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Evaluating Potential of Estrogen and Melatonin in Alzheimer's Disease: Evidence from Cell and Fly Models

By Amber Lin

AUTHOR BIOGRAPHY

Amber Lin is a high school senior who is passionate about researching natural treatments for chronic diseases such as cancer and Alzheimer's Disease. Her interest in neuroscience was sparked after she witnessed elderly residents struggling with dementia at senior centers, motivating her to search for a solution. In her free time, she likes to play piano, play golf, and write poetry.

ABSTRACT

Alzheimer's Disease (AD), an age-related neuroimmune disorder that is the seventh leading cause of death in the United States, has risk factors of reduced postmenopausal estrogen and the APOE4 allele, which causes inefficient lipid transport. Biomarkers of β-amyloid (Aβ) buildup, reduced nitric oxide (NO), increased caspase-3, and lipid droplet (LD) accumulation arise. Current drugs target symptoms temporarily and cause side effects like cognitive decline. Estrogen and melatonin, both able to cross the blood-brain barrier, offer potential as natural treatments. Estrogen degrades Aβ and inhibits caspase-3; melatonin reduces Aβ aggregates and neuroinflammation. We hypothesized that estrogen and melatonin could reduce LDs, prevent cell death via NO pathways, lower caspase-3 levels, and improve movement in Aβ-transgenic *Drosophila melanogaster*. Molecular docking analyzed their interactions with key AD proteins. *In vitro* assays on Aβ-treated human cell lines as AD models evaluated their effects. *In vivo* tests assessed motor function in Aβ-mutant flies. Estrogen and melatonin have high binding affinity with AD proteins including Aβ, confirming interaction with proteins. They had the same protein binding sites, suggesting synergy. They statistically significantly ($p < 0.05$) reduced LDs and caspase-3 levels in HTB-11 cells and improved cell proliferation under Aβ conditions, with diminished effects under inhibited NO synthase. Treated Aβ flies displayed improved mobility compared to untreated ones. These findings support the hypothesis that estrogen and melatonin have protective effects against AD *in vitro* and *in vivo*. They reduce LD buildup and enhance NO production, highlighting their potential as natural treatments in conjunction with other AD treatments.

Keywords: *Alzheimer's Disease, β-amyloid, estrogen, melatonin, APOE4, caspase-3, Drosophila melanogaster, molecular docking, aging*

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INTRODUCTION

Alzheimer's Disease

Alzheimer's Disease (AD) is a neurodegenerative disorder that is the seventh leading cause of death in the United States ("Alzheimer's Disease Fact Sheet", n.d.). It affects both the nervous system and the immune system (Wu et al., 2021). The main risk factor is old age, in which most cases of AD develop after age 65 (Bagit et al., 2021; "Thinking About", 2023). This phenomenon is known as late-onset AD (LOAD), which makes up the majority of AD cases. Its causes, genetic or non-genetic, are not well-determined, with multiple factors being explored (Kanekiyo et al., 2014).

Besides old age, another main risk factor is the sex, with female patients being disproportionately affected by AD compared to male patients (Wang et al., 2016). This phenomenon can be explained by reduced estrogen levels in female patients undergoing menopause, which has been researched to be correlated with AD (Wang et al., 2016). A third major risk factor is owning one copy or two copies of allele 4 of the apolipoprotein E gene (APOE) (Riedel et al., 2016). APOE is a gene that transcribes the apolipoprotein E protein (ApoE), which transports triglycerides and cholesterol (Riedel et al., 2016).

Risk factors often lead to development of AD biomarkers. The APOE4 gene regulates β-amyloid (Aβ) deposition, a main biomarker of AD (Kanekiyo et al., 2014). Aβ is a protein generated from amyloid precursor protein (APP), and it aggregates and has increased deposition in AD patients (Kanekiyo et al., 2014). Aβ levels are increased during neuroinflammation, a second biomarker (Akbar et al., 2021). Neuronal cells treated with Aβ had increased neuroinflammation through promotion of inflammasome expression (Nopporat et al., 2023). Aβ peptides are formed after proteolysis of APP, a third biomarker (Akbar et al., 2021). Decreased nitric oxide (NO) is a fourth main characteristic of AD (McCarty, 1998).

Furthermore, caspase levels are increased in AD patients (Vassar, 2007). Caspases, which are enzymes, cleave APP to create Aβ peptides (Vassar, 2007). In AD patients, the levels of caspase-3, one type of caspase, increase (Vassar, 2007). Caspase-3 knockout has been researched to cause neuronal apoptosis in mice, while other caspase knockouts did not produce the same effect, making caspase-3 a principal actor of apoptosis (Vassar, 2007). Thus, caspase-3 levels were measured in AD models within the experiment, described in the Materials and Methods.

Current Drug Treatments for AD and Limitations

Current treatments include using artificial drugs that are approved by the Food and Drugs Administration that are designed to ameliorate cognitive symptoms of AD ("How Is", 2023). For example, donepezil and cerebrolysin has been researched to synergistically increase the brain derived neurotrophic factor (BDNF), a protein involved in memory, which is notably decreased in AD patients. In AD patients that are

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APOE4 carriers, BDNF increased more, resulting in greater cognitive repair. However, both donepezil and cerebrolysin do not work as well as individual treatments without their synergistic results (Alvarez et al., 2016). In addition, donepezil has been researched to downregulate caspase-3 in mental health disorders, but not AD (Li et al., 2018). This demonstrates that current drugs have not been applied at large to risk factors of AD.

In the greater context of AD treatments, current drugs have modest effects because they are unable to pass the blood-brain barrier. To compensate, more of these drugs are administered, which can cause unfavorable side effects (Passeri et al., 2022). Nausea, diarrhea, vomiting, and depression are some common results, with bleeding of the brain and cognitive decline occurring in recent cases ("How Is", 2023; Villain et al., 2022). Most importantly, these drugs are used to treat symptoms rather than causes of AD, making them temporary solutions (Passeri et al., 2022).

Therefore, other drugs need to be explored as prevention for AD, focusing on its risk factors. These drugs should minimize side effects, be able to cross the blood-brain barrier to exert its full effectiveness, and be able to target the biomarker of AD.

Lipid Droplet Formation in Alzheimer's Disease

Research has been done on the induction of lipid droplets (LDs) caused by Aβ in microglial cells (Prakash et al., 2023). In AD, LDs are made of triglycerols in its core and cholesterol surrounding it. The nearer the proximity to the Aβ plaques, the greater the amount of LDs formed (Prakash et al., 2023). Microglia with a lot of LDs lack Aβ phagocytosis, which is the digestion of Aβ (Prakash et al., 2023).

LD production has been researched to happen before amyloid plaques and neurofibrillary tangles do in mouse models of AD, meaning they are a potential indicator of AD (Ralhan et al., 2021). Neurons transfer toxic LDs that they produce during oxidation to glial cells, which are neuroprotective. As the ApOE proteins are involved in lipid transportation in the brain, and APOE4 causes inefficiency at lipid transfer from glial cells to neurons, APOE4 expression reduces lipid transfer so toxic LDs accumulate in the neurons, which is a characteristic of Alzheimer's Disease (Yang et al., 2023; Ralhan et al., 2021). There has yet to be a study on the build-up of LDs in neurons, as opposed to microglial cells.

Estrogen and its Effects

Estrogen is mainly produced by both female and male reproductive systems in humans, though at lower levels in males (Rush, 2024). Estrogen, researched to be a neuroprotective hormone, is usually in its biologically active form, estradiol (Bagit et al., 2021). Estradiol is able to cross the blood-brain barrier, which is impaired in AD (Bagit et al., 2021; Montagne et al., 2017). Estradiol can bind to estrogen receptors ERα and ERβ through different types of signaling, allowing it to modify neuronal cells through genetic transcription (Bagit et al., 2021).

