**The Effects of Increased Net Reactive Oxygen Species on Mitophagy**

1. **Introduction**

As the global human population continues to age and live longer lives, both developed and developing countries have begun to feel the negative impact of neurological disorders. These disorders are an epidemic that ignore race, age, education, income, and sex. There has been some evidence that people with long-term alcohol abuse use have higher chances of developing dementia in old age compared to people who drink more casually or occasionally (Lin et al 2006).

Reddy et al (2013) speculated that alcohol-induced oxidative stress can alter the brain mitochondria. Oxidative stress contributes to aging, which is a major risk factor for dementia. This is because mitochondria are the primary regulators of cell death. The key feature of neurodegeneration is the lack of dysfunctional mitochondria being disposed of. Reactive oxygen species (ROS) are natural by-products of mitochondria in cells when producing energy. Lin et al (2006) speculated that an excess of ROS in a cell can damage mitochondria and cause a build-up of these dysfunctional structures. One of the regulatory processes preventing the build-up of dysfunctional organelles is autophagy, which is the process of breaking down cytoplasmic structures. Cells use autophagy to prevent the build-up of dysfunctional organelles by signaling them for regulated destruction by lysosomes. The build-up of these dysfunctional organelles can cause cell death.

This imbalance of ROS in a cell can cause a phenomenon called “net ROS” and is thought to be the main contributors to aging. When there is not an even ratio of ROS being produced and removed, there can be a surplus of ROS in the cell (Lin et al 2006).

Ethanol increases oxidative stress in a cell and provides a growth-inhibiting environment. This increase of reactive oxygen species can cause conformational changes of important proteins through protein oxidation. These conformational changes of the proteins can disrupt the proper regulatory processes of the cell.

Atg-32 and Atg-11 are specific proteins located in the cytoplasm that have been identified to regulate mitophagy in *saccharomyces cerevisiae* (yeast). Atg-11 is a protein that initiates the phagophore assembly site (PAS) where cytosolic membrane vesicles are formed. The phaghophore assembly site is where the autophagosome is made. Atg-11 then interacts with the mitochondrial residential receptor Atg-32, forming a complex and then brings in mitochondria to the PAS. The autophagosome membrane forms around the mitochondria and then proceeds to fuse with a lysosome where the cargo (mitochondria) is degraded.

**Figure 1: Overview of Mitophagy Mechanism. Adapted from Figure 6 of Ref Aoki et al 2011**

The purpose of the experiment described in this proposal is to determine if a surplus net ROS will decrease the level of affinity of Atg-32 to Atg-11 (Aoki et al 2011).

1. **Experiment**

The aim of this experiment is to determine if higher concentrations of ethanol exposure will decrease levels of Om45-GFP processing by yeast cells in response to ethanol. If the phosphorylation of Atg-32 is necessary for mitophagy, then I would expect that higher net levels of ROS would decrease levels of GFP processed because the kinase processing Atg-32 has a higher chance of being oxidized and becoming dysfunctional. The correlation of GFP processing and levels of mitophagy is described in section II.A.

I will produce Atg-32 protein expressing Om45-GFP to measure the levels of mitophagy in the yeast cells and introduce them to different levels of ethanol as their environment. My control group will be yeast cells not in ethanol. The levels of mitophagy will be measured by the amount of GFP processed using a Om45-GFP processing assay as described by Aoki et al (2011).

II.A Measuring Levels of Mitophagy in Yeast

This method was employed by Aoki et al (2011) to detect for the evidence of changing rates of mitophagy in yeast induced by nitrogen starvation. Aoki et al (2011) speculated that the phosphorylation of Ser-114, a specific amino acid structure on Atg-32, might be important for mitophagy and for the Atg-32 affinity for Atg-11. They tested this idea by producing phosphorylation-deficient Atg-32 mutants expressing Om45-green fluorescent protein(GFP) and inducing the yeast cells to undergo mitophagy by nitrogen starvation.

Om45 is a mitochondrial outer membrane protein that has no known function. When Om45 is tagged with GFP, the complex hovers around the mitochondrial outer membrane. Each group of mutants was had a different amino acid that was phosphorylation-deficient. One group was the wild-type, one group was deficient in Ser-114, one group was deficient in the Ser-119, and one group was deficient in both Ser-114 and Ser-119.

**Figure 2: Data from Aoki et al (2012) experiment showing the bands formed after levels of GFP processed were measured for each of the mutant groups**

When the yeast cells underwent mitophagy, Om45-GFP is sent to the cell’s vacuole and then broken down. GFP is stable within the vacuole, so it is released as an intact protein. Aoki et al used an antigen called anti-GFP in order to detect the specific protein being monitored. After the induction of mitophagy, the samples are then put on a gel and run through a machine that separates the specific proteins into portions of the gel using electricity. The processed GFP were detected as bands that migrated from the Om45-GFP band. Om45-GFP processing assay is explained in greater detail in Kanki et al (2009). By measuring the levels of GFP processed from Om45-GFP, they were able to correlate that to levels of mitophagy.

