HIGH SCHOOL EDITION
Journal of Student Research
Volume 11 Issue 2 (2022)

The Molecular Mechanism of Vitamin D3 in Mitigating Pathological Formation of Parkinson's Disease

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ABSTRACT

Parkinson's Disease (PD) has been in the spotlight of research for decades, but despite efforts aiming to discover treatments, there is still no effective therapy that targets PD pathology. Vitamin D3, however, has been identified as a promising chemical in mitigating the effects of alpha synuclein (α-syn), a hallmark protein in diagnosing PD. Mitochondrial complex I is a protein complex that produces oxidative stress, an effect that can lead to symptoms of PD. Rotenone, a chemical which induces PD-like effects was used to set a PD model. This study focused on the anti-PD effects of vitamin D3 in attenuating rotenone through mitochondrial complex I. Treatments were tested by measuring cell proliferation, rotenone induced necrosis, and rotenone induced apoptosis. Varying concentrations of vitamin D3 proved to reduce cytotoxicity substantially. Caspase activity in apoptosis decreased with vitamin D3 presence, suggesting that cell death was mitigated. In addition to using in vitro rotenone PD models, molecular docking was used to depict vitamin D3, rotenone, and mitochondrial complex I interactions. To our knowledge, this was the first study to use vitamin D3 and mitochondrial complex I in a molecular docking analysis in addition to utilizing rotenone to study PD. Docking established a connection between mitochondrial complex I, rotenone, and vitamin D3 by displaying a strong bond between molecules. This ultimately suggested that vitamin D3 can indirectly mitigate oxidative stress by attenuating rotenone and α-syn through mitochondrial complex I, thus limiting the presence of α-syn, which is seen in elevated amounts in PD.

Introduction

Parkinson's Disease

Parkinson's Disease (PD) is one of the most common neurodegenerative disorders, hindering motor capabilities and muscle control (DeMaagd, 2015). In 2015, 6.9 million people worldwide were affected by Parkinson's with numbers projected to double to 14.2 million by 2040 (Dorsey, 2018). The most recent study analyzing PD prevalence in the United States was conducted in 1978 (Parkinson's Foundation). There is currently no definite cure for Parkinson's Disease although there are many drugs and surgeries that improve quality of life. Certain drugs may bring more dopamine to the brain while others may cause nerve cells to react to dopamine, but there is no universal drug to counter the effects of Parkinson's (U.S. Departments of Health and Human Services).

Many influences are involved in the formation of PD including a combination of both genetic and environmental factors. Of all PD cases, about 10 to 15% are a result of genetics (Parkinson's Foundation). There are a handful of gene mutations that are known to inflict PD like SNCA, PARK2, and LRRK2, but exactly how these genes do so is

being further researched (Johns Hopkins). In addition to genetic causes, environmental factors also play a role in the development of PD. Certain occupations may come with more vulnerability towards PD. For example, factory workers with longitudinal exposure to trichloroethylene, a contaminant in groundwater, were observed to have a significantly high number of cases of PD (Parkinson's Foundation). Additional studies with pesticides including paraquat and rotenone have shown these substances increasing PD risk in farmers (Pouchieu et al., 2018).

Despite PD affecting the physical mobility of humans, the origin of the disease stems from the lack of dopaminergic neurons in nerve cells (DeMaagd, 2015). Alpha-synuclein (α-syn), coded by the SNCA gene, is a vital protein in the pathology of PD that regulates neurotransmitter release (Gómez-Benito, 2020). The aggregation of α-syn is closely associated with the loss of dopaminergic neurons in PD as mutations in the SNCA gene cause the release of α-syn (Gómez-Benito, 2020). α-syn is then expressed in the form of Lewy bodies, proteins that induce problems affecting brain functions such as movement, memory, and behavior. In these Lewy bodies is the oligomeric α-syn species, the most toxic molecules responsible for cell death in PD and provocation of oxidative stress (Gómez-Benito, 2020). These oligomeric bodies are shown to restrain mitochondrial complex I, triggering the death of dopaminergic cells due to mitochondrial depolarization (Marella et al., 2006; Rcom-H'cheo-Gauthier et al., 2017). Interactions between α-syn and mitochondria are associated with increases in nitric oxide and oxidative modification which induce oxidative stress (Grünewald, 2018). Oxidative stress in mitochondrial complex I can lead to neurodegeneration that heavily influences the progression of PD.

Vitamin D_3

Cholecalciferol, also known as vitamin D3, boosts calcium and supports bones in the body. Studies have shown that this substance plays a significant role in treatment for Parkinson's. Ever since its discovery, vitamin D3 has been used as a supplement to cure diseases related to bone disorders. It was originally researched for a cure to rickets, a disease indicated with softened, deformed bones and muscle spasms (Nature News, 2002). Rickets became increasingly problematic in the late 1700s when more Europeans lived in the sunlight-deprived, polluted cities, and a cure for the disease was not discovered by scientists until the 1890s. It was discovered in the 1920s by Sir Edward Mellanby that irradiated foods served as a practice to prevent rickets (Deluca, 2014). However only in the 1930s was the real reason behind the success of these foods identified. Uncontestedly, Vitamin D was declared as the compound responsible for curing rickets as a result of studies on the chemical structures of cholesterols by Askew and colleagues (Askew et al., 1930; Nature News, 2002).

Vitamin D3 has been known to be beneficial in studies not only for bone health but also brain development. This vitamin's neuroprotective effects prevent the formation of inducible nitric oxide synthase (iNOS), which catalyzes the production of nitric oxide free radicals, which can do oxidative damage to cells (Rimmelzwaan et al., 2016). It also aids in the synthesis of glutathione, an antioxidant that protects cells from damage by balancing harmful substances like nitric oxide free radicals (Rimmelzwaan et al., 2016). In addition to its protective properties, vitamin D boosts neuronal growth.

