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Inhibition of TORC1 Pathway in *Saccharomyces Cerevisiae* to Induce Autophagy Using Graphene Oxide

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Abstract

Alzheimer's Disease (AD) is a neurodegenerative brain disorder that affects close to six million people in the US alone. The disease is characterized by amyloid beta plaque buildup in the brain which limits proper communication and connection between neurons. Patients with AD were shown to have lower rates of autophagy in neuronal cells. Autophagy is the cellular process by which waste buildup is broken down through lysosomal activity. In mammals, the activated mTORC1 pathway inhibits autophagy in the presence of growth factors and amino acids. To explore the potential of using autophagic activity to break down amyloid beta plaques, *Saccharomyces cerevisiae*, which conserves mTORC1 as TORC1, could be used as a model organism. This project focuses on the inhibitory effectiveness of Graphene Oxide (GO) on the TORC1 pathway in *S. cerevisiae*. Firstly, the cytotoxicity of GO to the *S. cerevisiae* species was tested to ensure GO did not hamper cell proliferation by growing *S. cerevisiae* in a medium which included various concentrations of GO. Then, a fluorescent marker, Rosella, which indicates autophagic activity was used to measure the effects of GO. The *S. cerevisiae* was transformed with the Rosella plasmid to give it fluorescent properties, following which the *S. cerevisiae* was grown in the presence of various GO concentrations. The resultant emissions revealed the relationship between the concentration of GO and the induction of autophagy through inhibition of the TORC1 pathway. By inducing autophagy, GO could prove to be the key to breaking down amyloid beta plaques and treating AD.

Keywords Alzheimer's Disease, Autophagy, Amyloid Beta Plaque, mTOR Pathways

1. INTRODUCTION

1.1. Alzheimer's Disease

Alzheimer's Disease (AD) is a neurodegenerative brain disorder that is also classified as a chronic form of progressive dementia [1]. In the US alone, there are almost six million people [2] living with AD with an estimated 500,000 new diagnoses per year [2]. The brains of people diagnosed with AD are characterized by amyloid beta protein plaques and tau protein tangle buildups. These protein aggregates can block neurotransmission between neurons which can result in cell death [3]. Over time, patients with AD experience a decrease in brain matter and cognitive function as neurons die off at a rapid rate due to intense plaque buildup in critical areas [3].

With no cure, the global prevalence of AD is growing with an estimated 60% of the annual 10 million new cases of dementia coming from the disease [4]. Furthermore, the causes behind plaque buildup and the direct relationship between beta amyloid plaques and dementia is still unclear. As the WHO recognizes AD and dementia as public health priority, initiatives to understand and cure AD are increasingly vital to human health [4].

1.2. Autophagy, mTOR Pathways, and Amyloid Beta Plaque Decomposition

The mammalian mTOR is a serine/threonine kinase that is present in two complexes, mTORC1 and mTORC2. mTOR stands for mammalian Target Of Rapamycin, referencing a drug which is known to inhibit the pathway [5].

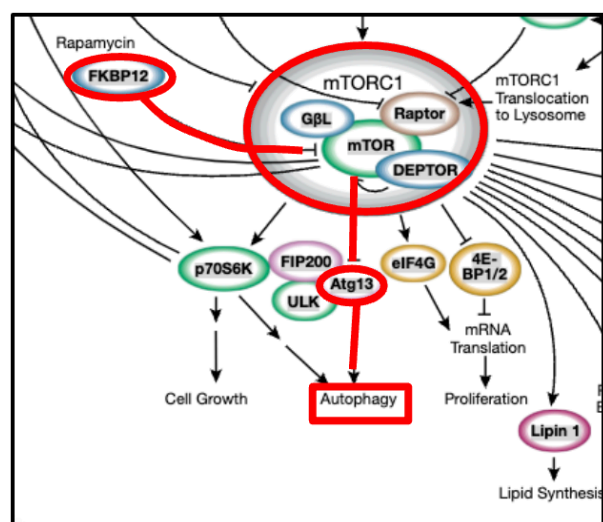


Figure 1. The diagram of the mTORC1 pathway showing the activation of autophagy caused by inhibition of the phosphorylated ATG13 in the presence of Rapamycin.