What they observed was that the wild-type yeast cells had a darker band in the gel, which indicated high levels of GFP processing. They observed a lighter band in the gel for Ser-119 deficient, which means less GFP processing compared with the wild-type. The yeast cells with Ser-114 deficiency and with both Ser-114 and Ser-119 deficiency had no band shown, indicating that none to very little GFP was processed. These bands corresponding to each group can be all seen in Figure 2.

1. **Discussion**

If all goes well and my predictions are correct, high levels of net oxidative stress due to ethanol exposure will cause a decrease levels of GFP processing, which could mean that there is a decrease in levels of mitophagy. This data could be used to provide a greater link between the development of dementia and long-term alcohol consumption.

The biggest problem behind this experiment is that there is very little knowledge about the specific mechanisms of mitophagy. What we have learned so far about the proteins responsible were only very recent findings. The identity of the kinase (only designated as X in Figure 1) that phosphorylates Ser-114 has not been identified yet, but we just know that it happens and that is it necessary for the regulation of mitophagy.

There will be great difficulty in actually interpreting the results because of this unknown kinase. Even if we are able to determine if GFP processing decreases, we do not have the knowledge yet to determine to what extent the effects of the decreased levels of mitophagy actually stems from. There could also be many other factors as a result of using ethanol. Free oxidative species are not the only results of ethanol exposure.

Neurological disorders such as dementia are difficult to diagnose and treat because much of human brain function is still widely unknown. If the results of this experiment suggest that increasing levels of net ROS can increase the risk for developing dementia later in life for a human being, then physicians can work their way towards providing more options to diagnose and prevent the disorder. The science community will also make progress towards decreasing the frequency of neurological disorders and integrating better treatments world-wide.

**References**

Almansa, I., A. Fernandez, C. Garcia-Ruiz, M. Muriach, J. Barcia, M. Maranda, J. Fernandez- Checa, F. Romero. 2009. Brain mitochondrial alterations after chronic alcohol consumption. J Physiol Biochem 65: 305-312

Aoki, Y., T. Kanki, Y. Hirota, Y. Kurihara, T. Saigusa, T. Uchiumi, D. Kang. 2011. Phosphorylation Serine 114 on Atg32 mediates mitophagy. MBoC 22: 3206-3217

Ashrafi, G., T. Schwarz. 2013. The pathways of mitophagy for quality control and clearance of mitochondria. Cell Death and Differentiation 20: 31-42

Bailey, S. M. 2003. A Review of the Role of Reactive Oxygen and Nitrogen Species in Alcohol- induced Mitochondrial Dysfunction. Free Radical Research 6: 585-596

Dolganiuc, A., P. Thomes, W. Ding, J. Lemasters, T. Donuhue Jr. 2012. Autophagy in Alcohol- Induced Liver Diseases. Alcoholism: Clinical and Experimental Research 36: 1301-1308

Kanki, T., D. Kang, D. Klionsky. 2009. Monitory mitophagy in yeast. Autophagy 8: 1186-1189

Knorre, D., K. Popadin, S. Sokolov, F. Severin. 2013. Roles of Mitochondrial Dynamics under Stressful and Normal Conditions in Yeast Cells. Oxidative Medicine and Cellular Longevity 1: 1-6

Kondo-Okamoto, N., N. Noda, S. Suzuki, H. Nakatogawa, I. Takahashi, M. Matsunami, A. Hashimoto, F. Inagaki, Y. Ohsumi, K. Okamoto. 2012. Autophagy-related Protein 32 Acts as Autophagic Degron and Directly Initiates Mitophagy. J Biol Chem 287: 10631- 10638

Kurihara, Y., T. Kanki, Y. Aoki, Y. Hirota, T. Saigusa, T. Uchiumi, D. Kang. 2012. Mitophagy Plays an Essential Role in Reducing Mitochondrial Production of Reactive Oxygen Species and Mutation of Mitochondrial DNA by Maintaining Mitochondrial Quantity and Quality in Yeast. J Biol Chem 287: 3265-3272

 <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3270981/?report=classic>

Lin, M., M. Beal. 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443: 787-795

Okamoto, K., N. Kondo-Okamoto, Y. Ohsumi. 2009. A landmark protein essential for mitophagy. Autophagy 5: 1203-1205

Reddy, V., P. Padmavathi, G. Kavitha, B. Saradamma, N. Vaaradacharyulu. 2013. Alcohol- induced oxidative/nitrosative stress alters brain mitochondrial membrane properties. Mol Cell Biochem 375: 39-47

Schreiner, B., H. Westerburg, I. Forne, A. Imhof, W. Neupert, D. Mokranjac. Role of the AAA protease Yme1 in folding of proteins in the intermembrane space of mitochondria. MBoC 23: 4335-4346

Wager, K., C. Russell. 2013. Mitophagy and neurodegenernation: The zebrafish model system. Autophagy 9: 1-17

Wang, K., M. Jin, X. Liu, D. Klionsky. 2013. Proteolytic processing of Atg32 by the mitochondrial i-AAA protease Yme1 regulates mitophagy. Autophagy 9: 1-9