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When women were treated with estrogen, there were contradictory effects, with APOE4-positive women having worse cognitive performance compared to APOE4-negative women. In addition, there are contradictory effects of the estrogen receptors, with ERα upregulating APOE mRNA and ERβ downregulating APOE RNA. Due to these contradictory effects, scientists have theorized that APOE4-positive brains, unlike APOE2 or APOE3 brains, use two sources of fuel: ketone bodies and ATP. Reliance on ketone bodies causes the brain to use up white matter as sources of ketone, but estrogen use suppresses this reliance on ketone bodies and guides the brain to rely only on ATP. Thus, there could be potential positive effects when using estrogen to treat APOE4-positive brains (Riedel et al., 2017).

Estrogen has been researched to induce nitric oxide synthase (NOS), which produces nitric oxide (NO), in vascular endothelium, a tissue which makes up most of the brain (McCarty, 1998). There are three types of NOS: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS) (Fan et al., 2018). The amount of eNOS is controlled by gene transcription as well as estrogen receptors that act as transcription factors (Chambliss & Shaul, 2002). During the menstrual cycle, when there is an increase in estrogen produced and an increase in vasodilation of the endothelium, it correlates with an increase in the NO produced and exhaled (Chambliss & Shaul, 2002). Also, the estrogen receptors ERα and ERβ are able to control eNOS activation, causing NO to be released. Past research indicated men undergoing coronary artery bypass surgery with estrogen levels greater than 100 nM caused relaxation, which was inhibited with removal of the endothelium or an NOS antagonist. Also, estrogen suppresses iNOS expression in microglial cells, and estrogen induces eNOS (Karpuzoglu & Ahmed, 2006; McCarty, 1998). However, there is lack of research on estrogen-mediated impact on nNOS in AD.

In SH-SY5Y cells, neuroblastoma cells, induced with Aβ42, estradiol treatment was able to modulate cell apoptosis by reducing the increased reactive oxygen species (ROS) production (Pan et al., 2020). Furthermore, estrogen strongly inhibits caspase-3 (Peri & Serio, 2008).

Currently, there is no research on estrogen's effects on LDs.

Melatonin and its Effects

Melatonin is produced by the pineal gland of the human body and manages the circadian rhythm. Melatonin levels have been researched to decrease with older age, which is a risk factor of AD ("Melatonin: What It Is", 2022). Melatonin has been able to reduce multiple aspects of AD such as inflammation, oxidative stress, tau protein hyperphosphorylation, and formation of Aβ aggregates (Andrade et al., 2023).

Melatonin was able to prevent glucose-induced processing of APP in SH-SY5Y cells (Nopparat et al., 2022). Also, melatonin has been researched to decrease Aβ levels in rats treated with streptozotocin, which induces AD (Andrade et al., 2023).

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Melatonin binds to and inhibits apoE4, as well as suppresses PI3K and pSer9 on GSK-3β among other pathways, so that Aβ aggregation is reduced (Chen et al., 2020). Also, melatonin was researched to undo the effects of apoE4, which increased fibril formation, as well as decrease the neurotoxicity produced by combination of Aβ and apoE4 (Li et al., 2020).

Furthermore, melatonin has an effect on neuroinflammation found in AD. Aβ causes the NLRP3 inflammasome, which recruits caspases, to promote caspase-1, thus inducing neuroinflammation. Pretreatment of melatonin reduced immunostaining of caspase-1 in the SH-SY5Y neuroblastoma cell line (Nopporat et al., 2023). In male mice, melatonin decreased the levels of caspase-3, causing an inhibition of cell apoptosis (Waseem et al., 2017).

Melatonin has been researched to have varying effects on nNOS. At physiological levels, melatonin inhibits nNOS in the neuronal cells of the hypothalamus; however, during nerve injury, nNOS is upregulated by melatonin as a protective effect (Fan et al., 2018). More research needs to be done to elucidate melatonin's effects on nNOS in an AD model.

In addition, there has been no research conducted on melatonin's effects of LD accumulation in AD.

Problem Statement, Hypothesis, and Rationale

As stated earlier, current artificial AD drugs have harmful side effects, are inefficient because they cannot cross the blood-brain barrier, and are temporary because they treat AD symptoms, not causes. In addition, LOAD, which makes up the majority of AD cases yet does not have determined genetic factors. Thus, there is an urgent need to explore other compounds as potential AD treatments. Estrogen and melatonin, natural compounds that can cross the blood-brain barrier, have been researched to have potential anti-AD effects.

Thus, we presented the question "How are estrogen and melatonin able to modulate aspects of Alzheimer's Disease induced by the β-amyloid peptide?"

We hypothesized that melatonin and estrogen are able to reduce LD production, reverse cell death through the nitric oxide-mediated pathway, reduce caspase-3 levels in Aβ-induced neuronal and immune cells, and improve impairment of movement in transgenic Aβ *Drosophila melanogaster* flies. The AD pathways that estrogen and melatonin target are promising yet have not been well-researched.

Researchers have found that many of LOAD's potential genetic risk factors are involved in Aβ homeostasis, making it the defining biomarker of AD (Hampel et al., 2021). The pathways that estrogen and melatonin target in this paper all address the defining biomarker, specifically on the expression, trafficking, and degradation of Aβ:

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First, we explored Aβ expression through the Aβ pathway and the NO pathway, as NO is induced by Aβ. Second, Aβ trafficking is explored through the LD pathway and through APOE4 levels, since APOE4 has been researched to cause lipid transfer inefficiency. Third, Aβ degradation is explored through the caspase-3 in the apoptosis pathway. We aim to see how well estrogen and melatonin are able to target these pathways and in turn, ameliorate AD.

METHODS

Molecular docking

Estrogen and melatonin files were downloaded from databases found on the PubChem website. PDB files of proteins were downloaded from the Protein Data Bank: Aβ peptide, amyloid precursor protein (APP), APOE4, BACE-1, COX-2, ERα, ERβ, NF-kB, and NO synthase (NOS). Using PyRx, the downloaded proteins were opened and autodocked, and the "Make Macromolecule" button was clicked. Estrogen and melatonin were individually docked as ligands. The binding affinity table and the 3D structure of the macromolecule-protein complex were downloaded. The downloaded 3D structure was opened in the BIOVIA Discovery Visualizer application, and the estrogen or melatonin molecule was made the ligand. The binding sites' 2D diagram was screenshotted.

Dilution of chemicals

β-estradiol (Eβ), purchased from Sigma-Aldrich (St. Louis, MO) and melatonin, purchased from Cayman Chemical Company (Ann Arbor, MI), were solvated in Dimethyl Sulfoxide (DMSO), purchased from Sigma-Aldrich, and serially diluted with Minimal Essential Medium (MEM), purchased from Sigma-Aldrich. Eβ is one of two isoforms estradiol, the other being Eα, and Eβ is the most physiologically active estrogen in the human body ("Estradiol", 2022). For the 24-well plate, Eβ was diluted to 74 μM, 7.4 μM, and 0.74 μM and melatonin was diluted to 86 μM, 8.6 μM, and 0.86 μM. For the 6-well plate, Eβ was diluted to 18.5 μM, 1.85 μM, and 0.185 μM and melatonin was diluted to 21.5 μM, 2.15 μM, and 0.215 μM. For the 96-well plate, Eβ was diluted to 370 μM, 37 μM, and 3.7 μM and melatonin was diluted to 430 μM, 43 μM, and 4.3 μM.

Oil Red O staining

The first Oil Red O staining was conducted to observe the effects of Eβ on cells not affected by AD. HTB-11 cells purchased from ATCC (Gaithersburg, MD) and COLO320 cells also purchased from ATCC, were seeded into a 24-well plate, then treated using 10.0μL of solutions of Eβ at varying concentrations over 24 hours. HTB-11 cells, which are neuroblastoma cells, were used to model neuronal cells. COLO320 cells, which are colorectal cancer cells, were used to model gut cells affected by AD. The reason that COLO320 cells were used is to monitor the effects of Eβ on cells not primarily affected by AD.