Both mTORC1 and mTORC2 regulate cell proliferation, autophagy and other growth-related processes in response to growth factors, hormones, and glucose [5]. Particularly mTORC1, activated in the presence of amino acids, phosphorylates protein ATG13; this protein inhibits the activation of autophagy, a process by which intracellular lysosomal activity decomposes aggregates.

In patients with AD, lysosomal activities in the brain related to autophagy have been shown to be impaired [6]. Lower levels of Amyloid beta protein clearance through autophagy contributes to plaque buildup and progression of AD in these patients [7]. Therefore, this experiment explores how inducing autophagy through inhibiting the mTORC1 pathway could help break down Amyloid beta plaques and prevent progression of AD.

1.3. *Saccharomyces Cerevisiae*

Saccharomyces cerevisiae (*S. cerevisiae*) is a species of yeast commonly referred to as Brewer's Yeast. *S. cerevisiae* is an appropriate model organism for autophagy-related research because it conserves a particular regulatory pathway with mammalian cells called mTOR as TOR [8]. Furthermore, *S. cerevisiae* can easily be cultivated and transformed while also maturing quickly and requiring little maintenance. The *S. cerevisiae* strain that was obtained for this project was genetically transformed in order to use fluorescent markers to detect autophagy [9].

1.4. Graphene Oxide

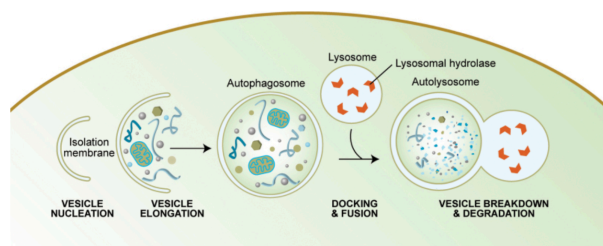


Figure 2. The diagram of the autophagy shows the step-by-step process of how autophagy helps break down intracellular molecules.

Graphene Oxide (GO) is made up of a layered carbon structure with functional groups containing oxygen [10]. It has been shown to have promising properties for numerous applications including field-effect transistors, biomolecule sensors, graphene batteries, and conductive films. GO has also been shown to be a potential inhibitor to various intracellular pathways [11]. Particularly, supporting evidence shows that GO could induce autophagy in human microglia and neurons [7]. In this experiment, GO is tested as a potential inhibitor of the TORC1 pathway in *S. Cerevisiae* to induce a dose dependent autophagic response.

1.5. Rosella Plasmid

Rosella is a fluorescent reporter of autophagy which is composed of green and red markers. When autophagy is induced emissions of specific wavelengths can be detected after excitations using a spectrophotometer. By transforming the yeast with a Rosella plasmid, this method can be used to detect induced autophagy. 10 μ L of the Plasmid required for the transformation was obtained from the Dohlmán Laboratory at UNC Chapel Hill in an Eppendorf.

2. MATERIALS AND METHODS

2.1. *S. cerevisiae* Strain

The WB BY4741 strain of *S. cerevisiae* was obtained from Dohlmán Laboratory at UNC Chapel Hill with the following selection: his3D1 leu2D0 ura3D0 met15D0 LYS2. All the strains were maintained at 30 $^{\circ}$ C in an incubator on the YPD plates upon which they were shipped and were periodically used to inoculate dispersions.

