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# Hypoglycemia, Hyperglycemia and Astaxanthin: An *in Vitro* Alzheimer's Disease Model

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## Abstract

Alzheimer's Disease is projected to increase to 30 million people in the next 30 years and the rate of diabetes mellitus is projected to rise also. Hyperglycemia is commonly observed in patients with diabetes mellitus, and hypoglycemia is a common consequence due to insulin therapy. Previous research has shown a potential link between Alzheimer's disease and diabetes. This study sought to determine if Astaxanthin (ATX) could prevent mitochondrial dysfunction from the compounded effects of amyloid $\beta$ (A$\beta$) plaque and hypoglycemia or hyperglycemia. Growth patterns, ATP production, and ROS generation were examined in 2 μM, 5 μM, 25 μM (hypoglycemic groups), 2 mM, 5 mM (normal groups), and 25 mM glucose (hyperglycemic group), and then treated with or without ATX or A$\beta$. When hypoglycemia groups and the hyperglycemia group were treated with ATX, their growth patterns were either comparable to control or increased. ATX and A$\beta$ treated cells demonstrated increased growth patterns over cells treated with A$\beta$ alone. A$\beta$ alone treated groups overall had significantly less growth than controls ($p < 0.05$). Hypoglycemic groups produced overall low levels of ATP, and the hyperglycemia group produced high levels of ATP. Cells cultured with A$\beta$ demonstrated low levels of average fluorescence generated by ROS production as determined by MitoSox assay while ATX groups actually produced higher to normal levels of ROS. Cells grown in the presence of A$\beta$ and ATX generally produced more ROS than just A$\beta$ groups. Thus, hypoglycemia and hyperglycemia do appear to compound the effects of A$\beta$ on hippocampal cells. ATX treatment demonstrated promise with increased cellular growth, which promoted usage of ATP by the cell and ROS production. This growth was present even in the presence of A$\beta$, suggesting that ATX is able to overcome the negative effects of A$\beta$.

## 1. Introduction

Alzheimer's Disease (AD) is a neurodegenerative disease involving the progressive loss of memory and mental function that affects approximately 5.8 million people in the United States in 2019 [1]. Despite the prevalence of this disease, few treatment options are available, and no cure exists [2]. A hallmark of AD is the presence of amyloid beta peptide ($A\beta$) deposition in the brain [3]. $A\beta$ peptides are known to increase reactive oxygen species (ROS) production [4] [5] [6]. $A\beta_{1\text{-}42}$ has also been shown to enhance insulin receptor substrate phosphorylation, which an antioxidant known as astaxanthin was able to reduce and to reverse the cognitive and memory deficits caused by amyloidogenic effects in the hippocampus [7]. $A\beta$ increases the number of mitochondria with mutated mDNA, contributing to higher oxidative stress [8] [9] [10].

ROS are produced from the partial reduction of $O_2$ at the end of the electron transport chain. When $O_2$ is only partially reduced, it produces reactive oxygen species, such as superoxide anion, hydrogen peroxide, and a hydroxyl radical, that change the structure of proteins, lipids, and nucleic acids and lead to cell death [5] [11].

ROS production may vary due to the presence of high blood glucose levels (hyperglycemia), typical of Diabetes Mellitus (DM). DM is a risk factor for Alzheimer's Disease; connecting diabetes-related mechanisms to actual AD pathology [12] [13]. Interestingly, hypoglycemia and hyperglycemia have been shown to cause an increase in ROS production and cognitive dysfunction [14] [15]. The signaling pathways triggering hyperglycemia also play a part in DM symptoms by increasing ROS, oxidative stress, and cellular death and necroptosis [16] [17] [18]. Hypoglycemia in type 1 diabetes damages working and long-term memory as a result of oxidative stress [19]. Hyperglycemia is a major contributor to ROS production and other damage in type 2 diabetes [20].