Because of its neuroprotective effects, vitamin D3 has been widely used in studies pertaining to Parkinson's. A relationship between PD and vitamin D3 deficiency highlights the effects of hypovitaminosis and PD. In studies using rotenone in an in vitro PD model, vitamin D3 was shown to reverse rotenone induced effects by increasing autophagy markers and preventing α-syn aggregation (Jang et al. 2014). In separate studies with PD patients, vitamin D serum has been correlated with a decrease in PD risk and motor severity based on the Unified Parkinson's Disease Rating

Scale, but the pathogenesis has not been found (Luo et al., 2018; Peterson et al. 2013). Some studies may show that vitamin D3 does not have an effect on PD, there is no unanimous conclusion as to whether or not the supplement has definite effects in PD research.

Neuroinflammation, oxidative stress, and mitochondrial dysfunction have all been noted as causes of α-syn folding, and vitamin D3 may be a solution to mitigating this trigger (Gómez-Benito, 2020). Previous studies showing the relationship between a vitamin D binding protein calbindin-D28k and the exclusion of α-syn aggregation prove the effectiveness of the vitamin in mitigating the severity of PD. Calbindin-D28k-positive neurons resulted in a decline of α-syn aggregates and an increase in α-syn occurred with the suppression of calbindin-D28k expression and lack of calcipotriol, a synthetic form of vitamin D (Rcom-H'cheo-Gauthier et al. 2017).In this study, vitamin D3 could successfully decrease α-syn aggregation, a key hallmark in PD.

Rotenone

First discovered in South America and Southeast Asia hundreds of years ago, rotenone is a naturally occurring compound from the roots and seeds of tropical plants (Radad et al. 2019). One of the most universally used insecticide and pesticide, it is a substance used both in the fields as well as in the lab. Although it has been used as a pesticide for decades, rotenone has only recently been brought into the attention of researchers. In 2000, Betarbet *et al* discovered the similarity of rotenone's toxic features and Parkinson's (Beterbet et al. 2000). Rotenone's primary site of its mitigating effects is the respiratory chain mitochondrial complex I (Grünewald, 2018). Because of its Parkinson's-like toxic effects, it serves as a commonly used alternative to the disease in research studies.

Research Purpose

Previous research has shown separate studies of benefits of vitamin D3 in treating PD and rotenone induced oxidative stress, but the link between the two is not fully known. This study aimed to investigate the correlation between vitamin D3 and its effects on rotenone and mitochondrial complex I in an in vitro PD model.

Materials and Methods

Materials and Dilutions

All chemicals and testing tools used were acquired from Sigma Alrich unless otherwise specified. To obtain varying dilutions of vitamin D3, 1 1,000 IU tablet of VD_3(25 µg) was dissolved into ethanol. This dilution represented the VD_3 x1 dilution, or 65 µM dilution. Tubes were then prepped with Minimum Essential Media (MEM) for the dilutions of the VD_3 x10 (6.5 µM), VD_3 x100 (0.65 µM), and VD_3 x1000 (0.065 µM) respectively. The other dilutions were made in a serial dilution series. For the rotenone dilutions, the same process was done to obtain Rot x1 (1268 µM), Rot x10 (126.8 µM), and Rot x100 (12.68 µM). Rot x1, the highest concentration, had 5mg in 10mL, thus resulting in a 1268 µM solution. In addition to these dilutions, samples of Rot x10,000 (0.1268 µM) and Rot x50,000 (0.0254 µM) were made for further tests.

Cell Culture

To prepare HTB-11 cells, old media was discarded and trypsin was added, and cells were incubated at 5% CO_2 and 37 °C to break down the proteins in the cells. New media was then added to neutralize the trypsin, then used to rinse the cells from the walls of the flask and inserted into a tube. The tube was then centrifuged in a Corning Centrifuge set at 3000 rpm for 4 minutes, leaving a cell pellet in the bottom of the tubes. 10 mL of new media was then added into 2 new tubes and 4 mL of fresh media was added to the tube with the cell pellet. After being mixed into a homogenous solution, the liquid was split and 2 mL was piped into each of the 2 new tubes. Then, the cells were poured into a tray to be transferred to a 96-well plate with a multichannel pipette.

MTT Cell Proliferation Assay

A 24-hour colorimetric MTT assay was performed to determine the survival rates of the HTB-11 cells under PD induced conditions. 5 µL of the different dilutions of vitamin D3 and rotenone were inserted into their respective columns. 0.65 µM vitamin D3 and 12.68 µM rotenone were combined to form one sample in the plate amongst the other individualized samples. After being incubated for 24 hours, 10 µL of MTT assay solution was added to each well and incubated for 1.5 hours. Following the incubation period, 80 µL of dimethylsulfoxide (DMSO) was added in order to fully dissolve the colored formazan crystals set on the bottom of the wells. With the wells being thoroughly mixed 10 minutes later, the absorbance levels were read with a Bio-Rad iMark Microplate Absorbance Reader. The program Microplate Manager 6 (MPM6) (Bio-Rad) was set at 595 nm to record the relative transparency, which served as a proxy for the cell survival rate.