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The Oil Red O staining was conducted according to the instructions of Sigma-Aldrich, the manufacturer of the Oil Red O stain ("Product Information"). The cells were then covered with 250 µL distilled water and viewed using a microscope purchased from OMAX (Kent, WA). 4 photos were taken of each cell well using Amscope. The number of cells and the density of each well were counted using the ICTN plugin of ImageJ.

This was repeated for a second Oil Red O staining 24-well plate of U937 cells, which were treated with 10.0µL of Eβ; Lipopolysaccharide (LPS), purchased from Sigma-Aldrich; and Aβ, purchased from Apex Scientific (Maynooth, Ireland) at varying concentrations, individually and aggregately, over 24 hours. As AD is a neuroimmune disease, U937 cells were used to model immune cells affected by AD. LPS and Aβ are used to mimic characteristics found in AD patients, such as Aβ plaques, and LPS levels being higher in AD patients (Zhan et al., 2018). Thus, it creates an AD model. This second Oil Red O staining was done to determine the effects of Eβ treatment on cells affected by AD through the cell count. The rest of the procedures of the Oil Red O staining were identical to the first trial.

A third Oil Red O staining was conducted on a 6-well plate of HTB-11 cells, treated with 10.0 µL of Eβ and β-amyloid at varying concentrations, individually and aggregately, over 24 hours. This Oil Red O staining was done to determine the cell count, the cell area, the LD count in cells, and the LD count on dendrites (as the magnification for the 24-well cells were too small to count LDs) of HTB-11 cells. 1 mL was used, instead of 250 µL, for all treatments of the Oil Red O staining procedures in the third trial.

MTT Assay

HTB-11 cells purchased from ATCC (Gaithersburg, MD) and were seeded into 96-well plates, then treated using 5.0µL of solutions of estradiol, melatonin, and NOS Inhibitor at varying concentrations, individually and aggregately, over 24 hours. This treatment simulates the short-term effect of estradiol and melatonin on HTB-11 cells.

After cells were incubated, cell proliferation was assessed using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described in past protocols (Park et al., 2021). A slight change made was that a wavelength of 595 nm was used to spectrophotometrically evaluate the resultant solution. The process was repeated for another plate of HTB-11 cells from ATCC, treated with solutions for 96 hours to simulate long-term effect.

Drosophila Melanogaster Climbing Assay

Wild-type *Drosophila melanogaster* and Aβ mutated *Drosophila*, one vial of each, were purchased from Bloomington Drosophila Stock Center (Bloomington, IN). The following treatments were added to one of three empty plastic vials: 50 µL of 1.0 mM melatonin, 50 µL of 0.90 mM estradiol, and the last was a Control.

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One cup of *Drosophila* medium, purchased from the Carolina Biology Supply Company (Burlington, NC), using the provided measuring cup, was poured too.

Paper squares were taped over the three vials and small holes were made in the squares. After 24 hours, the paper squares were un-taped and 2 mL of distilled water was poured into each vial. Ten wild-type *Drosophila* were transferred from the stock vial to each of the three vials. After 48 hours, the flies within each vial were moved to new separate empty vials and taped with double-sided tape to two wooden slabs. A ruler was taped between the slabs. The vials were tapped on the table three times each so that the flies fell to the bottom of the vials. The number of flies able to pass the 5 cm mark within 10 seconds was recorded.

These procedures were repeated for a second trial with the following treatments: wild-type *Drosophila* treated with 50 µL DMSO (Control), Aβ *Drosophila* treated with 50 µL DMSO (Negative Control), Aβ *Drosophila* treated with 50 µL of 1.0 mM melatonin, and Aβ *Drosophila* treated with 50 µL of 0.90 mM estradiol. DMSO was added to the Control and Negative Control because the melatonin and estradiol were both dissolved in DMSO, and this was not taken into consideration in the first trial.

Cell Caspase Assay

The Caspase-3 Colorimetric Assay measures caspase-3's activities to assess whether melatonin and estradiol is able to alleviate apoptosis induced by Aβ. Caspase is an enzyme that controls apoptosis (Fan et al., 2005): when activated, caspase will execute apoptosis, so since Aβ is able to induce apoptosis, caspase will be activated. HTB-11 cells were seeded in a six-well plate and treated with varying concentrations of melatonin, estradiol, and Aβ, individually and aggregately. Caspase activity was measured using instructions of the Caspase Colorimetric Apoptosis Assay kit, purchased from Fisher Scientific (Hampton, NH). Finally, the percent change of the caspase activity was calculated.

ELISA Assay

After the HTB-11 cell cultures were seeded into each of 6 wells on a plate, 20 µL of melatonin, berberine, and a combination of both were added to 22 of the wells in various concentrations, and the remaining 1 well were designated as the control group. After incubation overnight at 37 degrees Celsius, medium was removed from each cell well, and 500 µL of trypsin, purchased from Thermo Fisher Scientific, was pipetted in each well. The plate rested for three minutes before 1 L of Minimum Essential Medium (MEM), purchased from Thermo Fisher Scientific, was pipetted in each well. The cell was washed three times with PBS before the solution was put into six labeled Eppendorfs and centrifuged for three minutes. The medium from each Eppendorf was then disposed of in a waste bottle and the samples were frozen for one week at a temperature below -18 degrees Celsius. Then, following the protocol of the Human APOE4 ELISA Kit bought from Boster Bio (Pleasanton, CA), the APOE4 levels were measured using a microplate reader at 450 nm.

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RESULTS

Estrogen and Melatonin effect on neuroblastoma, colorectal, and lymphoma cell survival

A first Oil Red O staining for a 6-well HTB-11 plate was conducted to determine whether estradiol had adverse effects on cells not affected by characteristics of Alzheimer's Disease. When treated with estradiol, the HTB-11 cell count increased by 0.61% for 1.85 μM estradiol and by 16.28% for 18.5 μM estradiol (Figure 1a). The HTB-11 cell count decreased by 5.61% for 0.185 μM estradiol. There were no significant p-values for all concentrations of estradiol, indicating that estradiol had no adverse effects to HTB-11 cells. There is a dose-dependent upwards trend.

When treated with estradiol only, the COLO320 cells showed an increase in cell count for all estradiol treatments: 7.08% for 0.185 μM estradiol, 17.10% for 1.85 μM estradiol, and 6.14% for 18.5 μM estradiol (Figure 1b). Similar to the HTB-11 cells, there were no significant p-values for all concentrations of estradiol treating COLO320 cells, indicating that there are no adverse effects on COLO320 cells.

