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"Experiments using human mixed cortical neurons"

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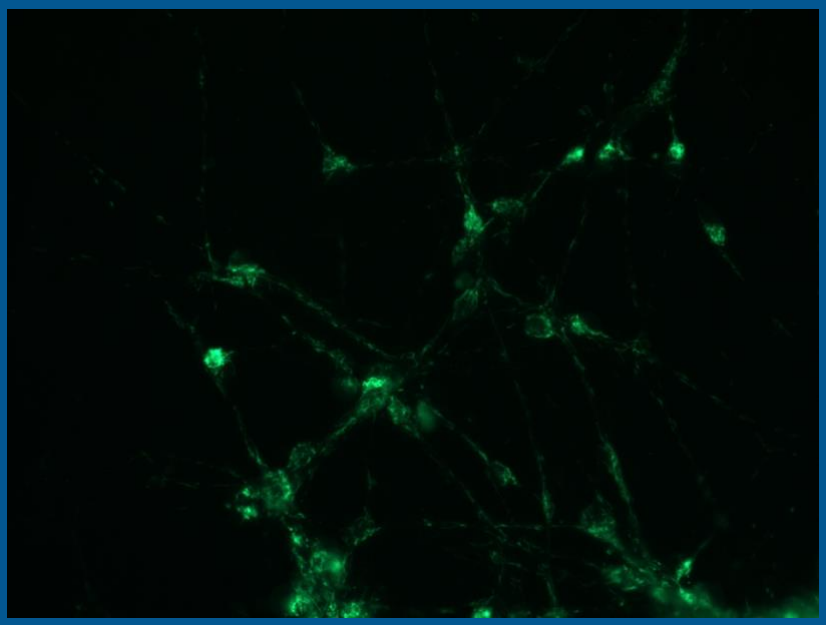

Evidence that oxidative stress leads to a change in mitochondrial charge in hiPSC-derived mixed cortical neurons: implications for the pathogenesis of Alzheimer's disease.

Nicholas P. Kelly

BIO 324 / Neurobiology
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Introduction

Dementia is a general term which refers to the degradation in cognitive ability serious enough to complicate activities of daily living (Cenini & Voos, 2019). Alzheimer's disease is the most common type of dementia. It is globally presented in 24 million people ages 65 and older (Cenini & Voos, 2019). Alzheimer's disease is a neurodegenerative disease with subtle onset which leads to eventual impairment of behavioral and cognitive functions (Weller & Budson, 2018). As of 2020, it is the sixth leading cause of death in the United States (Cenini & Voos, 2019). Typically, Alzheimer's disease is characterized by an accumulation of amyloid-beta plaques and tau-tangles in the brain (Moreira et al, 2009). However, recent advances in the study of neurodegeneration suggests that mitochondrial dysfunction plays a heavy role in the pathogenesis of Alzheimer's disease (Wang et al, 2020).

In the current study, we began with human induced pluripotent stem cells (hiPSCs) that were initially differentiated into neural progenitor cells. Further differentiation resulted in human neurons, which are our disease relevant cells (Morris spoken words, September 16, 2020). These neurons were then split up into separate chamber well slides to stimulate various conditions. This system then gave us a suitable environment to begin our experiment. Primarily, our study is focused on the mitochondria that are present in our human neurons. We are interested in oxidative stress and its effect on mitochondria in the mixed cortical neurons present in our culture. Oxidative stress and it's components are critical in determining what sort of factors play a role in developing certain diseases relevant to mitochondrial function. While we know that mitochondrial dysfunction does indeed play an intriguing role in Alzheimer's disease pathogenesis, we still do not know with certainty what causes this sort of dysfunction in human neuronal mitochondria (Moreira et al, 2009).

In order to better understand why some mitochondria may become dysfunctional in aging adults, we tested the hypothesis that oxidative stress leads to a lingering change in mitochondrial charge in mixed cortical neurons in culture. We used human neurons differentiated in culture from hiPSCs that were first stained in dichlorofluorescein (DCF) to quantify cellular oxidative stress (Wang & Joseph, 1999). Next, we stained neurons with rhodamine 123 (R123) to measure membrane polarization in our cell's mitochondria (Scaduto & Grotyohann, 1999). Lastly, these

cells were imaged with a Nikon Eclipse inverted microscope using both transmitted light microscopy and fluorescence microscopy.