Astaxanthin (ATX) is a carotenoid produced by aquatic microorganisms that is then ingested into marine wildlife, including salmon, shrimp, lobster, and crayfish. ATX can cross the blood-brain barrier and scavenge ROS in the brain [21]. ATX does accumulate in the hippocampus 4 and 8 hours after a single dose and increases over time [22]. ATX has also been shown to be neuroprotective against $A\beta_{1\text{-}42}$-mediated toxicity in PC-12 neuronal cells and against hypoglycemic conditions [14] [23]. Under normal blood glucose levels, ATX was found to ameliorate the mitochondrial dysfunction caused by $A\beta$ [14]. This paper looks at the effectiveness of ATX in reducing oxidative stress in neurons as determined by ROS and ATP production under hypoglycemic and hypergly-

cemic conditions.

## 2. Materials and Methods

### 2.1. Cell Culture

A mouse hippocampal cell line (NE-4C) was purchased from ATCC (CRL-2925), along with Eagle's Minimum Essential Medium (EMEM) and Fetal Bovine Serum Albumin (FBS). The EMEM was supplemented with Pen Strep at a concentration of 0.99% to reduce microbial growth. Once the cells were thawed, the EMEM and Pen Strep medium was mixed with FBS to a final concentration of 15%. This total 20 mL medium was prepared and warmed in a 37°C water bath. Warm EMEM/FBS medium was then added drop-by-drop to the cell solution in a 1/10 dilution and mixed. Cells were then spun down at 1K for 5 minutes, and the supernatant was removed, and warm EMEM/FBS medium was added to the cell pellet in a 1/8 dilution and mixed. The cells were incubated at 37°C, and the medium was changed every two to three days. Once the desired cell population was reached, the cells were split and frozen in a −80°C freezer.

### 2.2. Neuronal Differentiation

For the ATP and MitoSOX assays, the cells were thawed and then differentiated. Neuronal differentiation was accomplished using retinoic acid (RA) at a concentration of $10^{-7}$ M for 24 hours [24], then cells were washed in warm PBS at 37°C. During the 24-hour retinoic acid exposure, the cells were grown in the EMEM/FBS medium in an incubator at 37°C. After 24 hours, the cells were washed with warm PBS at 37°C and challenged based on their conditional groups, either G, GA$\beta$, GATX, or GA$\beta$ATX for 2 μM, 5 μM, 25 μM, 2 mM, 5 mM, and 25 mM glucose groups. The cultures were exposed to the group condition outlined above for 24 hours, washed with PBS at 37°C, and then, either a luciferase assay or MitoSOX assay was performed.

For the GA$\beta$ or GA$\beta$ATX conditional groups, A$\beta_{1-42}$ was obtained from Sigma Aldrich and was then dissolved in sterile DMSO to make a 0.5 mM stock solution. Cells in the GA$\beta$ and GA$\beta$ATX groups were incubated with a concentration of 2 μM A$\beta_{1-42}$ for 24 hours. Astaxanthin was also obtained from Sigma Aldrich and dissolved in sterile DMSO to make a 0.1 μM stock solution. Cells were then incubated with ATX at a concentration of 10 nM for 24 hours. Cells in the GATX and GA$\beta$ATX groups were incubated with a concentration of 10 nM ATX for 24 hours.

### 2.3. ATP Luciferase Assay

Cells for this assay were grown in Eppendorf tubes. Under hypoglycemic conditions, only two challenges were performed due to the results obtained from controls. Based on the exposure groups, the cells were incubated with their specific glucose and/or treatment exposure, 2 μM of A$\beta_{1-42}$, or 10 nM ATX for 24 hours.

Glucose challenge and glucose with ATX exposure groups were tested. This was due to the very small luminescence of the groups compared to ATP concentrations. Intracellular ATP in the cells of the different exposure groups were measured utilizing Firefly Lantern Extract from Sigma-Aldrich and ATP hydrate from Sigma-Aldrich. 100 μl experimental and control samples were boiled for 3 minutes at above 95°C. Samples were then removed from the boiling water and cooled briefly, and the boiled sample was transferred to the scintillation vials. This solution was then diluted with 1 mL double deionized water.