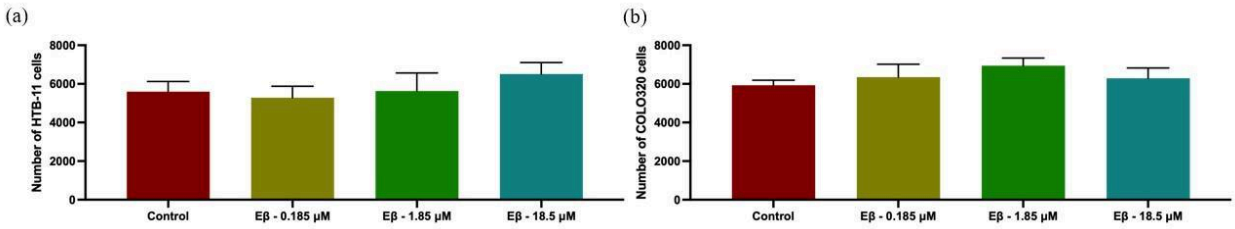

Figure 1: Estradiol Effect on HTB-11 Cell Survival and COLO320 Cell Survival. Number of HTB-11 cells and COLO320 cells, treated with Estradiol. An Oil Red O staining assay was conducted on HTB-11 cells and COLO320 cells. Error bars represent Standard Deviation. (a) HTB-11 Cell Survival. (b) COLO320 Cell Survival. * = $0.05 > p > 0.01$, ** = $0.01 > p > 0.001$, *** = $p < 0.001$

Knowing that estradiol does not cause harmful effects on HTB-11 and COLO320 cells, a second Oil Red O staining was conducted on a 24-well plate of U937 cells. When treated with LPS and Aβ, the U937 cell adhesion significantly increased by 335.92% (Figure 2a). When treated with LPS only, the U937 cell adhesion significantly increased by 213.96% (Figure 2a). When treated with LPS and estradiol, the U937 cells showed a significant reduction in cell adhesion for 7.4 μM estradiol, significantly increasing with a percentage of 173.85%, and a reduction in cell adhesion for 74 μM estradiol with a percentage of 75.62% (Figure 2a). When treated with Aβ, the U937 cells showed a significant increase in cell adhesion with a percentage of 157.49% (Figure 2b). When treated with 7.4 μM estradiol and Aβ, U937 cells showed a decrease in cell adhesion with a percentage of 61.78%, the largest decrease of all treatments (Figure 2b). All graphs follow a dose-dependent downwards trend in terms of percentage of increase. When comparing the LPS treatment to the LPS and 74 μM estradiol, as well as the Aβ to the 7.4 μM estradiol and Aβ, there is significance for both (Figure 2).

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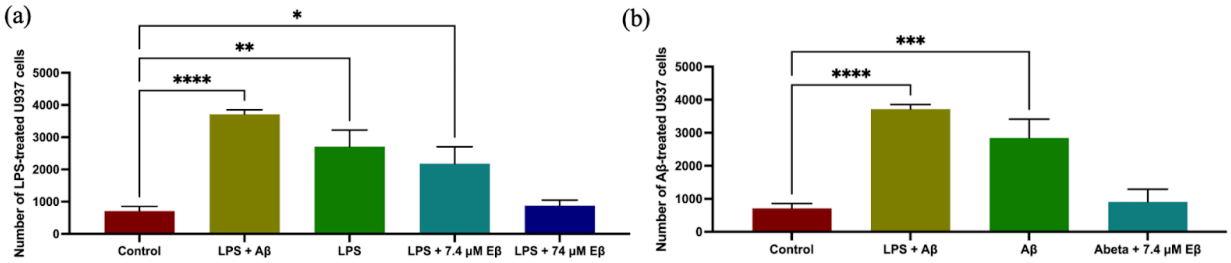

Figure 2: LPS and Aβ Effect on U937 Cell Survival. Number of U937 cells, treated with LPS, Aβ, and Estradiol treatment. An Oil Red O staining assay was conducted on U937 cells. Error bars represent Standard Deviation. (a) LPS-Treated U937 Cells. (b) Aβ-Treated U937 Cells. * = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = p < 0.001

Another Oil Red O assay was conducted to quantify the cell adhesion and lipid formation within HTB-11 cells; thus, a 6-well plate was used so that it could be viewed with 500x magnification of the microscope.

The cell adhesion of the HTB-11 cells was measured. When treated with Aβ alone, the HTB-11 cells showed an increase in cell adhesion with a percentage of 20.17% (Figure 3a). When treated with 18.5 μM estradiol and Aβ, the HTB-11 cells showed a decrease in cell adhesion with a percentage of 16.60% (Figure 3a). When treated with 21.5 μM melatonin and Aβ, the HTB-11 cells showed a decrease in cell adhesion with a percentage of 16.80% (Figure 3b). Notably, when treated with 21.5 μM melatonin alone, there is a decrease of 32.01%, the largest decrease of all treatments (Figure 3b). There is a downwards trend for estradiol (Figure 3a).

In addition, the cell area of the HTB-11 cells was measured. When treated with Aβ alone, the HTB-11 cells showed a decrease in cell area with a percentage of 2.13% (Figure 3c). When treated with 18.5 μM estradiol and Aβ, the HTB-11 cells showed an increase in cell area with a percentage of 5.96% (Figure 3c). When treated with 21.5 μM melatonin and Aβ, the HTB-11 cells showed an increase in cell area with a significant percentage of 13.39%, the greatest increase of all treatments (Figure 3d). All graphs show an upwards trend in terms of percentage of increase.

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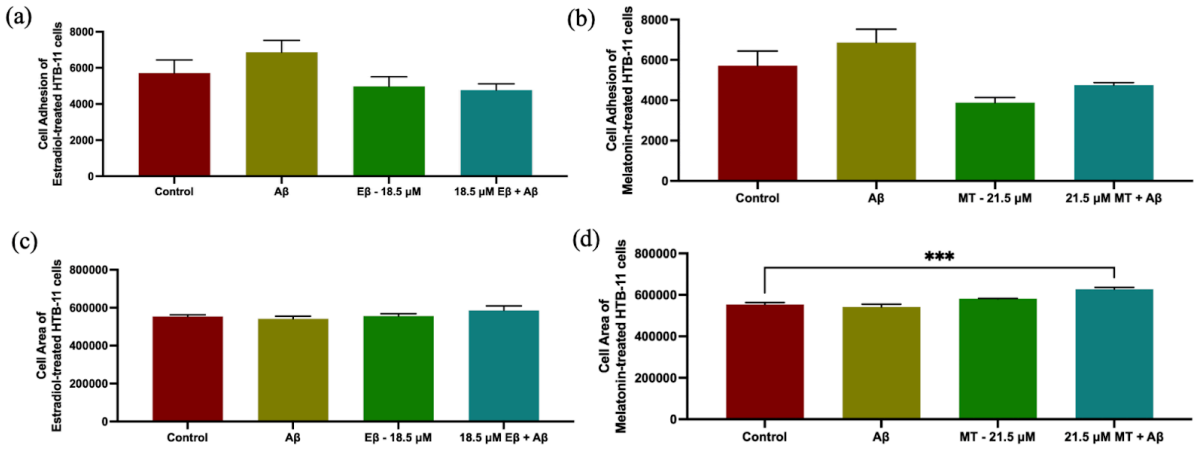

Figure 3: Estradiol and Melatonin Effect on HTB-11 Cell Adhesion and Area. Cell area of HTB-11 cells with Estradiol and Melatonin treatment. An Oil Red O staining assay was conducted on HTB-11 cells. Error bars represent Standard Deviation. (a) Cell Adhesion of Estradiol-Treated HTB-11 Cells. (b) Cell Adhesion of Melatonin-Treated HTB-11 Cells. (c) Cell Area for Estradiol-Treated HTB-11 Cells. (d) Cell Area for Melatonin-Treated HTB-11 Cells. * = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = p < 0.001

Estrogen and Melatonin effect on neuroblastoma lipid droplet (LD) count

Continuing from the results of the previous Oil Red O staining, when treated with Aβ alone, the HTB-11 cells showed an increase in LD count with a percentage of 20.65% (Figure 4a). When treated with 18.5 μM estradiol and Aβ, the HTB-11 cells showed a significant decrease in the LD count with a percentage of 30.98%, the greatest decrease of all treatments (Figure 4a). When treated with 21.5 μM melatonin and Aβ, the HTB-11 cells showed a significant decrease in the LD count with a percentage of 30.32% (Figure 4b). All graphs follow a downwards trend in terms of percentage of increase. When comparing the Aβ treatment to the 18.5 μM estradiol and Aβ treatment, as well as the 21.5 μM melatonin and Aβ treatment, there is significance for both (Figure 4a, Figure 4b).

