of protein is a desirable trait, it becomes a major problem when unwanted proteins need to be broken down for revival or performance purposes of the organism. For example, when some cells are not working properly anymore, they need to be broken down, removed, and recycled to form new functional cells. The tough job is made possible by the help of proteases that use their acidic, basic, and nucleophilic catalytic property to expedite the hydrolysis of the peptide bonds.

Ubiquitin is a regulatory protein composed of seventy-six amino acids[6]. As the name suggests, ubiquitin is present in all tissues of eukaryotes. It was first discovered by Goldstein in 1975 and the concept of the Ubiquitin Proteasome System was later developed during the late 1970s and 1980s. Ubiquitin works within the Ubiquitin Proteasome System, responsible for the removal of ubiquitin tagged proteins. The pathway includes three catalyzing steps with at least one enzyme taking part in each step[5]. E1, E2, or E3 are the three enzymes taking part at each of the stages in the pathway. E1 refers to the first, E2, second, and E3, the third enzyme in the pathway.

First of all, enzyme E1 selects and activates the ubiquitin molecule and targets the molecules that require degradation. Next, the E2 (also known as the ubiquitin conjugating enzyme or UBC) removes ubiquitin molecules from E1s, recruits an E3 molecule, and transports the ubiquitin molecules either to the targeted substrate or the E3. The E2 continues doing this till enough ubiquitin molecules are bound to the substrate for degradation to take place. The E3 then attaches to and transfers the ubiquitin molecules to the target substrate and finally the process of degradation begins.

Apart from the fact that ubiquitin in humans and in *C. elegans* are 98% similar, these organisms are a great model organism also due to their ability for genetic modification, ease of maintenance, short and prolific life span[7,8]. Large populations of *Caenorhabditis elegans* can be grown in a short amount of time. It takes roughly three days to obtain one day adults to be used in the experiment.

The main focus of the experiment is to observe the localization of ubiquitin in the intestine of *C. elegans* and to help us do that, a protein called green fluorescent protein

(GFP) is tagged with the ubiquitin in the intestine. The GFP, as the name suggests is a small piece of protein that gives out green light when placed under a fluorescent microscope. The green fluorescent protein was first obtained from jellyfish and injected into *Caenorhabditis elegans* by use of genetic engineering. In 1987, a scientist named Douglas Prasher had the ground-breaking idea to use GFP as a tracer molecule for small molecules such as proteins that are too small to be seen even under an electron microscope. He suggested inserting a gene for constructing GFP into the gene for a protein so that the cell would produce a GFP attached to the protein.

The two intestinal strains of *C. elegans* used in this experiment were the ERT 261 and ERT 264 (mutant). To make the ERT 261 strain, GFP was first fused with ubiquitin (denoted by GFP::ubiquitin). Next, the intestinal-specific *vha*-6 promoter (genetic element that will initiate transcription of proteins only in the intestine) is fused to GFP at the N-terminus of ubiquitin (denoted by *vha*-6p::gfp::ubiquitin) and this construct is injected into *C. elegans*. Therefore, we can denote ERT 261 by: *vha*-6p::gfp::ubiquitin. Likewise, the same construct was injected with a mutant version of ubiquitin without its last two C-terminal glycines into *C. elegans* to make ERT 264. ERT 264 is denoted by: *vha*-6p::gfp::ubiquitinΔGG. As a result of the deletion mutation, ERT 264 is not able to form long chains of ubiquitin while ERT 261 is able to.

a. ERT 261

b. ERT 264

Fig 1: Genetic structure of ERT 261 (top) and ERT 264 (below)

This experiment asks three important questions:

- ✓ Does oxidative stress also result in spheres in the nucleus of intestinal cells of *C. elegans* like those observed under salt stress in the Boyd Lab?
- ✓ How does the percentage of nuclei with spheres compare with increasing levels of oxidative stress?
- ✓ What percentage of intestinal cells show nuclei with spheres after a recovery period of up to 24 hours is allowed?