Materials and Methods

Materials: 50mM of dichlorofluorescein (DCF) in DMSO, catalog number D6883-50mg (Sigma-Aldrich), open and made in October of 2018, and 10mg/ml of Rhodamine 123, catalog number R8004, were purchased for use. Images were gathered using a Nikon Eclipse TS100 inverted microscope equipped with phase optics and an LWD 40x/0.55 Ph1 ADL objective a Diagnostic Instruments (DI) Idea 3.0 Mp Color Mosaic camera with a DI 0.5x C-mount adapter driven by DI Spot software version 4.6.1.26 on an Apple iMac7.1 computer using OS X 10.5.8.
Primary Culture of Stem Cells: Human Induced Pluripotent Stem Cells (hiPSCs) were obtained through EverCell Bio, INC. located in Natick Massachusetts. hiPSCs were differentiated and grown using standard culture protocols for hiPSCs (Morris, 2020).
Treatment of Cells: The neurons were transferred and plated on a chamber slide and treated through reduction in feeding frequency. A control set of neurons were fed normally every other day according to protocol. An experimental set of neurons were starved for five days until the individual chamber slide wells were re-fed. The experimental set of wells were then refilled and fed with 250ul of L-15 Neuronal Growth Media (L15 NGM) until dyed.
Cell Dyes: The stock solution of 50mM DCF was diluted to create a working solution. A 1:1000 dilution of DCF was transferred into growth media for a target concentration of 50uM. 1.1 ul of DCF was added to 1.1 ml of growth media to create our working solution. 250ul of this working solution was added three times into three individual control wells and incubated in an air incubator at 37° Celsius for 20 minutes to test the dye. The remaining 250 ul of this solution was then added into the experimental well and was incubated fully covered from the light for 20 minutes. Post incubation period, the wells were washed three times using Hanks Buffered Salt Solution (HBSS) and were covered, and then set aside for imaging.

For Rhodamine 123, a 1:20 dilution of the original 10 mg/ml stock was done by adding 10ul R123 into 200ul L-15 media to create a 500ug/ml intermediate stock. A 1:100 dilution was then performed to create a target working stock concentration of 5ug/ml. 6 ul of R123 was added into 600 ul of growth media. 250ul of this solution was then added twice, once into each experimental well. R123 was left in the well and incubated in the air incubator fully covered from the light for 10 minutes. Post incubation period, the treated wells were washed 10 times each. Six of the washes were done with HBSS while the last 4 were done with L-15 without growth factors.
Fluorescence imaging: The individual DCF and R123 stained cells were imaged using a Nikon Eclipse TS100 inverted microscope equipped with phase optics and an LWD 40x/0.55 Ph1 ADL objective, a Diagnostic Instruments (DI) Idea 3.0 Mp Color Mosaic camera with a DI 0.5x C-mount adapter driven by DI Spot software version 4.6.1.26 on an Apple iMac7.1 computer using OS X 10.5.8. The cells were taken from the incubator and imaged quickly to minimize temperature change and light exposure. Images of the cells were first taken using transmitted light microscopy optimized for phase contrast. The image setting for Spot was under "brightfield" with no unique changes to the image attributes. Images were acquired once the cells were in focus and clear. Once the transmitted light images were acquired, the Spot software settings were changed to "GFP" with gain set to 1, exposure set to 500 ms, and gamma set to 0.5. For certain images, the exposure was increased to help outline the fluorescence mitochondria and was noted within the image file name. The light was then shut off and the blue fluorescence light

was quickly activated for the time necessary for Spot to acquire an image. Any region that was imaged was only imaged once to minimize bleaching.

Image analysis and data collection: Images that were captured using the Spot software were individually uploaded to ImageJ (Version 1.53, Java 1.8.0_172) on Mac OS X. One control image for DCF, one control image for R123, one experimental image for DCF, and one experimental image for R123 were opened into ImageJ. Images for the DCF wells were analyzed for brightness. Images were taken at random after moving the chamber slide well to a suitable position for imaging. Cell bodies were defined as being round structures with protruding dendritic and axonal formations. Under the general toolbar, all visible cell bodies were encircled using the circular tool drawing selection. The circles were placed within the cell bodies at the point of highest brightness in order to minimize the background frequency that was being applied to our data. Once encircled, the analyze menu in ImageJ was opened and "measure" was selected in order to obtain a mean and a max brightness value over the encircled area. This method was done for both images. A mean of the max brightness values was collected for n = 29 cell bodies that displayed fluorescence activity for ROS in the control well and n = 9 cell bodies that displayed fluorescence activity for ROS in the experimental well. This data was further analyzed via a histogram to display presence of oxidative stress in both cultures.