The LD formation on dendrites of HTB-11 cells was also quantified. When treated with Aβ alone, the HTB-11 cells showed an increase in LD count with a percentage of 25.96% (Figure 4c). When treated with 18.5 μM estradiol and Aβ, the HTB-11 cells showed a decrease in LD count with a percentage of 23.08%, the greatest decrease of all treatments (Figure 4c). When treated with 21.5 μM melatonin and Aβ, the HTB-11 cells showed a decrease in LD count with a percentage of 21.15%. All graphs follow a downwards trend in terms of percentage of increase (Figure 4d).

When comparing the HTB-11 cells treated with 18.5 μM estradiol and Aβ to the HTB-11 cells treated with Aβ only, there are much fewer LDs per cell (Figure 4f, Figure 4e).

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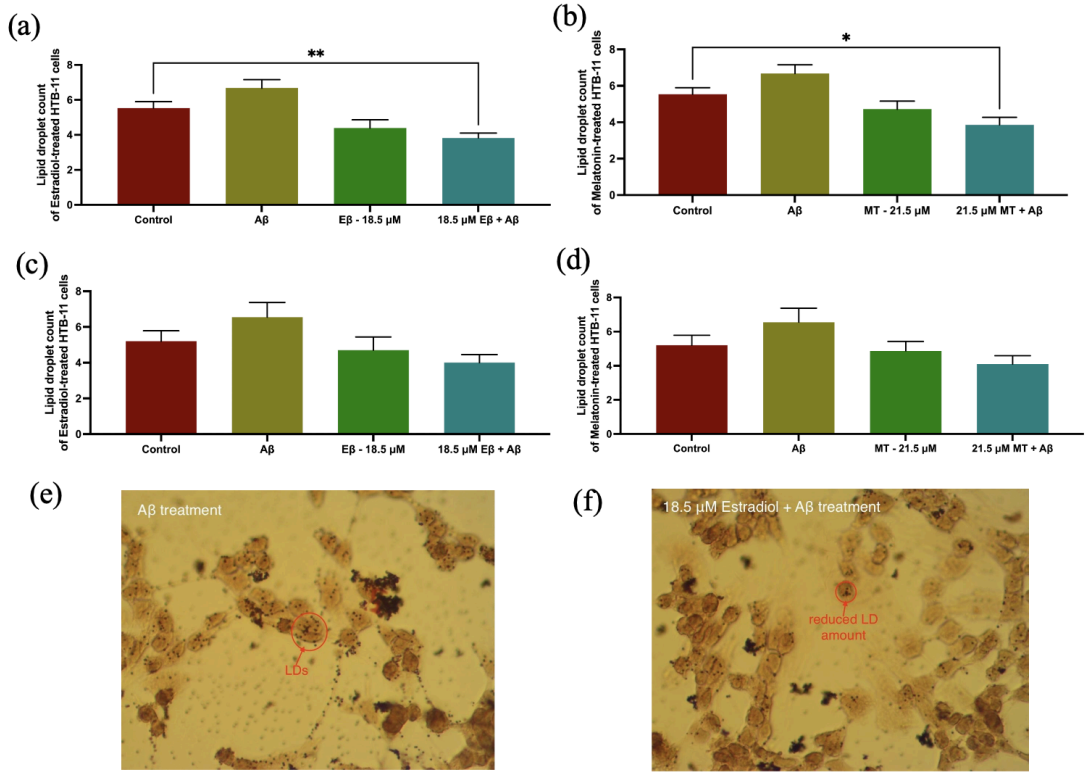

Figure 4: Estradiol and Melatonin Effect on HTB-11 LD Count. Number of LDs in HTB-11 cells with Estradiol and Melatonin treatment. An Oil Red O staining assay was conducted on HTB-11 cells. Error bars represent Standard Deviation. (a) LD Count in Estradiol-Treated HTB-11 Cells. (b) LD Count in Melatonin-Treated HTB-11 Cells. (c) LD Count on HTB-11 Estradiol-Treated Dendrites. (d) LD Count on Melatonin-Treated HTB-11 Dendrites. (e) Image of LDs in HTB-11 cells, Aβ Treatment. (f) Image of LDs in HTB-11 cells, 18.5 μM Estradiol + Aβ Treatment. * = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = p < 0.001

Estrogen and Melatonin effect on caspase activity in neuroblastoma cells

For regulation of caspase-3 in HTB-11 cells when treated with Aβ, the HTB-11 cells showed a significant increase in caspase-3 with a percentage of 220.55%, the greatest increase of all treatments (Figure 5a). When treated with 1.85 μM estradiol and Aβ, the HTB-11 cells showed a significant reduction of increase in caspase-3 with a percentage of 71.23% (Figure 5a). When treated with 2.15 μM melatonin and Aβ, the HTB-11 cells showed a significant reduction of increase in caspase-3 with a percentage of 87.67% (Figure 5b). When treated with 2.15 μM melatonin, the HTB-11 cells showed a reduction of increase in caspase-3 with a percentage of 45.21%, the lowest increase in caspase-3 (Figure 5b). For the estradiol treatment, there is a downward trend in terms of percentage of increase (Figure 5a).

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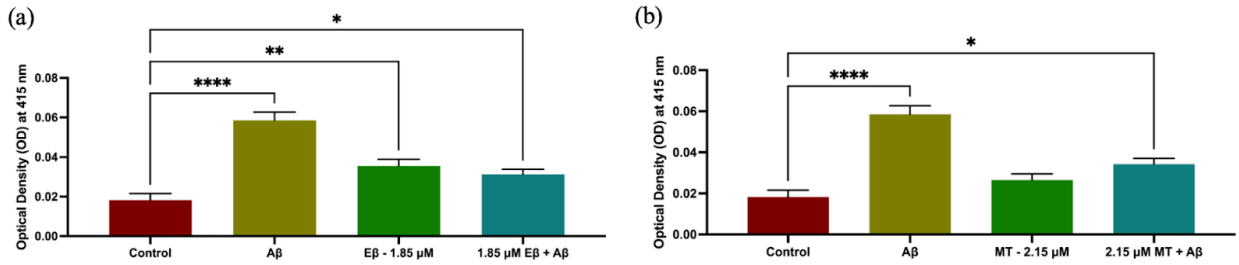

Figure 5: Estrogen and Melatonin Effect on Caspase Activity in HTB-11 Cells. Optical Density of HTB-11 cells, treated with Estradiol and Melatonin. A caspase-3 assay was conducted on HTB-11 cells. Error bars represent Standard Deviation. (a) Caspase-3 Levels in Estradiol-Treated HTB-11 Cells. (b) Caspase-3 Levels in Melatonin-Treated HTB-11 Cells. * = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = p < 0.001

Estrogen and Melatonin effect on cell proliferation in neuroblastoma cells

For the short-term effects of 24 hour treatment, when treated with Aβ alone, the HTB-11 cells showed a decrease in cell count with a percentage of 21.42% (Figure 6a). When treated with 3.7 μM estradiol and Aβ, as well as 37 μM estradiol and Aβ, the HTB-11 cells showed a decrease in cell count with a percentage of 21.06% and 21.67%, respectively (Figure 6a). When treated with 3.7 μM estradiol, Aβ, and NO synthase inhibitor, as well as 37 μM estradiol, Aβ, and NO synthase inhibitor, the HTB-11 cells showed a reduced decrease in cell count with percentages of 4.62% (which was the second smallest decrease of all treatments) and 6.28%, respectively (Figure 6a). When treated with 4.3 μM melatonin and Aβ, as well as 43 μM melatonin and Aβ, the HTB-11 cells showed a decrease in cell count with percentages of 12.73% and 11.00% (Figure 6b). When treated with Aβ, 4.3 μM melatonin, and NOS inhibitor, the HTB-11 cells showed a significant decrease in cell count with a percentage of 32.90% (Figure 6b).