To each of these vials, Firefly Lantern Extract was added. After the addition of the luciferase, the samples were capped and mixed and stood in the dark for five minutes. Measurements were performed using a scintillation counter with 1-minute readings on a wide band setting. Known concentrations of ATP were used for a standard curve to help calculate the ATP concentration in each sample. ATP concentrations were then plotted using this standard curve to determine differences between the different experimental groups.

### 2.4. MitoSOX Assay

Cells for this assay were cultured on cover slips within petri dishes. Cells were exposed to RA at $10^{-7}$ M for 24 hours for cellular differentiation. The RA was added into the EMEM/FBS medium. After the 24 hour-incubation period, the cells were washed with warmed PBS at 37°C. Based on the exposure groups, the cells were incubated with their specific glucose and/or treatment exposure group condition, 2 μM of $A\beta_{1-42}$, or 10 nM ATX for 24 hours. Before the end of the cell exposure period was over, the 5 mM MitoSOX stock solution was made from vials obtained from Thermo Fisher Scientific. At the end of the exposure period, cells were washed with warmed PBS at 37°C and incubated at 37°C with a 5 μM MitoSOX solution for 10 minutes. Cells were then removed from the incubator and washed three times with warmed PBS at 37°C, and then suspended in warmed PBS. Cells were then imaged using a fluorescent microscope. Obtained pictures were analyzed via Image Pro 6. Once images were taken, the cover slips were prepped and mounted on slides.

### 2.5. Data Analysis

Data was collected and analyzed using Image Pro 6 to obtain mean fluorescence values for the entire image, and three-point region analysis was performed on the brightest regions. Alterations in glucose and varied chemical challenges were compared by one-way analysis of variance. A p-value of $p < 0.05$ was considered statistically significant.

## 3. Results

Cell growth, ATP production, and ROS production were measured and analyzed for significant changes between the control and experimental groups. The following presents the results of the four groups under hypoglycemic and hyper-

glycemic conditions (G, GATX, GA$\beta$, and GATXA$\beta$) in 2 µM, 5 µM, 25 µM, 2 mM, 5 mM and 25 mM glucose.

## 3.1. Cell Growth

Cell growth was assessed based on the percentage of the total coverslip coverage. These coverslips appeared sheet-like in growth. Cell growth was not significantly different among the different glucose groups (Figure 1(a)), but cell growth was decreased among groups treated with A$\beta$ (Figure 1(b)). The A$\beta$ and ATX group grew better than the A$\beta$ group, but still showed growth difficulties compared to the control (glucose) and glucose ATX groups.

## 3.2. ATP Assay—Hypoglycemia

A Luciferase firefly assay was used to measure ATP output after growth. The 2 µM, 5 µM, and 25 µM glucose exposure groups had lower ATP concentrations than the zero ATP concentration standards, and the values were corrected to zero. Cells challenged with glucose demonstrated significantly different ATP output values. The hypoglycemic exposure groups, 2 µM G, 5 µM G, and 25 µM G demonstrated very low levels of ATP production when compared to the normal glucose concentrations of 2 mM G and 5 mM G.

## 3.3. ATP Assay—Hyperglycemia

When comparing the ATP production of the glucose-only groups, the 25 mM glucose group produced more ATP than the 5 mM group. The 25 mM glucose group produced 242.7% more ATP than the 5 mM group. The 25 mM glucose group with ATX produced 255.8% more ATP than the 5 mM group. The 25 mM glucose group with A$\beta$ produced 391.5% more ATP than the normal glucose group, the highest increase among the four test groups (control, GATX, GA$\beta$, and GATXA$\beta$). Finally, the 25 mM groups treated with A$\beta$ and ATX demonstrated high ATP levels, but the levels of ATP production fell between the 25 mM GATX and the 25 mM GA$\beta$ groups. Among the 5 mM G groups, the 5 mM control group produced the most ATP. Among the 25 mM G groups, the 25 mM GA$\beta$ group produced the most ATP.