Images for the R123 wells were analyzed for brightness. Images were taken at random after moving the chamber slide well to a suitable position for imaging. Under the general toolbar, individual mitochondria were selected, and then small circles were placed within the mitochondria using the circular drawing tool in ImageJ. Mitochondria were defined by their position relative to the axon, their threshold fluorescence, and the presence of an ovoid shape. The individual mitochondria were also selected by witnessing a 4-fold brightness increase across 4 pixels at the mitochondrion's edges. The analysis was mainly done on dendritic mitochondria to eliminate bias. Under my explicit criteria, dendritic mitochondria will represent the mitochondria within the culture as a whole. The mitochondria on the cell bodies were over-expressed and layered on top of each other on different focal planes making it unsuitable and inaccurate for analysis of brightness values. The mitochondria were then analyzed by placing the circle tool on ImageJ within the mitochondrial body in order to reduce background fluorescence. These circles within the mitochondria were then analyzed by selecting the analyze menu in ImageJ and by selecting "measure". Most visible mitochondria that fit the criteria listed above were all analyzed so long as they were within the microscopes field of view. To eliminate brightness bias, images of over 155 brightness pixels were removed from the dataset as they were over-exposed and could create inaccurate results.

Results

To test the hypothesis that oxidative stress leads to a change in mitochondrial charge, human neurons were grown for 9 days in culture. Live cell imaging and fluorescence imaging of DCF for oxidation state and R123 for mitochondrial charge took place to show that both dyes work and that the oxidatively stressed conditions are met. Both dyes showed intense fluorescence brightness in both control dishes meaning that DCF was able to detect reactive oxygen species and that R123 was able to detect individual mitochondrial charges (Figure 1). Mitochondrial dysfunction was simulated through starving these cells for five days. Alzheimer's disease may have a certain pathogenesis originating from mitochondrial dysfunction. Starving these cells attempted to create an environment closely resembling an aged person with oxidatively stressed cells that could lead to mitochondrial dysfunction. After these five days passed, the cells were

again imaged both in live cell imaging conditions and in fluorescence imaging conditions. Cells were seen to be oxidatively stressed in both control and starved conditions in Figure 2 as shown by the outlined fluorescent cell bodies. Quantification of the brightness values for the DCF wells were later analyzed in Figure 4. These results show that this oxidative stress could lead to the large contrast seen between mitochondrial brightness in the outlined mitochondria in the neurons after being stained with Rhodamine 123 in Figure 3. Two histograms were created displaying brightness transmission levels for each set of dyes. Figure 4 displays the similar oxidative stress brightness environment in both control and DCF wells. Figure 5 displays the staggering difference in brightness between the mitochondria in the control group showing greater brightness versus the mitochondria in the starved group showing lesser brightness.