Thesis Statement

The first goal of the experiment is to discover whether or not the nuclei of intestinal cells in *C. elegans* form spheres under oxidative stress. If they do, then the second goal is to calculate what percentage of the total nuclei have them. The third goal is to study the relationship between the percentage of nuclei with spheres and different levels of oxidative stress, and, finally, to observe what happens to those percentages after a 24-hour recovery period is allowed.

Methodology

(a) Maintenance of *C. elegans* and the replication process:

C. elegans were grown at 25° C on Nematode Growth Media and seeded with *E. coli* (OP50) as their food source. Every two days, worms were picked and transferred at the L4 (larvae stage) onto new NGM (nematode growth media) plates. The cycle is repeated several times to obtain a considerable amount of nematodes always ready to be experimented. During the week, the newly picked plates are kept at 20° C or 25°C for faster growth and at 16° C during the weekend to avoid depletion of the nutrients and starvation.

(b) Stress application process:

One day adult *C. elegans* were immersed in 2ml of 500mM NaCl solution (used as positive control), M9 buffer (3.0 g monopotassium phosphate, 6.0 g disodium phosphate, 0.5 g sodium chloride, and 1.0 g ammonium chloride in 1 liter of water), and

5mM, 10mM, and 15mM concentrations of hydrogen peroxide solution. Next, each solution had a large amount of one day adult *C. elegans* immersed in the liquid. The same length of stressing period (60 minutes) was allowed for each solution. After 60 minutes, the nematodes were washed with M9 buffer for up to three times in order to prevent the organisms from experiencing any additional amount of stress. For the recovery period experiment, nematodes from each tube were washed and put in M9 for up to 24 hours.

(c) Use of AZ100 microscope:

Worms were anesthetized using a 250mg/ml tetramisole and egg buffer before being placed on a slide with 3% agarose and observed under the AZ100 microscope. A total of 36 nuclei have been observed in each solution for each strain. To obtain a random and unbiased sample, the worms were centered in a field at low magnification. The magnification was gradually increased until only about three nuclei were visible. The number of foci in each of the three nucleus were counted before moving onto the next worm.

(d) Data analysis:

A contingency table analysis was carried out to test the null hypothesis that foci formation in the nuclei of intestines of *C. elegans* under oxidative stress was independent of the kind of treatment used.

Data & Results

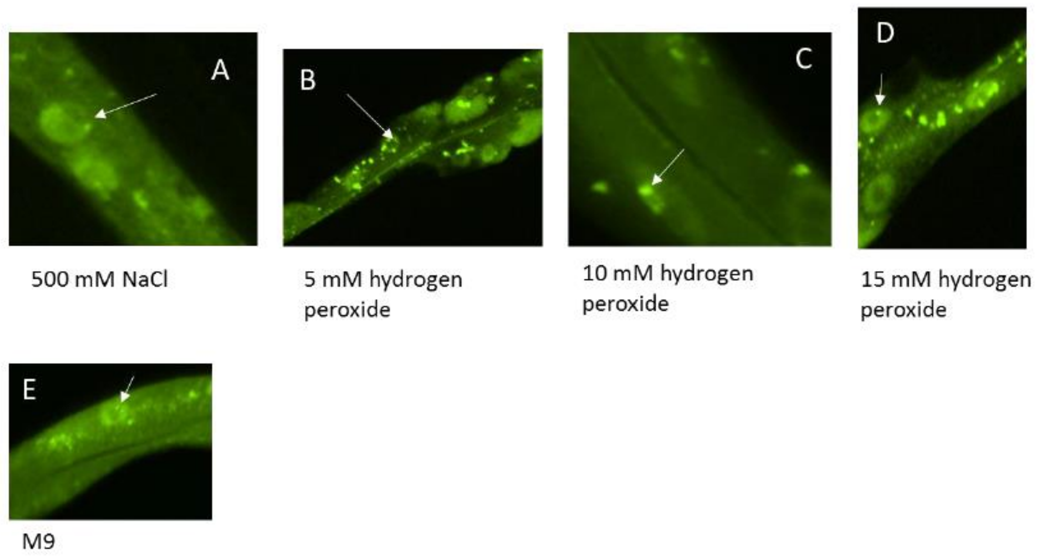

Fig 2.1 : ERT 261 under salt stress, different concentrations of oxidative stress, and unstressed conditions