LDH Assay

A CyQUANT lactate dehydrogenase (LDH) cytotoxicity assay was then conducted to test for necrosis. From a treated plate, media from each well was transferred to a new plate labeled for “released LDH”. In the treated plate, a lysis buffer was added to split the cells before an incubation period of 45 minutes. In the released LDH plate, LDH substrate was added to react with the LDH, and this interaction was confirmed with the observation of a color change. The plate was then placed in a container void of light at room temperature for 30 minutes. From the treated plate, the solution with the lysis buffer was inserted in a new plate. This plate represented the maximum amount of LDH that could have been released. After the LDH substrate was added, the plate was then set in a dark container for 30 minutes. To prevent the samples from colorizing further, 30 µL of stop solution was then added to both the released LDH and total LDH plates. Using the same MPM6 program as the MTT assay, two readings were done to determine valid values to reduce error. A 490 nm reading was done to read the color data and a 655 nm reading was administered to locate background interference, which were removed from the data set for more accurate numbers.

Caspase Assay

Prior to the caspase-3 assay, the cell samples were cultured in a 6-well plate with concentrations of 0.0254 µM ROT, 0.1268 µM ROT, 0.065 µM VD3, 0.065 µM VD3 + 0.0254 µM ROT, and 0.065 µM VD3 + 0.1268 µM ROT. The solutions were transferred from the wells into microtubes. In the wells, 500 µL of trypsin and 500 µL of media were added and thoroughly mixed before being piped into the tubes with their respective concentrations. After being centrifuged for 3 minutes, the tubes developed a cell pellet which was then lysed with 50 µL of buffer and combined. In a new 96-well plate, 50 µL of assay buffer and 45 µL of lysis buffer were mixed before 5 µL of the samples from the prepared tubes were added to their respective wells. For the apoptosis reaction to occur, 5 µL of caspase substrate was piped in. The plate was then read at 415 nm with a microplate reader. Multiple readings were conducted in 15-minute frames of an hour.

Molecular Docking

To explicate various PD-associated macromolecules with the ligands of substances such as α-syn, vitamin D3, and rotenone which affect the severity of Parkinson's, molecular docking using PyRx v.08 software (Source Forge) was conducted. Preprocessing before docking included loading the respective ligands and macromolecules into PDB files from protein data banks RCSB PDB and PubChem. SDF files from PubChem were converted into PDB files using the SMILES Translator and Structure File Generator (National Cancer Institute). Within docking, active binding sites were established by referring to native ligands co-crystallized with the respective macromolecule. The software then encased the ligand in the binding site within the selected border markings to ensure accuracy and viability of the docking positions. Post-docking, AutoDock Vina, an open-source program within PyRx, provided an analysis of binding affinities (kcal/mol) calculated using empirical scoring functions. From this execution, the most negative binding affinity conformations for each protein-ligand pair were identified.

Statistical Analysis

Significances of all results were analyzed using Student's t-test or an analysis of variance (ANOVA) test. Results with a p-value of <0.05 were assessed as statistically significant. Error bars represent a standard error of 5%.

Results

All treatments were performed using HTB-11 human neuron cells to create a neuronal model of Parkinson's. In this study, untreated HTB-11 cells were defined as the control and used to set the standard of no cell viability or cell ineffectiveness. Rotenone was administered at concentrations of 126.8 µM, 12.68 µM, 1.268 µM, 0.1268 µM, and 0.0254 µM. Vitamin D3 was administered at 6.5 µM, 0.65 µM, and 0.065 µM. Additional concentrations with an incorporation of both substances were also used: 0.65 µM VD + 12.68 µM ROT, 0.065 µM VD + 1.268 µM ROT, 0.065 µM VD + 0.1268 µM ROT, and 0.065 µM VD + 0.0254 µM ROT.

Rotenone Inhibition of Cell Proliferation

An MTT assay *(Fig. 1)* was done to measure the average cell survival rate through a colorimetric analysis. In comparison to the control, all the treated samples exhibited a decrease in survival. The vitamin D3 showed a higher survival rate at concentrations of 0.065 µM, 0.65 µM, 6.5 µM with rates of 91.69%, 86.10%, and 88.21% respectively. Rotenone showed lower survival rates at concentrations of 1.268 µM, 12.68 µM, and 126.8 µM at 52.19% ($p < 0.0001\%$) to 67.63% ($p < 0.01\%$) to 56.94% ($p < 0.0001\%$) respectively. There is no specific trend in the varying dilutions of rotenone and vitamin D3, suggesting that the concentration of either vitamin D3 or rotenone alone did not produce a significantly different cell survival rate. The sample with the least percent of survival was the combination of rotenone and vitamin D3(49.55%; $p < 0.0001$).