## 3.4. MitoSOXAssay—Hypoglycemia

Cells were imaged on a fluorescent light microscope at 5x intensity. High output of fluorescence indicated high stress and high ROS output. All cells are expected to fluoresce because any cell undergoing cellular respiration produces a small fraction of ROS through the process, but this assay identified cells producing abnormally high or low amounts of ROS. Some of the lowest levels of glucose produced the most fluorescence, indicating the most ROS production, indicating high stress. 2 µM G produced the greatest amount of fluorescence. When compared to the normal glucose group 5 mM G, the only groups showing a significant difference were the 5 µM G and 25 µM glucose concentrations.
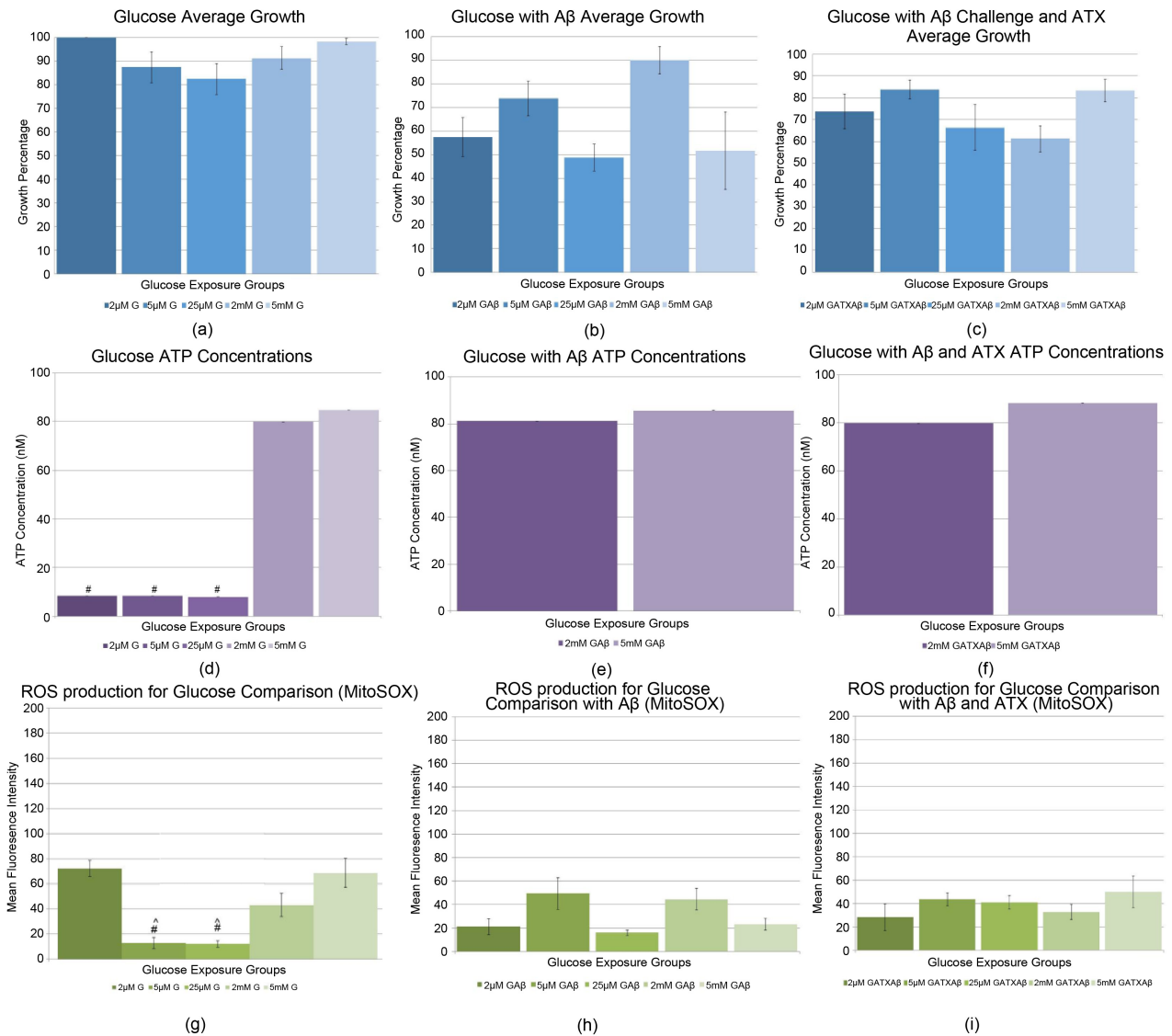
**Figure 1.** Cells challenged in hypoglycemia glucose concentrations demonstrate altered growth. (a) Cells exposed to different glucose concentrations did not show significant difference in growth. The 2 μM and 5 mM G groups grew the best, while the 5 μM G, 2 mM G and 25 μM G demonstrated poor growth. (b) Cells exposed to A$\beta$ exhibited decreased growth, and the 2 mM GA$\beta$ group grew the best. The 2 μM GA$\beta$ group demonstrated slightly decreased growth compared to the 2 mM GA$\beta$ group, while the 25 μM GA$\beta$ and 5 mM GA$\beta$ demonstrated notable decreases in growth. (c) Cells exposed to ATX grew well and showed statistical differences in growth. Cell exposed to both A$\beta$ and ATX, exhibited increased growth for all glucose