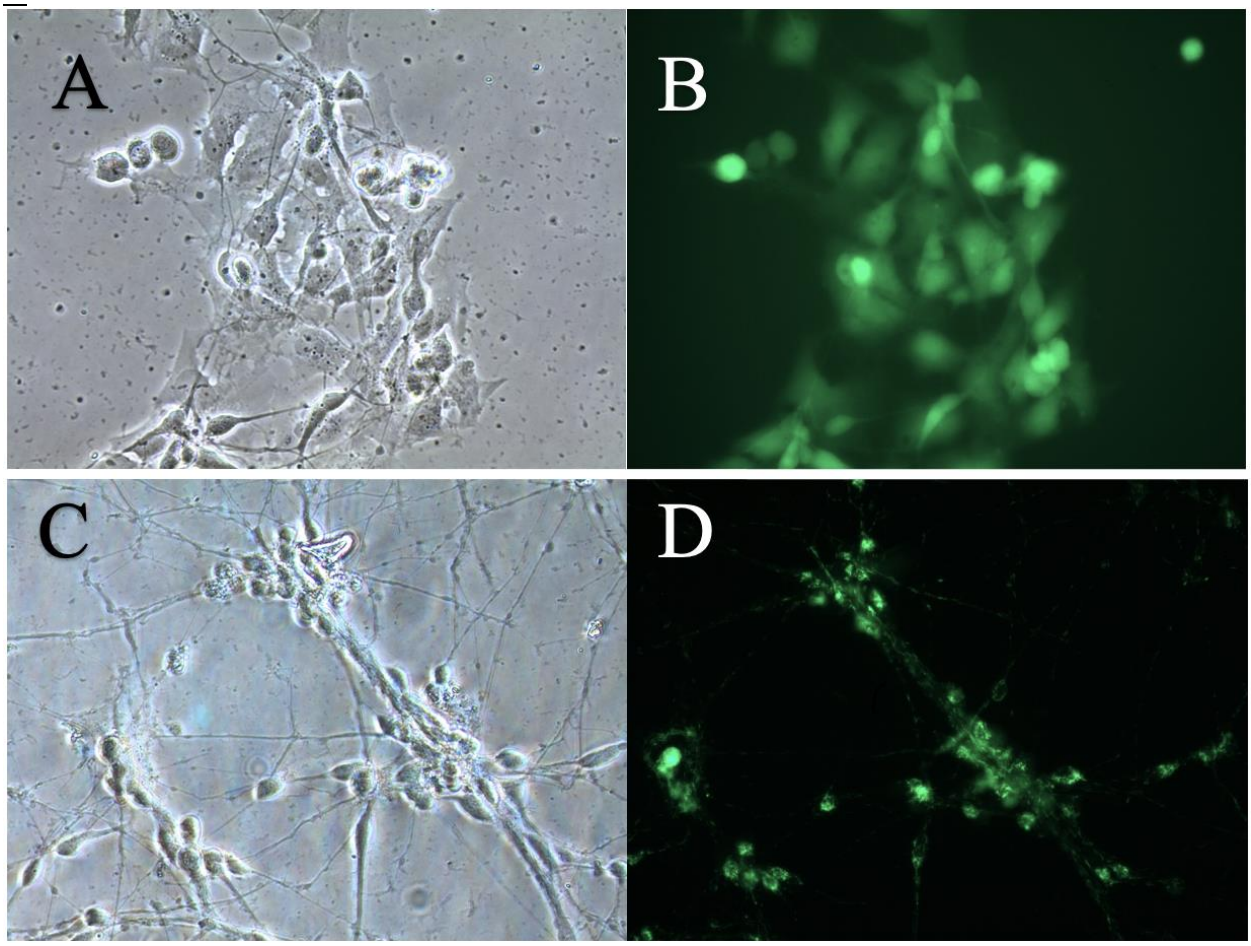

Figure 1: A sample set of images from the control wells. Image (A) is the transmitted light image of the control-fed neurons captured at 40x under phase 3. Image (B) shows the corresponding image of that same well's mitochondria after being stained with Dichlorofluorescein. Image (C) shows a similar transmitted light image captured at 40x under phase 2 of a different well of control-fed neurons. Image (D) shows the corresponding image of that same well's cell bodies after being stained with Rhodamine 123. Notice the brightness pattern for both reactive oxygen species (ROS) in image B and mitochondrial potential in image D.