2.2. Plasmid Transformation

To transform the yeast, 10mL of all buffers were formulated according to the following recipes:

2.2.1. Resuspension Buffer:

- 1 mL 10X TE pH 8.0
- 1 mL 1M LiAc
- 8 mL H_2O

Use HCl as needed for pH

2.2.2. 10XTE:

- 100 mM Tris-Cl pH 8.0
- 10 mM EDTA pH 8.0

Use HCl as needed for pH

2.2.3. Transformation Buffer:

- 100 μ L 10X TE
- 100 μ L 1M LiAc
- 800 μ L 50% PEG3350

2.2.4. 50% PEG3350:

- Dissolve 50g PEG3350 into 30ml H_2O
- Warm and stir
- Bring volume to 100mL

All solutions were immediately autoclaved or filter sterilized.

Under sterile conditions, 5mL of Luria Broth (LB) was inoculated using a sterile inoculating loop from the WB BY4741 plate. This dispersion was placed in a shaking incubator at 30°C until saturation. This dispersion was then added to 50mL of LB and incubated at 30°C until the OD_{600} was in the range of 0.8-1.2. After vortexing to ensure a homogenous solution, 45mL of the dispersion was separated in three 15mL centrifuge tubes. The yeast was then pelleted in a centrifuge at 3000 RPM for 5 mins before being washed in 15mL water (LB was siphoned off). This washing process was repeated and resulted in a 15mL H_2O dispersion. This yeast was then pelleted again and 1mL of Resuspension buffer was added after siphoning off the excess H_2O . The 1mL dispersion was transferred to an Eppendorf and vortexed to ensure homogeneity. The solution was then washed with 1mL of Resuspension Buffer after being pelleted at 18000 RPM for 30 seconds. To obtain an optimal number of yeast cells, the concentration of the solution was determined by counting 10 μ L of cells in a hemocytometer microscope slide. Using the concentration, the volume of the dispersion required to obtain 1-2 x 10⁷ yeast cells was determined.

5mL of Salmon Sperm was submerged in a hot water bath at 90°C for 5 minutes to denature before being cooled in an ice water bath until room temperature was reached. To the Eppendorf containing 10 μ L of the Rosella plasmid, 50 μ L of denatured Salmon Sperm, the determined volume of competent yeast cells dispersion, and 600 μ L of Transformation Buffer were added. After gently vortexing this solution to mix, it was incubated at 30°C for 30 minutes, then at 42°C for 20 minutes. Finally, this solution was washed twice with 1mL of H_2O after centrifugation for 30 seconds at 2500 RPM before a final volume of 200 μ L of H_2O was added. This solution of transformed *S. cerevisiae* was streaked onto SC-LEU-ampicillin plates to ensure strain selection.

2.3. Cytotoxicity Assay

To prepare the 4 concentrations of GO in LB used to test the cytotoxic effect of GO on *S. cerevisiae*, 6mg of GO flakes was added into 30mL of sterile LB. This dispersion was then sonicated for 3 hours. While vortexing, the 1.25mL wells were each filled with the 200 μ g/L broth. Then 22.5 mL of sterile LB was added to the solution, causing the concentration of GO to halve to 100 μ g/L. After the next 6 wells were filled with dispersion, 37.5 mL of sterile LB was added to bring the concentration down to 50 μ g/L. After the next 6 wells were filled with dispersion, 67.5 mL of sterile LB was added to bring the concentration down to 25 μ g/L. This concentration was used to fill the next column of 6 wells. Finally, plain LB was added to two columns as the control group (0 μ g/L) and the blank. Then, all the wells were inoculated with yeast except the blank column using a sterile inoculation loop. The plate was then placed for 72 hours in an incubating multimode microplate reader at 30°C with orbital shaking at 200 rpm. Optical density at 600 nm was measured automatically in five minute intervals.

2.4. Fluorescence Marking Assay

To prepare the two concentrations of GO in LB solutions, 50 μ g/L and 100 μ g/L, 25mg of GO flakes was added to 250mL of LB. This solution was sonicated for 5 hours in 30 minute intervals. After each interval, the solution was vortexed. Finally, the resultant dispersion was autoclaved. The 250mL dispersion was then split into two

125mL dispersions with the concentration of each being 100 μ g/mL. One of the dispersions was then added to 125mL of LB to reduce the concentration to 50 μ g/mL.