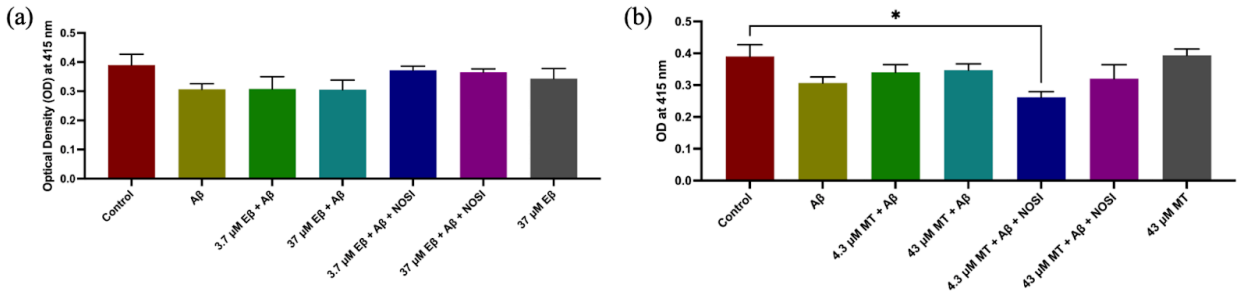

Figure 6: Short-Term (24 hours) Estrogen and Melatonin Effect on Cell Proliferation in HTB-11 Cells. Optical Density of HTB-11 cells, treated with Estradiol and Melatonin. An MTT assay was conducted on HTB-11 cells. Error bars represent Standard Deviation. (a) Cell Proliferation in Estradiol-Treated HTB-11 Cells. (b) Cell Proliferation in Melatonin-Treated HTB-11 Cells. * = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = p < 0.001

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For the long-term effects of 4-day treatment, when treated with Aβ alone, the HTB-11 cells showed a decrease in cell count with a significant percentage of 75.20% (Figure 7a). When treated with 3.7 µM estradiol and Aβ, as well as 37 µM estradiol and Aβ, the HTB-11 cells showed significantly reduced decreases in cell count with percentages of 29.66% and 43.60%, respectively (Figure 7a). When treated with 3.7 µM estradiol, Aβ, and NO synthase inhibitor, as well as 37 µM estradiol, beta-amyloid, and NOS inhibitor, the HTB-11 cells showed significantly reduced decreases in cell count with percentages of 72.25% and 69.68%, respectively (Figure 7a). When treated with 4.3 µM melatonin and Aβ, the HTB-11 cells showed a significantly reduced decrease in cell count with a percentage of 17.67% (Figure 7b). When treated with 43 µM melatonin and Aβ, the HTB-11 cells showed a significant increase in cell count with a percentage of 3.96% (Figure 7b). When treated with Aβ, 4.3 µM melatonin, and NO synthase inhibitor, as well as Aβ, 43 µM melatonin, and NOS inhibitor, the HTB-11 cells showed significantly reduced decreases in cell count with percentages of 70.38% and 71.77% (Figure 7b).

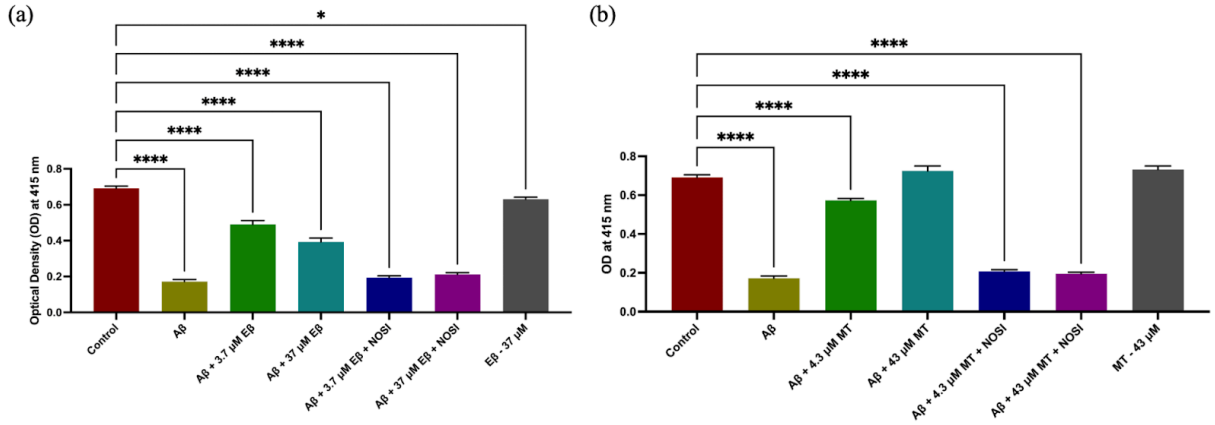

Figure 7: Long-Term (96 hours) Estrogen and Melatonin Effect on Cell Proliferation in HTB-11 Cells. Optical Density of HTB-11 cells, treated with Estradiol and Melatonin. An MTT assay was conducted on HTB-11 cells. Error bars represent Standard Deviation. (a) Cell Proliferation in Estradiol-Treated HTB-11 Cells. (b) Cell Proliferation in Melatonin-Treated HTB-11 Cells. * = $0.05 > p > 0.01$, ** = $0.01 > p > 0.001$, *** = $p < 0.001$

Estrogen and Melatonin effect on Drosophila climbing

Wild-type *Drosophila* flies were treated with 50 µL 0.9 mM estradiol and 50 µL 1.0 mM melatonin to observe if these two compounds had any adverse effects on the flies. It was determined that a lower concentration would be more suitable and avoid a large decrease in *Drosophila* activity. In the second trial, transgenic Aβ *Drosophila* flies were treated with 50 µL 0.9 mM estradiol and 50 µL 1.0 mM melatonin. The untreated Aβ flies showed a decrease in activity with a percentage of 14.81% (Figure 8). When treated with 0.9 mM estradiol, the Aβ flies showed a reduced decrease in activity with a percentage of 13.33% (Figure 8). When treated with 1.0 mM melatonin, the beta-amyloid flies showed a reduced decrease in activity with a percentage of 7.41%, the smallest decrease for all treatments (Figure 8).

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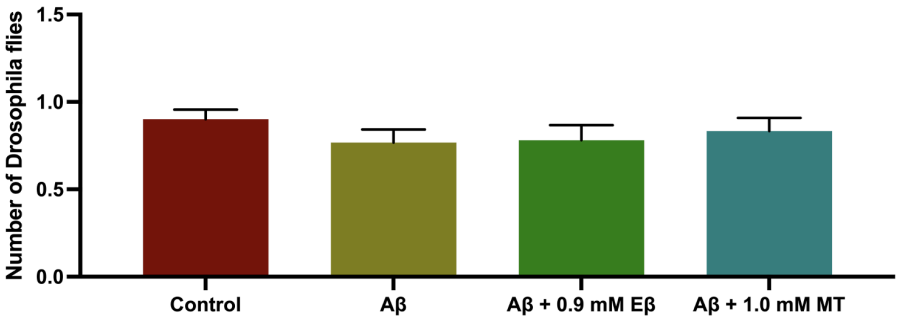

Figure 8: Estradiol and Melatonin Effect on *Drosophila* Climbing. Number of transgenic Aβ flies, treated with Estradiol and Melatonin, that passed the 5 cm mark in 10 seconds. An Oil Red O staining assay was conducted on HTB-11 cells. Error bars represent Standard Deviation. * = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = p < 0.001

Molecular docking of various proteins with Estrogen and Melatonin

For the molecular docking, the binding affinity values ranged from -8.4 to -4.0 Kcal/mol (Figure 9). Estrogen had higher binding values than melatonin for all of the proteins, indicating that estrogen binds more strongly than melatonin to these proteins. The highest binding affinity value is between BACE-1 and estrogen, which is -8.4. The second highest binding affinity value is between NO synthase and estrogen, which is -8.2. Other highest binding affinity values were between APOE4 and estrogen, which is -7.6; and between ERα and estrogen, which is -7.5. The binding affinity values between the Aβ peptide and melatonin as well as the Aβ peptide and estrogen were among the lowest, indicating that these molecules did not have strong binding. This was most likely because the whole Aβ peptide was too large to be completely loaded in PyRx, so the *in silico* binding was not complete. Thus, a higher value could have been yielded if the whole Aβ peptide was loaded. Despite this, the binding affinity shows that there is confirmed interaction between Aβ peptide and estrogen as well as Aβ peptide and melatonin. This result matches with the *in vitro* results of the Oil Red O stainings and MTT assays.