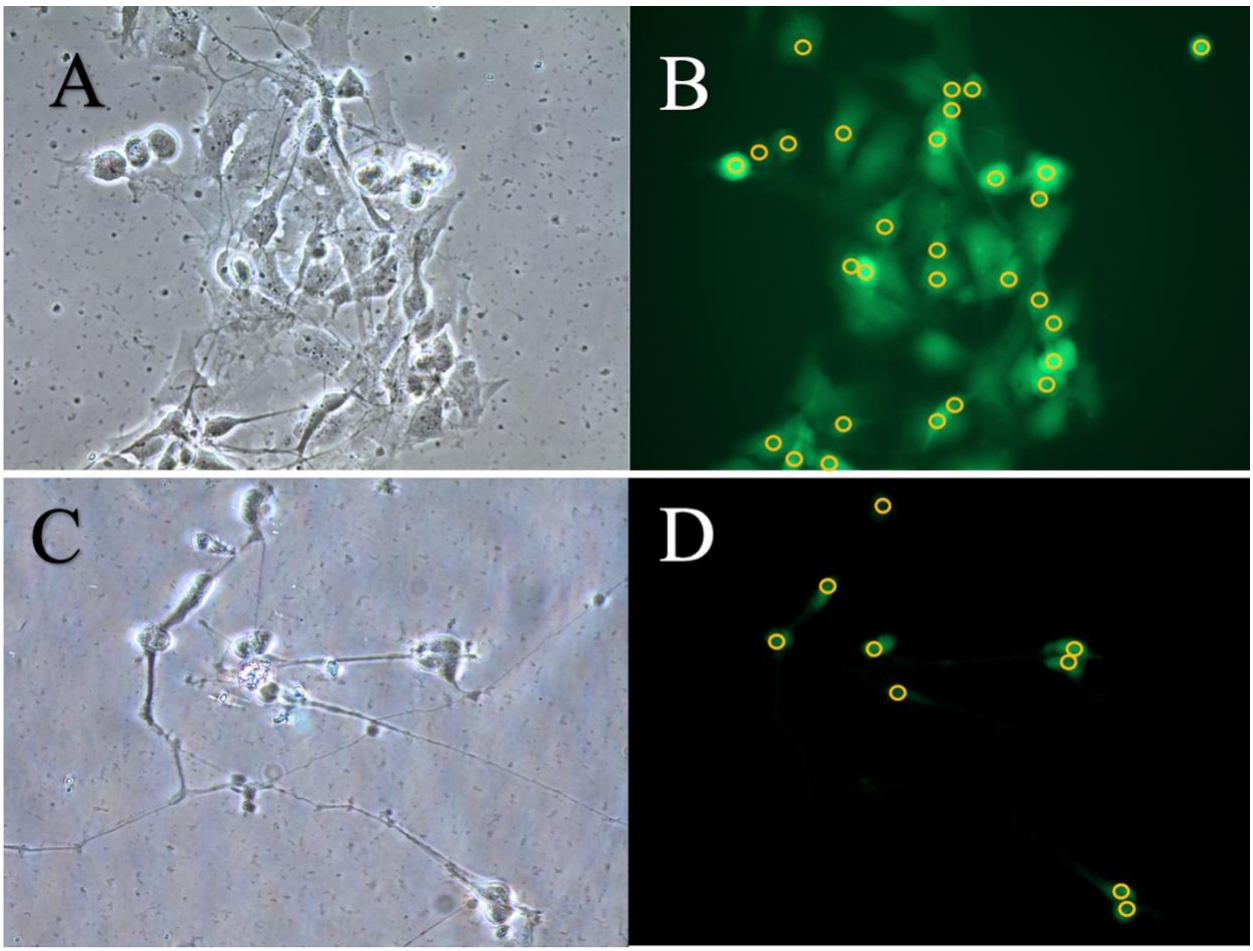

Figure 2: DCF dyed fluorescence images. In these fluorescence images, reactive oxygen species are fluorescing green within the cells. As outlined, individual cell bodies are the structures that are fluorescing, which suggests to us that there may be a reasonable presence of ROS within our neurons in both control (B) and experimental (D) wells. DCF is easily oxidized which causes fluorescence within the ROS in the cells. Notice the brightness between image B and image D.