concentrations. The 2 μM GATXA$\beta$, 25 μM GATXA$\beta$, 5 μM GATXA$\beta$, and 2 mM GATXA$\beta$ demonstrated decreased growth compared to the 5 μM group. (d) Hypoglycemia groups demonstrated very low levels of ATP concentration compared to the 2 mMG and 5 mM G groups. For the GA$\beta$, GATX, and GATXA$\beta$ groups, only 2 mM and 5 mM groups produced ATP concentrations high enough to be measured. (e) The 2 mM GA$\beta$ groups showed decreased ATP concentrations compared to the 5 mM GA$\beta$ group. (f) The 2 mM GATXA$\beta$ group demonstrated lower ATP concentration than the 5 mM GATXA$\beta$. (g) For ROS production, the 25 μM G and 5 μM groups demonstrated significant decrease in fluorescence compared to the 2 μM G group, which is the lowest glucose concentration and produced the most ROS. (h) All GA$\beta$ demonstrated decreased ROS production and were compared to 5 μM GA$\beta$ due to its low to normal levels of ROS production. The most significant decreases were seen for the 2 μM GA$\beta$ and 25 μM GA$\beta$, and the 2 mM group demonstrated a slight decrease. (i) A$\beta$ and ATX Group demonstrated lower levels of fluorescence and ROS output across all glucose concentrations. None of the groups were statistically different. 2 μM GATXA$\beta$ and 2 mM GATXA$\beta$ had the lowest ROS production. *indicates $p < 0.05$ when compared with the control (5 mM). #indicates $p < 0.01$ when compared with the control. ~ indicates $p < 0.05$ when compared to 2 μM. ^indicates $p < 0.01$ when compared to 2 μM.