For the 2D binding diagrams, there are multiple proteins in which estrogen and melatonin were bound to the same amino acids (Figure 10). For the Aβ peptide, estrogen and melatonin share a binding location at TYR A:10, with the same type of bond. For the amyloid precursor protein, estrogen and melatonin share a binding location at LYS A:510. For BACE-1, estrogen and melatonin share binding locations at TYR A:132 and THR A:133. These results show the possible synergistic effects of estrogen and melatonin.

Name of protein	Highest binding affinity to melatonin	Highest binding affinity to estrogen
Aβ peptide	-4.9	-5.7

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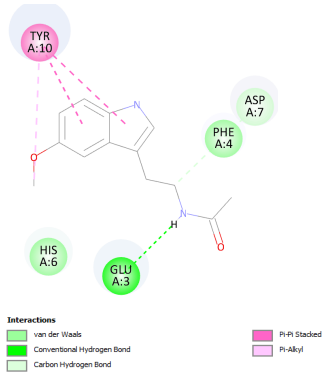
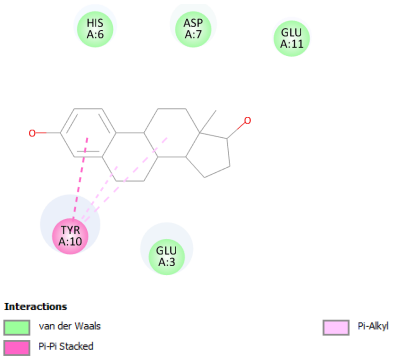
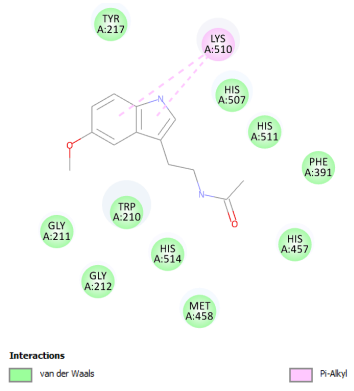
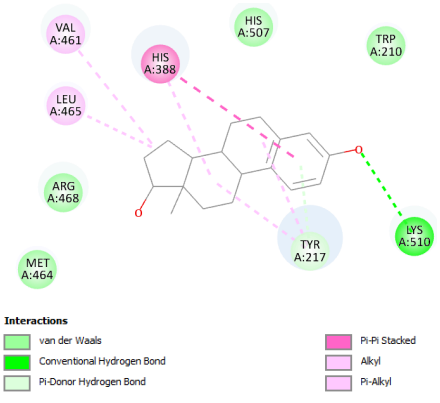
APOE4	-5.9	-7.6
BACE-1	-5.9	-8.4
ERα	-6.8	-7.5
NO synthase (NOS)	-6.4	-8.2

Figure 9: Estrogen and Melatonin Binding Affinities with Key Alzheimer's Disease Proteins

Name of protein	2D binding diagram to melatonin	2D binding diagram to estrogen
Aβ peptide		
APP		

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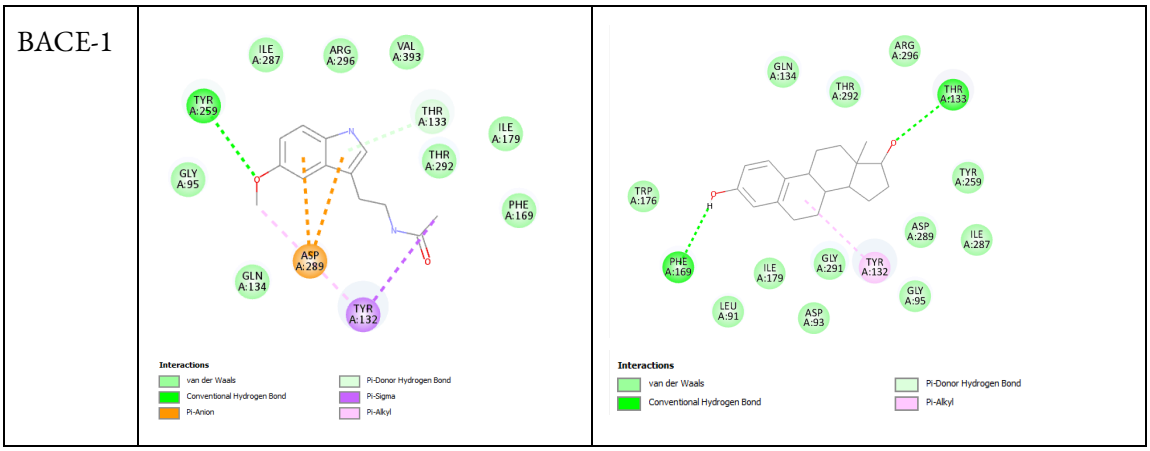

Figure 10: 2D Binding Diagrams of Estrogen and Melatonin to Key Alzheimer's Disease Proteins

Estrogen and Melatonin Effect on APOE4 Levels in HTB-11 Cells

For the ELISA assay, when HTB-11 cells were treated with 10 μM Aβ alone, the HTB-11 cells showed a significant increase in APOE4 levels with a percentage of 88.21% (Figure 11a). When treated with 10 μM Aβ and 18.5 μM estrogen, the HTB-11 cells showed a percentage of 4.72%, which is a great decrease in APOE4 levels compared to Aβ alone (Figure 11a). When treated with 10 μM Aβ and 21.5 μM melatonin, the HTB-11 cells showed a percentage of 8.02%, which is also a great decrease in APOE4 levels compared to Aβ alone (Figure 11b).

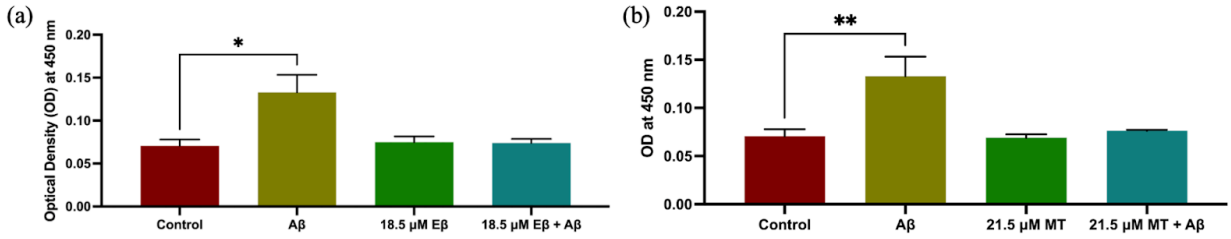

Figure 11: Estrogen and Melatonin Effect on APOE4 Levels in HTB-11 Cells. Optical Density of HTB-11 cells, treated with Estradiol and Melatonin. An ELISA assay was conducted on HTB-11 cells. Error bars represent Standard Deviation. (a) APOE4 Levels in Estradiol-Treated HTB-11 Cells. (b) APOE4 Levels in Melatonin-Treated HTB-11 Cells. * = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = p < 0.001

DISCUSSION

The aim of this study was to research the effects of melatonin and estrogen on *in silico*, *in vitro*, and *in vivo* models of AD. We hypothesized that melatonin and estrogen, individually and synergistically, are able to decrease production of lipids, reverse cell death, and reduce caspase-3 levels induced in neuronal cells by Aβ

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treatment through a nitric oxide pathway, as well as improve impairment of movement in transgenic Aβ *Drosophila*. Data was collected through molecular docking, Oil Red O stainings, MTT assays, caspase-3 assays, and *Drosophila* climbing assays. Our research sheds light on melatonin and estrogens' effects on HTB-11 and U937 cells, Aβ treated neuronal and immune cells as AD models. In addition, it furthers the potential of using natural hormones as treatment for AD.