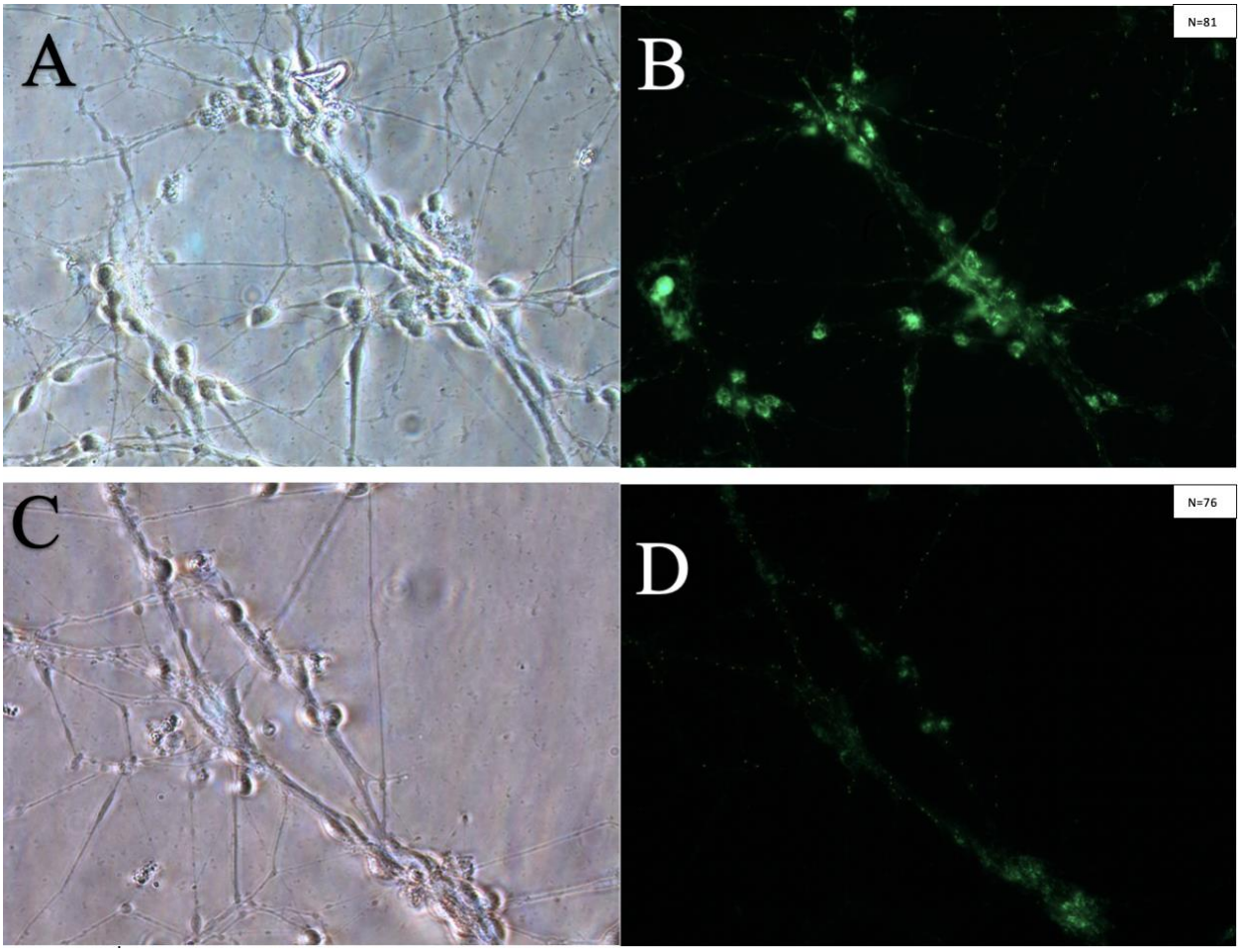

Figure 3: R123 dyed fluorescence images. In these fluorescence images, individual mitochondria are stained. Control well (image B) was stained with R123, imaged, and individual dendritic mitochondria analyzed on ImageJ for brightness data (requires zoom). Experimental well (Image D) was stained with R123, imaged, and individual dendritic mitochondria were analyzed on ImageJ for brightness data. As stated above, the dendritic mitochondria are representative of all the mitochondria in culture and were all selected according to defined criteria within the field of view. Notice the lessening of brightness in image D compared to image B. Loss of potential will result in loss of the dye, which includes fluorescence intensity (Chazotte, 2010). This loss of potential may be attributed to oxidative stress.