Into a 96 well black plate, 48 wells were filled with 200 μ L of the 100 μ g/mL GO-LB dispersion, while the remaining 48 wells were filled with 200 μ L of the 50 μ g/mL GO-LB dispersion. Finally, each well was individually inoculated with the transformed yeast and the plate was incubated for 24 hours at 30°C to initiate autophagic processes. The plate was then measured at 485/20 nm excitation and 528/20 nm emission with a resultant fluorescence reading for each well.

3. DATA/RESULTS

3.1. Cytotoxicity Assay

To determine if GO inhibits the regular growth and cell proliferation rates of *S. cerevisiae*, the yeast was grown in a growth medium which contained determined concentrations of GO. The Optical Density at 600 nm (OD_{600}) determines the level of yeast proliferation in the well with higher OD_{600} signifying more proliferation.

Figure 3 displays a clear logistic growth curve for the *S. cerevisiae* grown in all concentrations. There is a clear lag, logarithmic, and stationary phase regardless of concentration. In addition, the OD_{600} of the non-inoculated blank group remained at 0 which confirms the absence of any contamination. There was no significant difference in the rate or intensity of *S. cerevisiae* cell proliferation/growth when grown in different concentrations of GO.

3.2. Fluorescence Marking Assay

Once it was determined that there were no cytotoxic effects of GO on *S. cerevisiae* growth, GO was applied in the growth medium of the transformed *S. cerevisiae* to determine its effectiveness in inducing autophagy. The fluorescent readings at 485/20 nm excitation and 528/20 nm emission illustrate the ubiquity of autophagy induction in the samples.

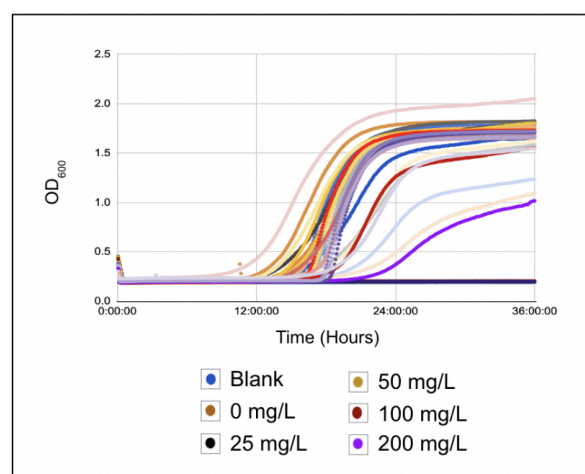


Figure 3. Dose dependent OD_{600} growth curve: The Optical Density of each well at 600 nm was measured every 5 minutes and plotted over time resulting in a logistic growth curve showing the proliferation of *S. cerevisiae* in all inoculated wells.

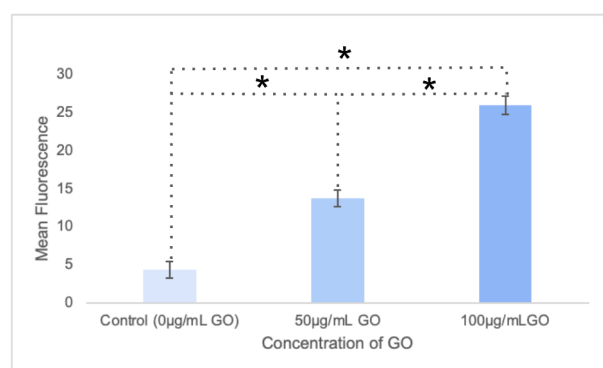


Figure 4. Fluorescent analysis of dose dependent autophagy induction: The fluorescence emission at 528/20 nm was recorded for 32 wells for each group including the control. The average of these values were plotted.