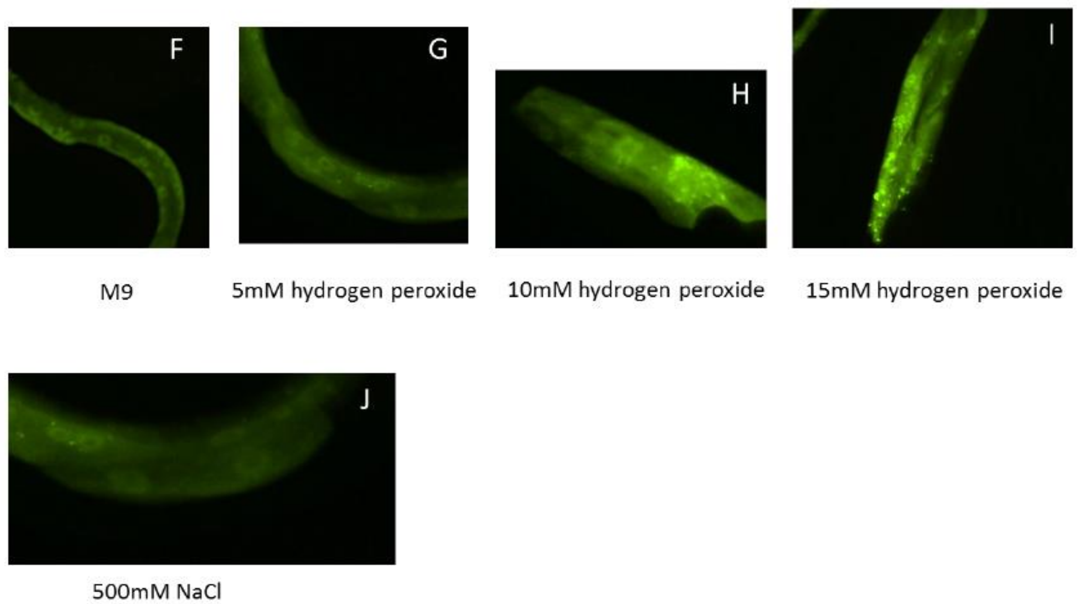

Fig 2.2: ERT 264 under salt stress, different concentrations of oxidative stress, and unstressed conditions

Figures 2.1 and 2.2 show ERT 261 and ERT 264 in M9, 500mM NaCl, and 5mM, 10mM, and 15mM hydrogen peroxide solutions. In figure 2.1, white arrows in A, B, C, D, and E are used to show the brighter green regions-the foci located in the bigger oval-shaped nuclei of the intestinal cells of *C. elegans*. In ERT 264 (Figure 2.2), the large oval-shaped nuclei are shown without foci.