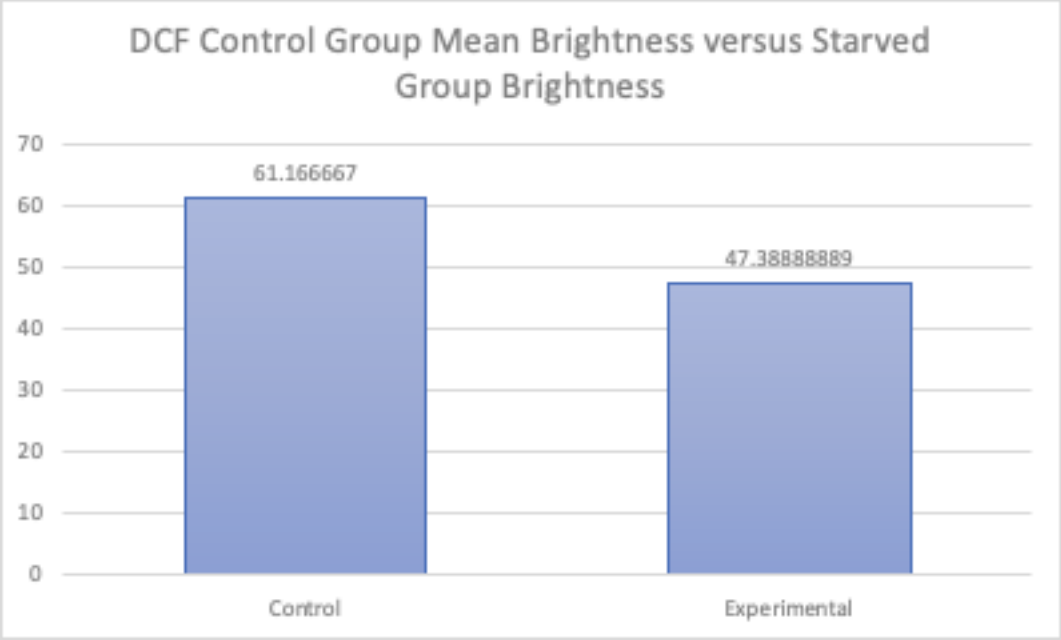

Figure 4: A bar graph relating DCF control group brightness versus DCF starved group brightness. In this image, brightness intensity from all outlined cell bodies within the field of view in each image was averaged and graphed. Notice that both cultures show to endure oxidative stress.

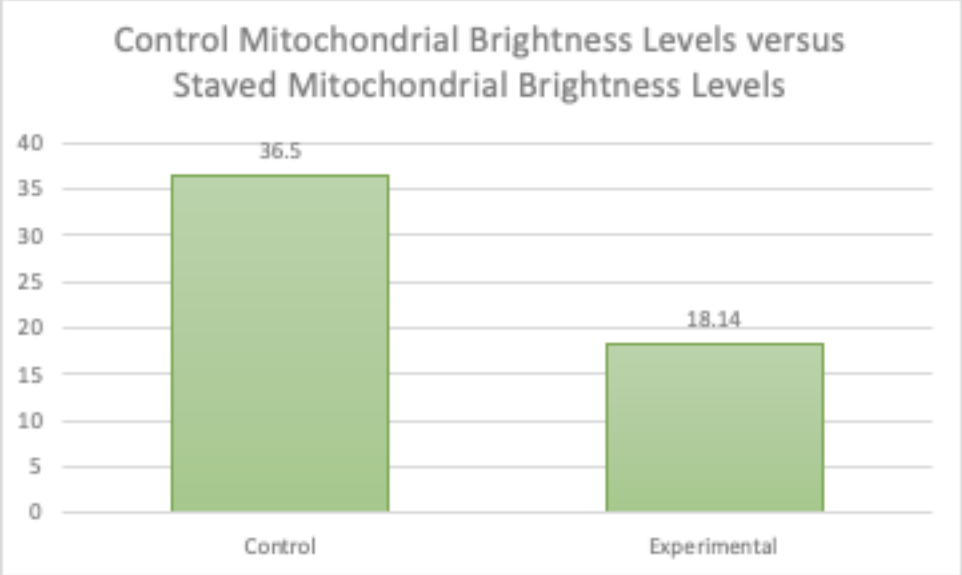

Figure 5: A bar graph relating mitochondrial brightness levels in the Control group versus mitochondrial brightness levels in the starved group. In this image, brightness intensity calculated from all dendritic mitochondria that fit the defined criteria in each image was averaged and graphed. Notice the greater average brightness level in the control group versus the much lower average brightness level in the starved experimental group.

Discussion and Conclusions

This study sought to test that oxidative stress will lead to a change in mitochondrial charge. The preliminary data generated throughout the imaging and analysis of our wells support this hypothesis.

A study in the Journal of Signal Transduction has shown that increased oxidative damage in cells is associated with aging (Cui & Zhang, 2012). This is important when relating to Alzheimer's disease. The vast majority of people that become diagnosed with Alzheimer's are 65 years and older (Cenini & Voos, 2019). Due to old age, oxidative stress could be a factor in the damaging of cells which would lead to mitochondrial dysfunction, and therefore progress the development of Alzheimer's disease (Cui & Zhang, 2012).