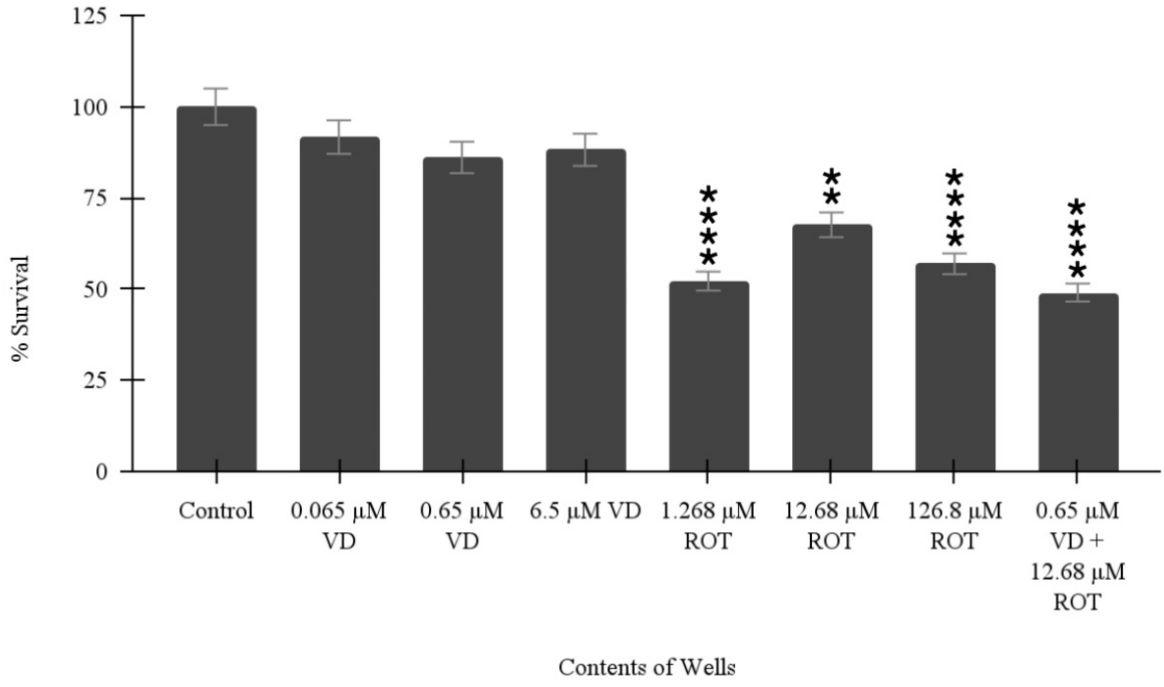

Figure 1. In the MTT proliferation assay, increasing concentrations of rotenone do not have an effect on the survival rate of cells. There was no trend in the concentrations of vitamin D3. The treatment with both rotenone and vitamin D3 showed the smallest survival percentage. (** = $p < 0.01$, **** = $p < 0.0001$). P values compared rotenone concentrations with the control.

Less Concentrated Rotenone Dilutions

Because the previous MTT assay did not yield definitive results, another assay *(Fig. 2)* was performed to determine the relationship between rotenone and vitamin D3. Four new treatments were utilized. Two individual dilutions of 0.1268 µM ROT and 0.0254 µM ROT were used in the assay. Additionally, two assays utilized 0.0254 µM ROT and 0.1268 µM ROT each mixed with 0.065 µM vitamin D3. The 0.1268 µM ROT dilution recorded a 66.32% survival rate. The 0.0254 µM dilution, however, resulted in a net increase in cell survival in comparison to the control and in contrast to the other dilutions of rotenone, displaying a 169.63% survival rate. When 0.065 µM vitamin D3 was combined with 0.0254 µM ROT and 0.1268 µM ROT, it decreased cell survival to 105.8% ($p < 0.001$) and 51.12%

respectively. When added to 1.268 µM ROT, vitamin D3 increased the cell survival slightly by 5.59% to a percentage of 34.94% ($p < 0.05$).

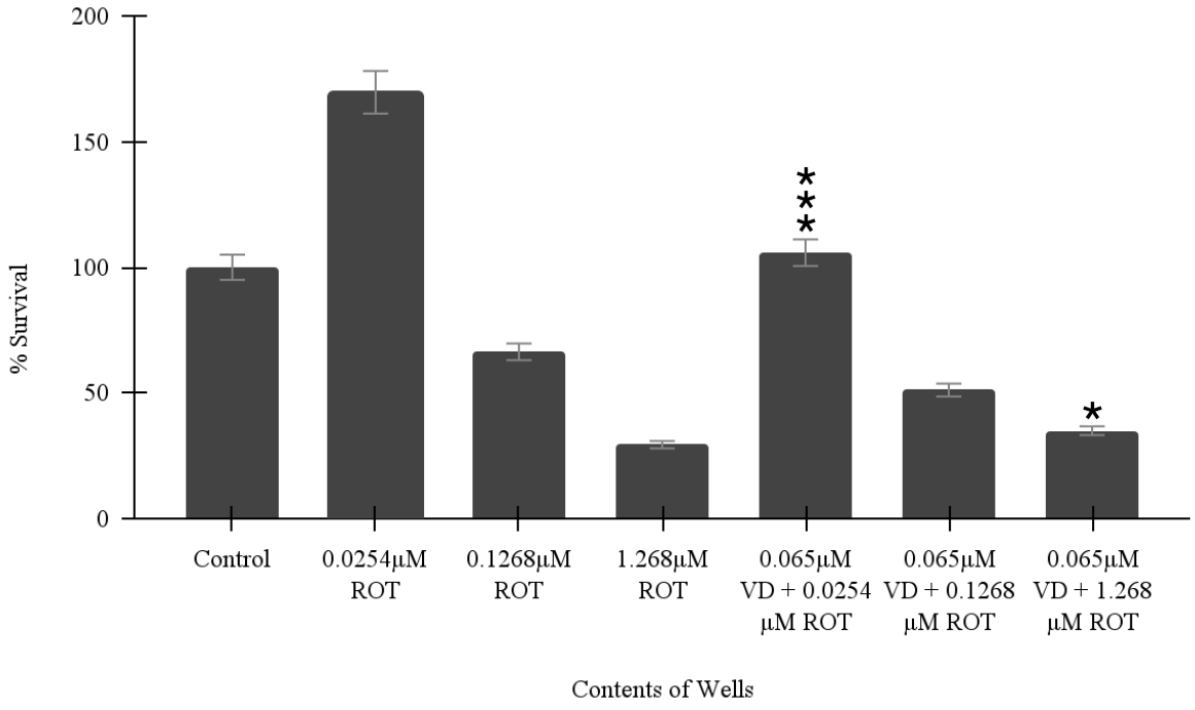

Figure 2. The second MTT assay done with less concentrated dilutions of rotenone yielded opposing results, exhibiting a smaller survival rate associated with the addition of vitamin D3 to rotenone. The less diluted a concentration, the larger the effect vitamin D3 had on reducing the survival rate. (* = $p < 0.05$, *** = $p < 0.001$). P values compared rotenone concentrations alone and rotenone concentrations with the addition of vitamin D3.