These results indicate that cell growth, ATP production, and ROS production was different among the different glucose groups. We also explored the other extreme of glucose concentration: hyperglycemia or high blood glucose. The following results come from treating the four experimental groups (G, GATX, GA$\beta$, and GATXA$\beta$) with 5 mM and 25 mM glucose levels.

### 3.5. MitoSOX Assay—Hyperglycemia

ROS production was determined by conducting MitoSOX (ROS) assays and measuring mean fluorescence (Figure 2). ROS production was different for groups with different glucose concentrations, A$\beta$ and/or ATX. The hyperglycemic
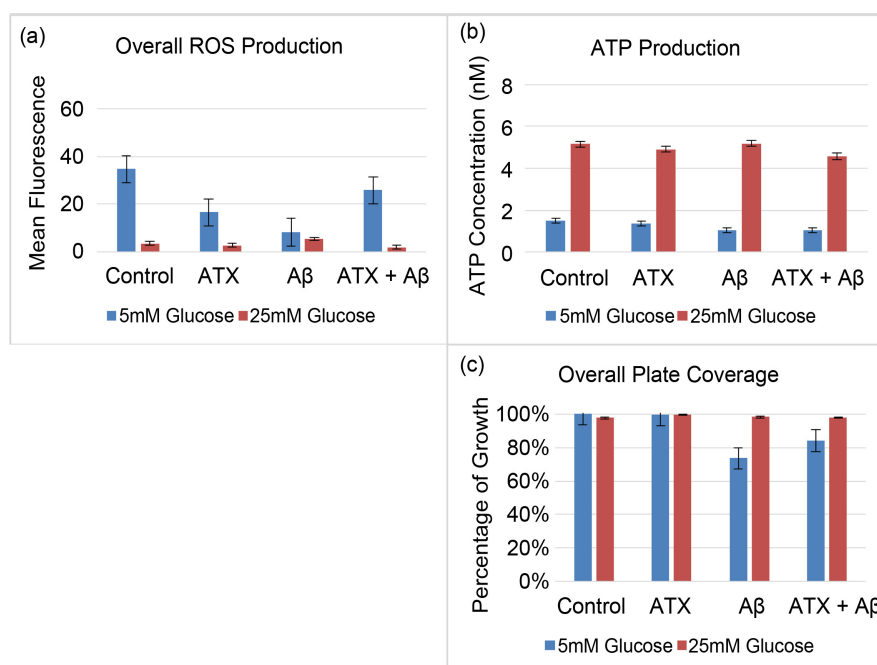


**Figure 2.** (a) Groups exposed to hyperglycemic concentrations produced higher ATP concentrations. The 25 mM G produced more ATP than the 5 mM G. The hyperglycemia group exposed to ATX produced high ATP concentrations. Hyperglycemia groups exposed to A$\beta$ produced very high levels of ATP, as the 25 mM group produced 391.5% more ATP than the 5 mM group. Hyperglycemia groups exposed to both A$\beta$ and ATX produced lower levels of ATP. Bars indicate standard error for each condition. ● indicates the 25 mM treatment is significant from its corresponding 5 mM equivalent. ▲ indicates that the 25 mM ATX was significantly different than the 5 mM ATX treatment. (b) ROS production was greatly decreased in the 25 mM glucose group. The 5 mM control group produced more ROS than the 25 mM control group. ROS production was significantly decreased in the 25 mM GATX group compared to the 5 mM GATX group. ROS production was slightly decreased in the 25 mM GA$\beta$ group compared to the 5 mM GA$\beta$ group. ROS production was greatly decreased in the 25 mM GATXA$\beta$ group compared to the 5 mM GATXA$\beta$ group. (c) Overall plate coverage varied between the two experimental groups. The hyperglycemic control group grew less than its 5 mM counterpart. The 25 mM GATX group did not grow significantly more than its 5 mM counterpart. The 25 mM GA$\beta$ and GA$\beta$ATX groups both grew significantly more than the 5 mM GA$\beta$ and GA$\beta$ATX groups. ✿ indicates the 25 mM treatment is significant from its corresponding 5 mM equivalent.

group produced 89.6% less ROS than the normal glucose group (Figure 2(b)). The 25 mM glucose group treated with ATX produced significantly less ROS than the 5 mM ATX group. The 25 mM glucose group treated with A$\beta$ produced 34.4% less ROS than the 5 mM A$\beta$ group. The 25 mM glucose group treated with ATX and A$\beta$ produced 91.9% less ROS than the 5 mM ATX and A$\beta$ group, but this difference was not statistically significant.