The data presented from this study is intriguing in the ongoing study of pathogenesis for Alzheimer's disease. The results with the DCF portion of the study seem to suggest that the cells are oxidatively stressed throughout the experiment. Since oxidative stress may be present, it may show that the damaged and starved cells are important factors in seeing the levels of reactive oxygen species in the culture. The results from the Rhodamine 123 portion of the study may also show that mitochondrial membrane could potentially have decreased consequently from the reduced feeding pattern. In the very bright and fluorescent control well, cells looked to be growing with no treatment and with regular feeding patterns. These findings may have resulted in the vivid and active mitochondria as shown by the R123 stained cells presented above. As the feeding frequency was reduced, these results suggest that the cells got damaged due to oxidative stress which may have further damaged mitochondrial membrane potential. These results seem to show that the reason the starved group results emit a much lower fluorescence was potentially due to much of the mitochondrial membrane potential being lost.

A valid shortcoming can be seen in our DCF image results. In the control set of images, high background noise was detected due to the DCF fluorescing in the media. Many more washes should have been done to reduce the background noise. This noise may have affected the results since higher brightness values within the controls could have unintentionally been analyzed. A different problem that occurred was during the starving of the cells. The air incubator within our lab has low humidity, and therefore our wells were re-fed after five days which ended the starving process prematurely. The wells should have been filled more than protocol standards in order to take the drying problem out of the equation.

Some future experiments that could be performed may include treating the cells with something that could simulate an oxidatively stressed environment. For example, the cells could have been treated with a very low concentration hydrogen peroxide (H2O2). H2O2 has been seen to induce oxidative stress in inflammatory and vascular cells in certain studies (Coyle & Kader, 2007). The cells could have also been starved for a longer time, so long as that the well that the culture is in does not dry up.

These preliminary data regarding oxidative stress and mitochondrial dysfunction may have lasting potential for further studies on neurodegenerative diseases. Alzheimer's disease affects many people worldwide and finding a cure or potential remedy to this disease would save countless lives.

References

Cenini, G., & Voos, W. (2019). Mitochondria as Potential Targets in Alzheimer Disease Therapy: An Update. Frontiers in pharmacology, 10, 902. https://doi.org/10.3389/fphar.2019.00902

Chazotte, B. (2010). Labeling Mitochondria with Rhodamine 123. CSHL Press. Retrieved November 18, 2020, from http://cshprotocols.cshlp.org/content/2011/7/pdb.prot5640.abstract Coyle CH, Kader KN. (Jan, 2007). Mechanisms of H2O2-induced oxidative stress in endothelial cells exposed to physiologic shear stress. ASAIO J. 2007 Jan-Feb;53(1):17-22. doi: 10.1097/01.mat.0000247157.84350.e8. PMID: 17237644.

Cui, H., Kong, Y., & Zhang, H. (2012). Oxidative stress, mitochondrial dysfunction, and aging. Journal of signal transduction, 2012, 646354. https://doi.org/10.1155/2012/646354

Moreira, P., Carvalho, C., Zhu, X., Smith, M., & Perry, G. (October 21, 2009). Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology. Retrieved November 18, 2020, from https://www.sciencedirect.com/science/article/pii/S0925443909002427

Morris, RL. (September 16, 2020). Spoken words.

Morris, R. L. (2020). Culturing of hiPSC-derived mixed cortical neurons: a procedure for Wheaton College BIO 324 Neurobiology, fall 2020. Wheaton Journal of Neurobiology Research (in press)

Scaduto, R. C., Jr, & Grotyohann, L. W. (1999). Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophysical journal, 76(1 Pt 1), 469–477. https://doi.org/10.1016/S0006-3495(99)77214-0

Wang H., Joseph JA. (September 26, 1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med. 1999 Sep;27(5-6):612-6. doi: 10.1016/s0891-5849(99)00107-0. PMID: 10490282.

Wang, W., Zhao, F., Ma, X. Perry, G., Zhu, X. (2020) Mitochondria dysfunction in the pathogenesis of Alzheimer's disease: recent advances. Mol Neurodegeneration 15, 30 (2020). https://doi.org/10.1186/s13024-020-00376-6

Weller, J., & Budson, A. (2018). Current understanding of Alzheimer's disease diagnosis and treatment. F1000Research, 7, F1000 Faculty Rev-1161. https://doi.org/10.12688/f1000research.14506.1