Rotenone Induced Necrosis

A 24-hour LDH test *(Fig. 3)* was done to determine whether cell death was due to necrosis with LDH release. Readings showed an inverse relationship between rotenone concentrations and cytotoxicity: the smaller the dilution, the more toxic the treatment would be. 1.268 µM ROT was notably less toxic, being 3.84% toxic in comparison to 0.0254 µM ROT. 0.0254 µM ROT and 0.1268 µM ROT were respectively 30.76% and 27.94% toxic. In every addition of vitamin D3 to a rotenone dilution, vitamin D3 decreased cytotoxicity. The decrease with 0.1268 µM ROT was the greatest, declining from 27.04% to 0.89% ($p < 0.0001$). All data suggest that vitamin D3 mitigates the toxic effect of rotenone.

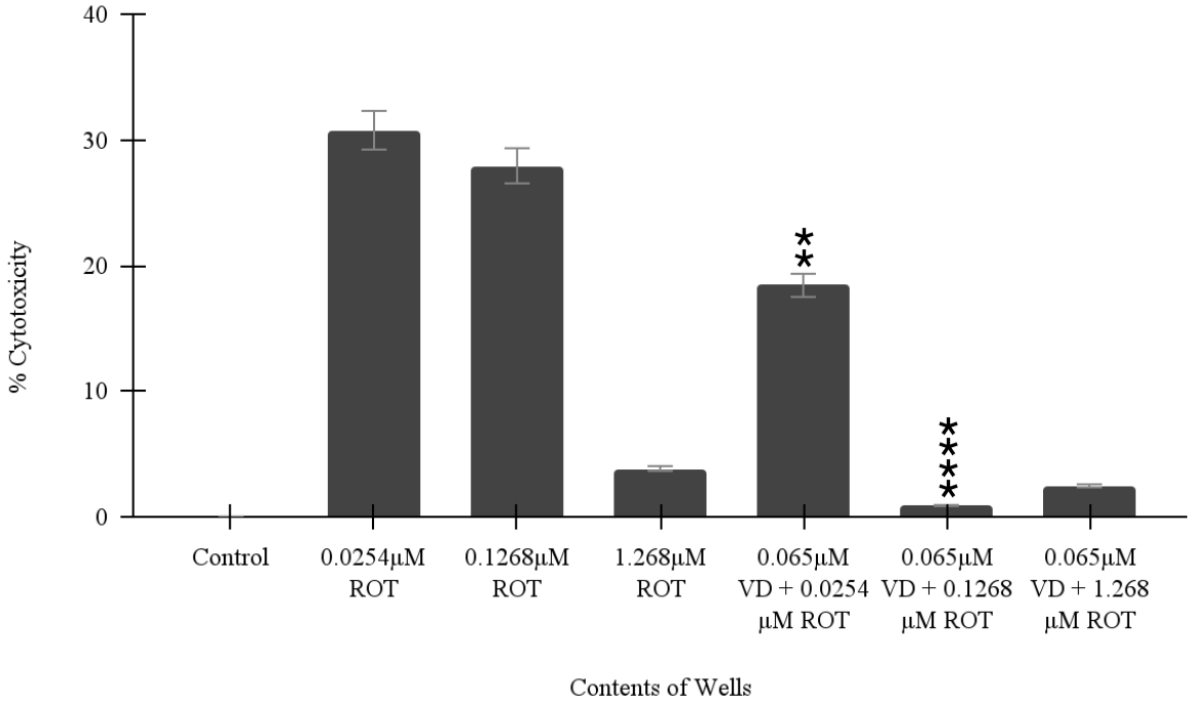

Figure 3. The LDH assay showed vitamin D3 reduces the toxicity of rotenone. More concentrated rotenone assays were more strongly affected by the inclusion of vitamin D3.
(** = $p < 0.01$, **** = $p < 0.0001$). P values compared rotenone concentrations alone and rotenone concentrations with the addition of vitamin D3.

Rotenone Induced Apoptosis

Previous studies have noted a significance in rotenone induced apoptosis and oxidative stress (Grünewald, 2018). In this study, a caspase-3 assay *(Fig. 4)* was performed to determine the effects of vitamin D3 on rotenone in apoptosis (Li et al. 2021). The assay produced varying results with the addition of vitamin D3. All the treatments indicated cell death, differing from 0% cell death in the control. Both individual rotenone treatments induced apoptosis, with 0.0254 µM ROT at 54.01% and 0.1268 µM ROT at 100.53%. Vitamin D3 displayed the lowest cell death percentage of 17.65%. When 0.065 µM VD was in combination with 0.0254 µM ROT, it reduced death by 31.55%--more than half of the original death rate. Vitamin D3 decreased the 0.0254 µM ROT cell death to 22.46%. Interestingly, 0.065 µM VD with 0.1268 µM ROT exhibited opposing results, increasing cell death by 77.01% to 177.54% ($p < 0.05$).