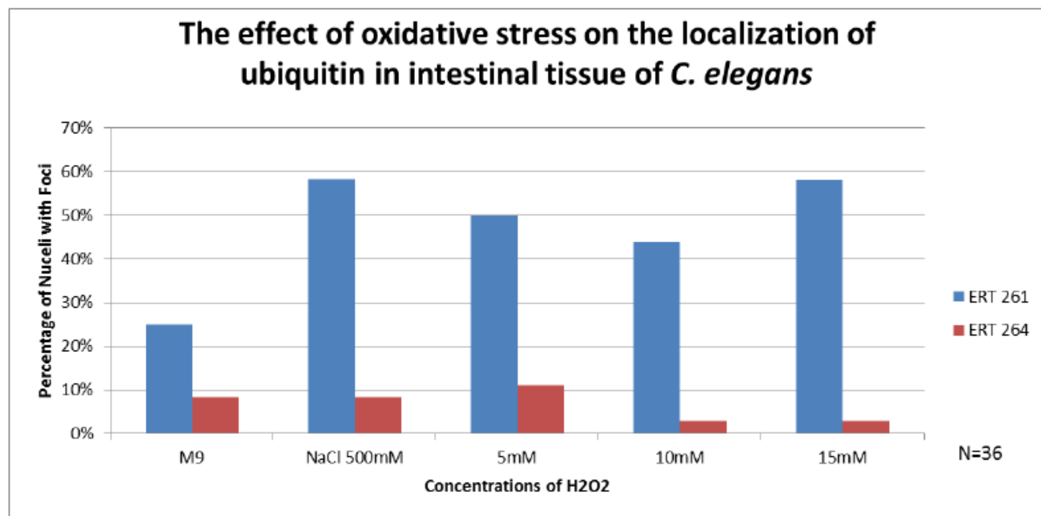

Fig 3.1: Percentage of nuclei with foci after 60 minutes in M9 buffer, salt and different concentrations of hydrogen peroxide

The graph above shows the percentage of nuclei with foci in the intestinal cells of *C. elegans* in M9 buffer, 500mM sodium chloride, and 5mM, 10mM, and 15mM of hydrogen peroxide concentration after 60 minutes of stressing.

In M9 buffer, 25% of the ERT 261 and 8% of ERT 264 had nuclei with foci. In 500mM sodium chloride solution, percentages of nuclei with foci were comparatively higher than that in M9, with a 58% in ERT 261 and 8% in ERT 264. At 5mM hydrogen peroxide, 50% of ERT 261 and 11% of ERT 264 had nuclei with foci which were

comparatively lower than those in 500mM NaCl. When the concentration of the hydrogen peroxide solution was increased to 10mM, a drop in the percentage of nuclei with foci was observed in ERT 261 to 44% and ERT 264 to 3%; a 6% and 8% decrease from the previous concentration of 5mM. Last but not the least, at 15mM hydrogen peroxide solution, 58% of the ERT 261 had nuclei with foci while that of ERT 264 showed the same 3% as in 10mM previously.