Figure 4 displays a significant dose dependent trend of fluorescence increasing with higher GO concentrations. A mean fluorescence reading of 4.38 was recorded for the control group; the 50µg/mL GO group had a mean fluorescence reading of 13.8; finally, the 100µg/mL GO group recorded a mean fluorescence reading of 26.0.

4. DISCUSSION AND LIMITATIONS

4.1. Cytotoxicity of Graphene Oxide to *S. cerevisiae*

Figure 3 demonstrates that GO does not have a significant cytotoxic effect on the cell proliferation of *S. cerevisiae*. The OD₆₀₀ of each well, regardless of GO concentration, traced the standard logistic curve of *S. cerevisiae* growth. Furthermore, the curves representing the 0 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L groups all overlapped throughout the 36 hours without significant variances, showing the same proliferation rate regardless of concentration. Therefore, higher GO concentrations did not have cytotoxic effects on *S. cerevisiae*. Because GO is not cytotoxic to *S. cerevisiae*, it can be tested as an autophagy inducer in *S. cerevisiae*.

4.2. Autophagic Response of *S. cerevisiae* to Graphene Oxide

Figure 4 demonstrates a dose-dependent effect of GO on autophagic rate of *S. cerevisiae* in the wells. When comparing either group which included GO, 50µg/mL or 100µg/mL, to the control group with no GO, there was a significant difference in the mean fluorescence reading. With both of these groups with GO compared to the control, there was significantly higher mean fluorescence indicating significantly higher autophagic activity. In other words, the presence of GO inhibited the TORC1 pathway and activated autophagy in the *S. cerevisiae*.

This effect also appears to be dose-dependent as greater concentrations of GO resulted in significantly higher rates of autophagy. This is shown in the significant difference in mean fluorescence between the 50µg/mL group and the 100µg/mL group. This dose dependence indicates that more quantity of GO is more effective in inhibiting the TORC1 pathway in *S. cerevisiae* and inducing autophagy.

The induction of autophagy at a dose dependent rate also incites a greater potential for amyloid beta plaques to be broken down with higher concentrations of GO.

4.3. Limitations

The scope of this study focused on inducing autophagy in *S. cerevisiae*, not directly on amyloid beta plaques. Therefore, though suggestions that amyloid beta plaques could be broken down at greater rates in the presence of GO due to autophagy can justifiably be made, more research is necessary for conclusive results.

Furthermore, cytotoxicity of GO to *S. cerevisiae* was determined for the chosen concentrations, however, higher/lower concentrations should be tested to determine the viable range for GO.

Another limitation is that the transformation of the yeast with the Rosella plasmid occurred over the course of three work days. Overnight, certain mixtures of ingredients from the middle of the protocol were left sitting which could have affected the quality of the produced *S. cerevisiae* strain.

5. CONCLUSIONS AND FUTURE WORK

The results from the cytotoxicity assay and the fluorescent indicator assay support that Graphene Oxide is a viable candidate to break down amyloid beta plaques. The initial cytotoxicity assay validated the results by providing evidence that GO was not cytotoxic to the *S. cerevisiae* and did not inhibit cell proliferation. By transforming the *S. cerevisiae* to include a fluorescent indicator of autophagy, the process by which amyloid beta plaques can be broken down, the inhibitory effects of GO on the TORC1 pathway which regulates autophagy became clear. The data strongly supports that autophagy is not only induced by the presence of GO, but also that greater concentrations of GO result in a dose dependent increase in autophagic response.

The next step is to determine the effects of GO directly on amyloid beta plaques in model organisms such as *Caenorhabditis elegans* or *Drosophila melanogaster* which can directly model said plaques. Moreover, the effects of a continuous reintroduction of GO into the growth medium could provide insight into the inhibition of the TORC1 pathway. Finally, Western Blot or ELISA assays which can test the presence and concentration of autophagy-related proteins (ATG 13) could further confirm the induction of autophagy.

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