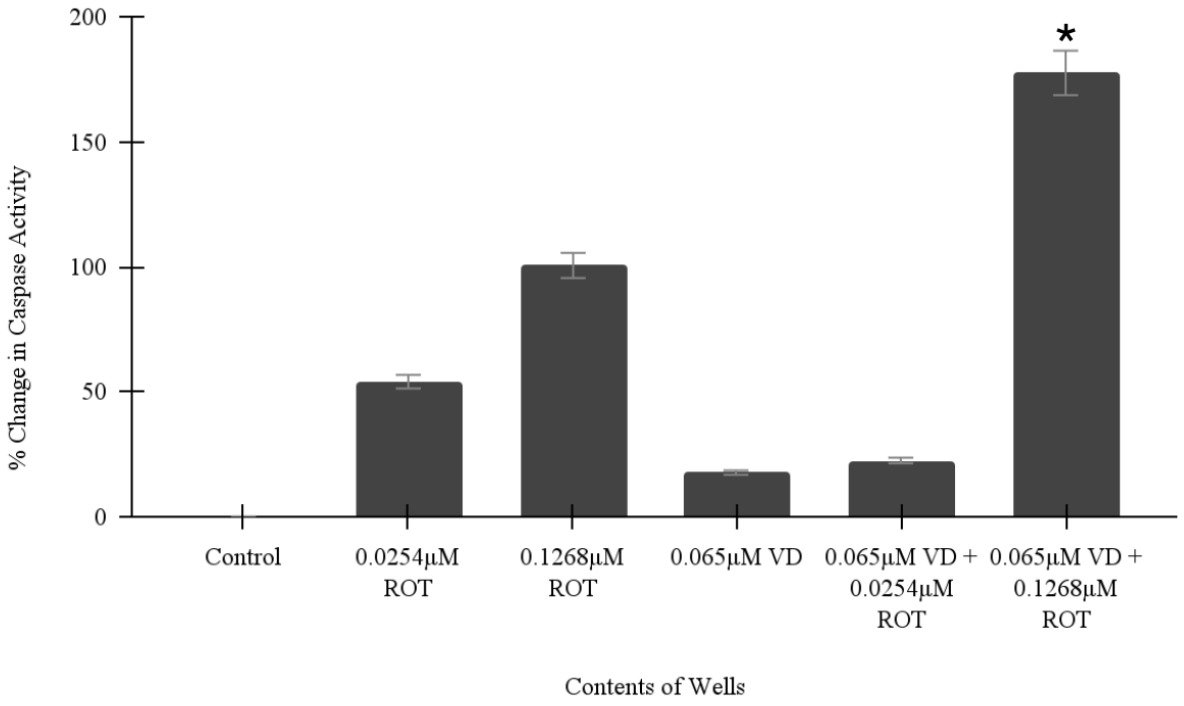

Figure 4. The Caspase 3 assay revealed vitamin D3 reducing the effects of a less concentrated sample of rotenone. In comparison, the higher concentration of rotenone increased toxicity with the supplement of vitamin D3 (* = $p < 0.05$). P values compared rotenone concentrations alone and rotenone concentrations with the addition of vitamin D3.

Molecular Docking

In order to produce an accurate representation of an in vivo ligand-macromolecule bond, molecular docking is used to provide realistic receptor sites and binding locations. Models displaying the binding affinities of mitochondrial complex I, rotenone, vitamin D3, and the vitamin D binding protein were tested. RMSD values were taken into consideration for accuracy, with lower RMSD numbers (<10) determining the more significant scores (Liebeschuetz, 2015). The averages were also calculated with the more accurate binding scores chosen by low RMSD values.

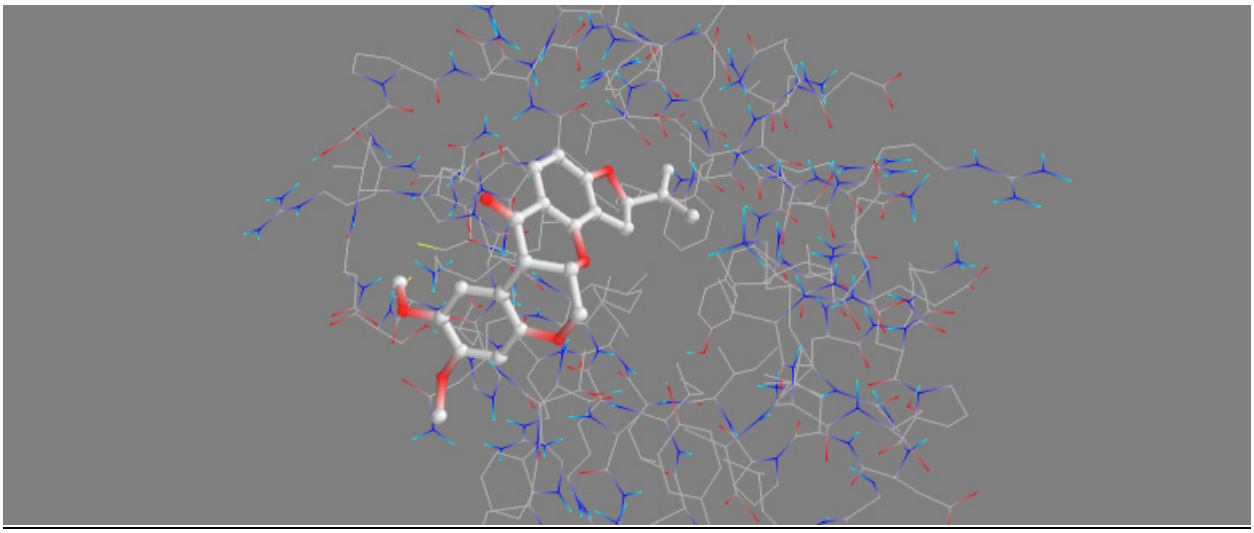

Figure 6. *Rotenone binds with mitochondrial complex I (1S3A).*

Docking was done to find the binding affinity between rotenone and mitochondrial complex I to verify rotenone induced oxidative stress. With the most negative binding affinity being -7.5 and the average being -6.08, the data showed strong binding affinities between rotenone and mitochondrial complex I. This further proved that rotenone can have an effect on the complex. Specifically, rotenone could cause oxidative stress which contributes to the pesticide's PD-like effects.