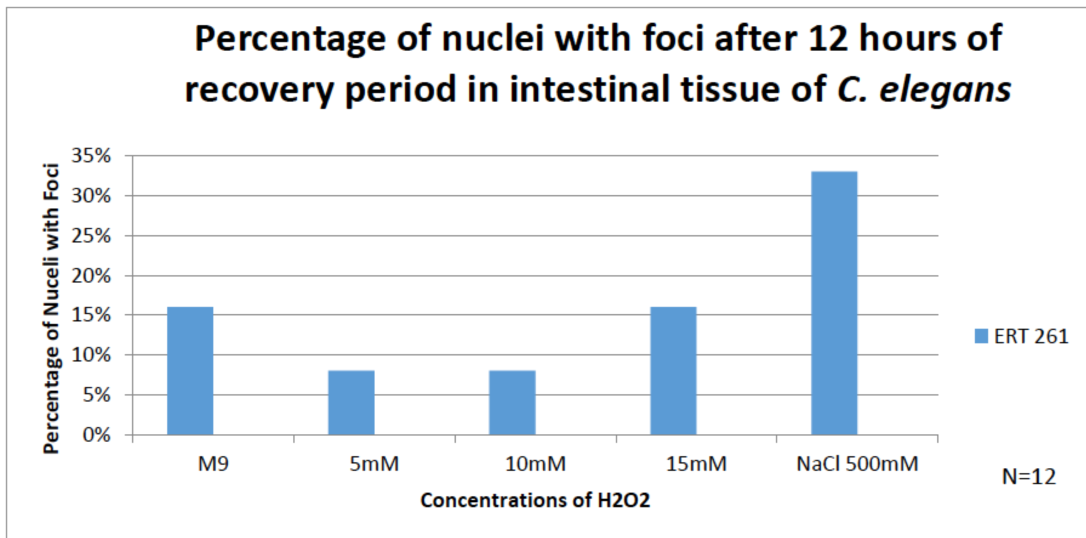

Fig 3.1: Percentage of nuclei with foci after 12 hours of recovery period

The graph above shows the percentage of spheres with foci observed in ERT 261 (wild- type) after twelve hours of recovery period. In M9, 16% of ERT 261 still had spheres. In 500mM NaCl, 33% ERT 261 showed spheres which is 7% more than it was in M9. In both 5mM and 10mM hydrogen peroxide solution, 8% ERT 261 had spheres while in 15mM, it increased to a 16%. When looked at after 24 hours, no spheres were observed due to very high auto fluorescence in the strain.

The contingency table analysis of all five treatments included had a Chi-square value (X^2) of 10.92 with a P = 0.001 (P is the probability of getting a result if the null

hypothesis is correct). However, since that probability is very small, it can be concluded that the null hypothesis is not correct. Therefore, treatment did have an effect on the formation of foci. When looking at the data, the M9 had result much different than the other treatments. So, M9 was removed from the data, and the data analysis was redone with the other four treatments where the $X^2 = 2.01$ with a $P = 0.156$. So, the conclusion here was that there is no evidence that the 500mM salt was different than the 5mM, 10mM, or 15mM NaCl solution treatment. All four of these treatments triggered the formation of foci but none of the treatments were different from each other. In other words, the four treatments were different from M9 but not different from each other.

Discussion

In this experiment, two strains of *Caenorhabditis elegans*, the ERT 261 and ERT 264 have been used. It was not known at the beginning of the experiment which strain was the mutated and which one the normal. After the collection of data from 36 individual nuclei for each solution in each strain, where the ERT 261 showed comparatively much higher percentages of nuclei with foci than the ERT 264, it was determined that ERT 261 was the normal strain forming poly ubiquitin chains under both salt and oxidative stress. The much lower percentages of nuclei with foci in ERT 264 was the evidence that it was the mutated strain where the di- glycine required to form poly ubiquitin chains was deleted preventing it from ubiquitin chain formation. Therefore, only ERT 261 was used in the last part of the experiment to observe the ubiquitin response after a 12 and 24 hour recovery period.

To eliminate errors and maintain uniformity throughout the experiment, *C. elegans* grown under the same conditions has been used for all the solutions and so no nematodes put in one particular solution was defective or different in any way from the rest. Also, it was made sure that all organisms were one day adults to eliminate any variations in results since they respond differently to the same stressors at different stages of the life cycle.

500mM sodium chloride has been used to serve as a positive control in the experiment showing that it responds to stress and forms spheres. Previous studies in Dr. Lynn Boyd's lab have pointed out that spheres do form under salt stress and a major component of these spheres is ubiquitin. In addition to the positive control, a negative control (M9 buffer) is included in the experiment which has shown lower percentages of nuclei with foci when compared to the stressed. The prime focus of the experiment was to study if foci appear in the nuclei of intestinal cells of *C. elegans* under oxidative stress and to cause that, hydrogen peroxide was used. Once it was observed that spheres do form under oxidative stress, more concentrations of hydrogen peroxide were used to observe how the rate of sphere formation relates to the level of oxidative stress. The contingency table analysis indicates that the lowest level of oxidative stress that was applied (5mM of hydrogen peroxide) was enough to initiate foci formation and that the amount of foci formation was not affected by higher levels of oxidative stress.

After 12 hours of recovery period was allowed, percentages of nuclear foci observed were 16% in M9, 8% in 5mM and 10mM hydrogen peroxide, 16% in 15mM hydrogen peroxide, and 33% in 500mM NaCl. This shows that *C. elegans* recovered as time was allowed after the stress had been minimized by washing and suspending them in

M9 buffer. However, after 24 hours, high auto fluorescence made it impossible to observe any foci in the intestinal tissue of *Caenorhabditis elegans* under oxidative stress.

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