For the molecular docking, melatonin and estrogen were seen to have high binding affinities as well as identical binding sites with key AD proteins (Figure 9, Figure 10). Both melatonin and estrogen have been researched to have synergistic effects with other compounds for treating some chronic diseases, but not in conjunction with each other (Ustandag, 2023; Nilsen, 2002). Thus, the identical binding sites of melatonin and estrogen to key AD proteins demonstrate their potential synergy. Furthermore, BACE-1 had both the highest binding affinity and identical binding sites for estrogen and melatonin (Figure 9, Figure 10). As BACE-1 is necessary for Aβ peptides to form, targeting BACE-1 using estrogen and melatonin could be explored to reduce Aβ (Hampel et al., 2020).

In the second Oil Red O staining, the U937 cells had the greatest increase in cell adhesion when treated with LPS and Aβ together, followed by a second greatest increase for LPS treatment alone and a third greatest increase for Aβ treatment alone (Figure 2). LPS has been researched to induce brain inflammation in aging mice models of AD (Zhao et al., 2019). The blood brain barrier, which regulates inflammation in the brain, is dysfunctional in an AD patient. When certain cell adhesion molecules are expressed, caused by AD, leukocytes are able to pass the endothelium of the blood-brain barrier and affect the neurovascular unit, which includes neurons. Thus, if cell adhesion was to be decreased, then Aβ deposition and tau hyperphosphorylation can be decreased in AD (Zenaro et al., 2017). Similarly, Aβ has been researched to mediate cell adhesion through modulating integrins in neuroblastoma cells (Sabo et al., 1995). Thus, when both compounds are combined, there is an increase in cell adhesion in U937 cells as seen in the experimental results, indicating that there is an increase in cell death.

When treated with LPS and 74 μM estradiol, the U937 cells had the lowest increase in cell adhesion, meaning that the estrogen contributed to decreasing cell adhesion induced by LPS (Figure 2a). Furthermore, when treated with Aβ and 7.4 uM estradiol, the U937 cells had a decrease in cell adhesion, the only treatment that caused a decrease in cell adhesion (Figure 2b). This demonstrates that the estrogen was able to reverse Aβ's effects and cause healthy cell proliferation rather than cell apoptosis.

The same results of decreased cell adhesion were seen in the HTB-11 cells when treated with Aβ combined with 18.5 μM estrogen as well as Aβ combined with 21.5 μM melatonin (Figure 3). These results align with a study in which a phytoestrogen was able to reduce the cell viability decrease of LPS and Aβ in mice astrocytes (Liu et al., 2011). Furthermore, these results align with the neuroprotective melatonin effect against Aβ in rat models (Hu et al., 2017). Surprisingly, 21.5 μM melatonin had the greatest decrease in cell adhesion, pointing to its possible effectiveness in increasing cell viability.

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In the third Oil Red O assay, the HTB-11 cells had the greatest increase in lipid droplet count when treated by Aβ (Figure 4a). In addition, there was a greatest decrease in lipid count when treated by Aβ and 18.5 μM estradiol and a second greatest decrease when treated by Aβ and 21.5 μM melatonin (Figure 4a, Figure 4b). There have been no studies that have discussed the increase of lipid droplets in HTB-11 neuronal cells before, so this is a new finding.

Furthermore, the dendrites of HTB-11 cells had the greatest increase in lipid droplet count when treated by Aβ, with the greatest decrease in lipid droplet count when treated by Aβ and 18.5 μM estradiol and the second greatest decrease in lipid droplet count when treated by Aβ and 21.5 μM melatonin (Figure 4c, Figure 4d). These lipid droplet counts demonstrate that estrogen and melatonin are able to reduce Aβ's effects on increasing lipid droplet count, even reversing the effects. Similarly, there have been no studies in lipid droplet increases in HTB-11 neuronal dendrites, so this is also a new finding.

HTB-11 cells had the greatest increase in caspase-3 activity when treated with Aβ, followed by the least increase when treated with 2.15 μM melatonin individually, the second least increase when treated with 18.5 μM estradiol and Aβ, and the third least increase when treated with 2.15 μM melatonin and Aβ (Figure 5). This demonstrates that melatonin was not able to reverse the effects of Aβ on the increase of caspase-3. Although there was a study done on decreasing caspase-3 activity in HTB-11 cells through an ERβ pathway (Nguyen et al., 2015), there has been no studies done on the effects of estrogen itself.

After 24-hour treatment, HTB-11 cells had the greatest decrease in cell proliferation after being treated by Aβ, followed by the greatest reduction in decrease when treated with 43 μM melatonin alone, followed by a second greatest reduction in decrease when treated by 3.7 μM estradiol, Aβ, and NO synthase inhibitor, and a fourth greatest reduction in decrease when treated by 43 μM melatonin and Aβ (Figure 6). This demonstrates that melatonin was not able to counteract the effects of decreased cell proliferation by Aβ too greatly, compared to if melatonin was just alone. In addition, the HTB-11 cells were most likely not treated with NO synthase inhibitor for long enough. Since NO is decreased in AD patients, and more NO is produced by NO synthase, NO synthase should reduce Aβ effects. NO synthase inhibitor decreases NO synthase activity. Thus, NO synthase levels probably were not decreased enough for estradiol's impact on Aβ to be reversed.

After 96-hour treatment, HTB-11 cells had the greatest decrease in cell proliferation after being treated by Aβ, with an increase in cell proliferation after HTB-11 cells were treated with 43 μM melatonin and Aβ (Figure 7b). In addition, cell proliferation all decreased when exposed to NO synthase inhibitors (Figure 7). This demonstrates that NO synthase is used by estrogen and melatonin in their function to counter Aβ's effects. In addition, melatonin has a stronger effect than estrogen in regards to countering decreased cellular proliferation, as caused by Aβ. Similarly, these were new findings.

In transgenic Aβ Drosophila flies, Aβ caused a reduction in fly activity, while estrogen and melatonin, both treated along with Aβ, had reduction in decrease of fly activity (Figure 8). However, estrogen and

melatonin were not able to completely reverse Aβ's effects of impairing fly movement. This might be due to low sample size of flies, which made all percentages of decrease non-significant. Again, these are new findings.

When treating HTB-11 cells, Aβ increased APOE4 levels, while melatonin alone had the greatest decrease in APOE4, while estradiol and melatonin, combined with Aβ, both had very reduced increase in APOE4 levels (Figure 11). This indicates estradiol and melatonin were able to reduce Aβ's effects of increasing APOE4. This is a new finding.

CONCLUSION

In conclusion, the hypothesis of this study is supported with evidence from *in vitro* and *in vivo* models suggesting anti-AD effects from melatonin and estrogen. Estrogen and melatonin were able to reverse Aβ effects in HTB-11 cells by increasing cell count and cell area, decreasing lipid droplet count inside the cell and on its dendrites, decreasing caspase-3 activity, and increasing cell proliferation.

One idea to explore in the future is comparing testosterone's effects to estrogen as potential treatments for Alzheimer's. Since AD has been studied to be a sexually dimorphic disease, and estrogen decrease is primarily found in postmenopausal women, it would be interesting to observe testosterone's effects on males with AD (Valencia-Olvera et al., 2023).

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