Table 1. Binding affinity between rotenone and mitochondrial complex I.

Compound	Binding Affinity	Mode	RMSD lower bound	RMSD upper bound
1S3A, ROT	-7.5	0	0	0
1S3A, ROT	-7.2	1	18.441	21.611
1S3A, ROT	-6.4	2	17.796	20.835
1S3A, ROT	-6.1	3	12.053	15.549
1S3A, ROT	-5.8	4	3.159	5.557
1S3A, ROT	-5.8	5	3.142	8.269
1S3A, ROT	-5.7	6	1.906	7.816
1S3A, ROT	-5.6	7	13.688	17.569
1S3A, ROT	-5.6	8	2.775	5.426

There is a high binding affinity between rotenone and mitochondrial complex I. A more negative binding affinity indicates stronger binding.

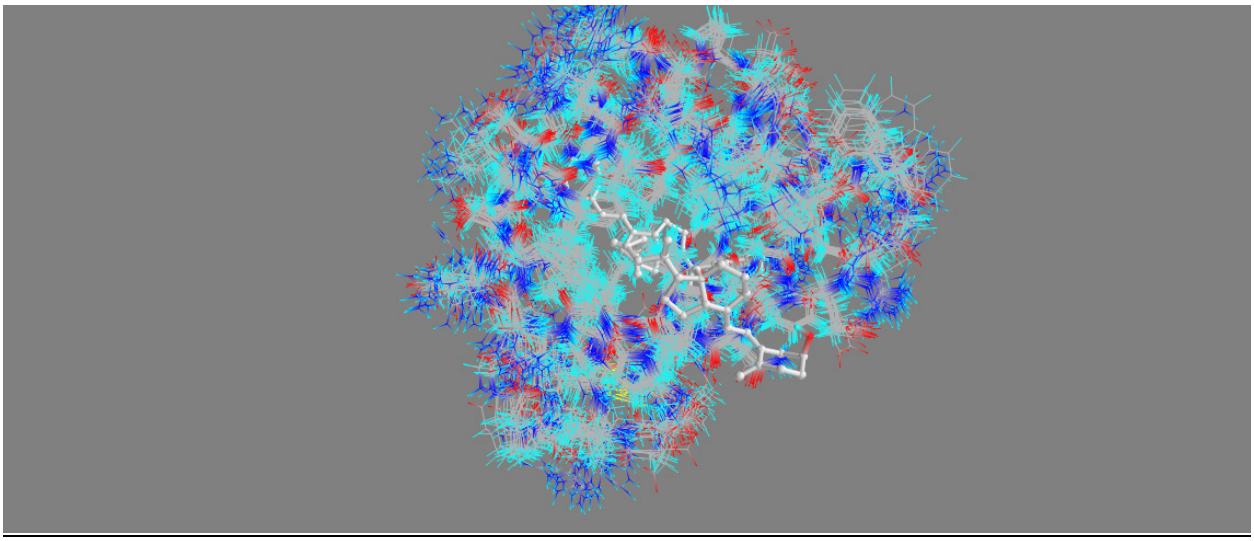

Figure 7. *Cholecalciferol (Vitamin D3) binds with mitochondrial complex I (1S3A).*

Molecular docking with vitamin D3 and mitochondrial complex I (Figure 7) was done to test how effective vitamin D3 could be in mitigating the effects of rotenone on mitochondrial complex I. The strongest binding affinity number was -6.6 with the average of the significant modes being -6.38. This average was the strongest result in all the molecular docking tests performed. The two highest scores following the top score of -6.6 also had low RMSD values, indicating significance and accuracy in the results.

Table 2. Binding affinity between rotenone and vitamin D3.

Compound	Binding Affinity	Mode	RMSD lower bound	RMSD upper bound
1S3A, VD	-6.6	0	0	0
1S3A, VD	-6.5	1	2.945	4.899
1S3A, VD	-6.5	2	1.797	2.862
1S3A, VD	-6.4	3	19.3	22.116
1S3A, VD	-6.2	4	19.828	23.212
1S3A, VD	-6.2	5	21.477	22.921
1S3A, VD	-5.9	6	12.798	15.627
1S3A, VD	-5.9	7	2.578	8.6
1S3A, VD	-5.9	8	19.94	22.291

There is a relatively high binding score between vitamin D3 and mitochondrial complex one. The low RMSD scores of higher binding affinities also amount to the accuracy of the pose (Liebeschuetz, 2015).