Among the 5 mM glucose groups, the control group produced the most ROS, and there was a significant reduction in ROS production observed in the 5 mM GA$\beta$ compared to the 5 mM control (p < 0.05). Among the 25 mM glucose groups, the A$\beta$ group produced the most ROS, followed closely by the control group. Overall, the 25 mM glucose groups produced less ROS than the 5 mM glucose groups.

## 4. Discussion

This paper sought to explore if astaxanthin could prevent mitochondrial dysfunction, observed through mitochondrial ROS output, and cell viability as observed through ATP production and cell growth. Under hypoglycemic conditions with A$\beta$ treatment, cells produced very low ATP concentrations, had increased ROS production, and decreased cell growth. Introduction of astaxanthin in the GA$\beta$ATX group indicated that ATX may prolong neural cell viability under these conditions.

The results varied among the three hypoglycemia groups (2 μM, 5 μM, 25 μM). In the severe hypoglycemia 2 μM glucose exposure group, the cells exposed to A$\beta$ exhibited decreased growth and decreased fluorescence in the MitoSOX assay. The cells therefore exhibited decreased ROS production, mirroring typical cell responses to A$\beta$. When exposed to A$\beta$, cells demonstrated increased mitochondrial dysfunction observed by high levels of ROS production. Cells also typically showed decreased cellular function with lower cell growth and low ATP production. When the 2 μM glucose exposure group was exposed to both A$\beta$ and ATX, cell growth increased as did mean average fluorescence compared to the 2 μM GA$\beta$ group. This indicates that the cells are likely not dead but may be in the process of dying, so ATX may help to prolong neuronal cell viability in the presence of A$\beta$. ATP assays were not conduced for GA$\beta$ or GA$\beta$ATX groups, as G and GATX groups produced very low ATP levels at 2 μM. This indicates that when glucose levels are exceedingly low, the cell is producing very low levels of ATP, possibly the bare minimum amount of ATP required for cell function.

In the 5 μM glucose exposure group, cell growth and ATP production were very similar to the 2 μM exposure group. The 5 μM GATX grew the best and produced the highest mean fluorescence average in the MitoSOX assay among the different glucose ATX groups. Abnormally high or low ROS levels indicate cell stress, but the levels observed among the 5 μM glucose groups indicate that the cells are flourishing and likely had more functional mitochondria to produce normal ATP levels. Similar to the 2 μM GA$\beta$ exposure groups, the 5 μM GA$\beta$

challenged group again demonstrated the lowest growth of all the 5 μM groups. The 5 μM GAβ demonstrated increased mean fluorescent levels compared to the 2 μM GAβ indicating a cell stress response to cell Aβ. The 5 μM GAβATX group exhibited both increased growth and mean fluorescence levels. This increase in mean fluorescence and hence ROS production was likely due to normal ATP production as opposed to cell stress.

The 25 μM glucose exposed group, similar to the other two hyperglycemia groups, grew the least and produced very little fluorescence. This decrease in fluorescence may be the result of decreased growth or increased cell death. The 25 μM G group exhibited the least growth when compared to lower glucose concentrations (2 μM and 5 μM) as well as very low ATP concentration and ROS production. The 25 μM GAβATX did however grow and produced low to normal fluorescence, showing that among the three hypoglycemic conditions, the addition of ATX to Aβ-exposed groups protected against ROS production and promoted cell growth.

The other two glucose groups (2 mM and 5 mM) grew well except for the 2 mM GAβATX group which exhibited the lowest ATP concentration and fluorescence production (indicating the cells were viable and using the ATP produced). The 5 mM GAβATX group demonstrate increased growth, increased ATP and normal mean fluorescence levels. The 2 mM GATX group grew the best among the 2 mM groups and provided normal ATP concentration and fluorescence production totals. The 5 mM GATX group exhibited decreased growth with slightly increased ATP concentration and low fluorescence production. The 5 mM GATX cells were therefore able to produce more ATP and hence more ROS, but the ATX group did not display a decrease in mitochondrial dysfunction compared to glucose exposed. The 2 mM GAβ group grew and produced the most ATP and fluorescence among the 2 mM groups, indicating the presence of cell stress after being exposed to Aβ. The ATP production indicated that the cells were making ATP, which would increase ROS production, but the cells did not appear to be using this ATP. The ATP that was produced but was not used may be stored in vesicles, a question that should be explored in future studies. The 5 mM GAβ group exhibited the lowest growth, ATP concentration, and ROS production of the 5 mM groups.

The hyperglycemic groups demonstrated increased cell growth and ATP production due to increased availability of glucose, but this availability also contributes to increased ROS. Among the 5 mM glucose groups, the 5 mM GAβ group exhibited the least growth and the lowest ROS production, along with low ATP concentrations. The low ROS production may relate to the lower rate of ATP production, and the low ATP and ROS levels indicate the cells had died or were dying when the Luciferase and MitoSOX assays were performed. Among the 5 mM glucose groups, the 5 mM GAβATX group ROS output was not statistically different from the 5 mM glucose control. This indicates that the mitochondria in the 5 mM GAβATX groups were functioning similar to the control, leading us to

conclude that ATX can protect the neuronal cells from A$\beta$ stress and mitochondrial dysfunction. The ATP concentration ranged 1 μM - 80 μM for sample.

The 25 mM hyperglycemic groups exhibited similar growth. The 25 mM GA$\beta$ and 25 mM GATX groups exhibited the most growth, and the 25 mM GA$\beta$ produced more ROS than the other groups, but the difference between the two groups was not statistically significant. ROS production was very low, and 25 mM groups produced more ATP than the 5 mM groups consistent with greater amount of glucose available for the cells to use. Despite this difference between glucose groups, none of the 25 mM glucose groups exhibited significant differences in ATP compared to one another.

## 5. Conclusions

The purpose of this paper was to demonstrate an *in vitro* model for Alzheimer's Disease could be used to investigate the effects of blood sugar concentration on the ability of Astaxanthin to function as an antioxidant. When examining both hypoglycemic and hyperglycemic conditions, we noted that during the hypoglycemic part of this study, a combination of A$\beta$ and hypoglycemia together cause an increase in ROS production, likely by increasing mitochondrial dysfunction. While ATX may aid in reducing ROS production and increasing growth of cells, its effects did not largely vary among glucose concentrations. Among the GA$\beta$ATX groups, the cells grew and produced ROS. An additional study may be required to explore the number of cells responsible for ROS production. Cell counts and cells assays will need to be performed. Our laboratory will proceed with cell death assays to test if cells are viable. The hyperglycemic part of this study indicated that ATX does bear neuroprotective effects against A$\beta$ and can improve mitochondrial function.

Our laboratory has therefore demonstrated Astaxanthin's ability to promote cell growth *in vitro* in hypoglycemic, normal glucose, and hyperglycemic solutions as well as when challenged by A$\beta$. The data presented in this study therefore supports ATX's role in promoting cell growth in both normal, DM (hypoglycemia and hyperglycemia), and AD (A$\beta$) disease states. This data also supports recent rat model studies demonstrating the neuroprotective effects of ATX. ATX has been shown to promote adult hippocampal neurogenesis in the hippocampus in a concentration-dependent manner and to increase spatial memory in mouse models [25]. ATX has been shown to reduce inflammation and reduce cognitive deficiency in diabetic mice [26]. ATX had been shown to be neuroprotective against hippocampal insulin resistance, a consideration for DM patients. Our study therefore supports previous research in the field calling for further investigation into the use of ATX as a potential treatment for Alzheimer's Disease.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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