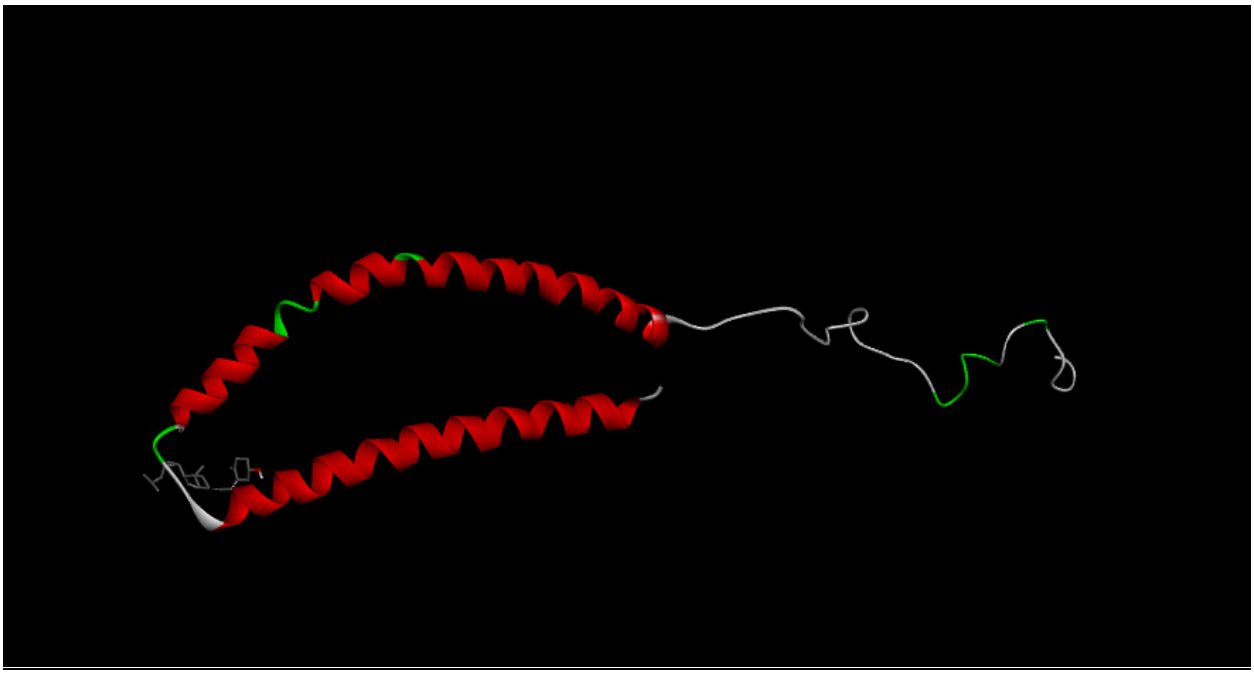

Figure 8. Vitamin D3 binds to a human micelle-bound α-syn (1XQ8).

Molecular docking was done in order to determine whether vitamin D3 could reverse the effects of α-syn, a test with both molecular structures was done. The highest binding value recorded was -5.5 and the average was -5.04, both indicative of strong binding affinities. Although the RMSD values were either very high or very low, the average reflected the binding affinities with lower RMSD values, resulting in a very accurate average.

Table 3. Binding affinity between alpha-synuclein and vitamin D3.

Compound	Binding Affinity	Mode	RMSD lower bound	RMSD upper bound
1XQ8, VD	-5.5	0	0	0
1XQ8, VD	-5.4	1	1.864	2.835
1XQ8, VD	-5.0	2	5.05	7.553
1XQ8, VD	-4.8	3	129.393	133.734
1XQ8, VD	-4.8	4	71.163	76.42
1XQ8, VD	-4.7	5	4.906	7.112
1XQ8, VD	-4.6	6	125.061	129.506
1XQ8, VD	-4.6	7	130.483	135.245
1XQ8, VD	-4.6	8	1.937	4.404

The negative binding affinities indicate a binding between the two molecules, a representation of a practical in vivo circumstance.

Discussion

This study aimed to analyze the effects of vitamin D3 on a rotenone induced PD model. Vitamin D3 mitigates the toxicity of rotenone through necrosis and apoptosis. The MTT assay showed increasing concentrations of rotenone associated with a lower survival rate. In the concentrations with vitamin D3 in combination with rotenone, the same correlation was observed, with the higher concentrations reducing cell survival. However, the addition of vitamin D3 in the samples decreased the survival rate when compared to the concentrations with only rotenone. Since the vitamin D3 induced substantial cell death, further assays were conducted to determine which type of death occurred.

In the LDH necrosis assay, vitamin D3 proved to reduce the toxicity of rotenone, having the most substantial effect at a concentration of 0.1268 μM ROT. From the 0.1268 μM ROT to the 0.065 μM VD + 0.1268 μM ROT, the cytotoxicity dropped from 27.94% ($p < 0.0001$) to 0.89%, exhibiting almost no cytotoxicity. Vitamin D3 reduced the toxicity of rotenone almost as substantially for the 1.268 μM ROT as well, and the only concentration that did not produce a major decrease in comparison to the other concentrations was the smallest dilution of rotenone, 0.0254 μM ROT. It is possible that the high cytotoxicity of 0.0254 μM ROT is consequently because of its small concentration. This may introduce antimicrobial effects of rotenone due to its relatively short half-life and thus causing exposure to be short term (Lazo et al., 2014; Perron et al., 2006). Rotenone has a very short half-life, so it is possible that exposing cells to a high concentration of it would not necessarily produce more substantial cytotoxicity than a more dilute concentration, but this proposal has not been confirmed (Lazo et al., 2014). Overall, vitamin D3 decreased the toxicity of rotenone especially in higher concentrations.

In the caspase-3 assay, vitamin D3 decreased caspase activity for the lower concentration of rotenone, 0.0254 μM ROT, but increased activity for the higher concentration, 0.1268 μM ROT. Parallel to other studies indicating the correlation between rotenone and apoptosis, the results also show rotenone mitigating cell death by apoptosis (Li et al., 2021). Rotenone can induce programmed cell death by producing reactive oxygen species (ROS) (Li et al., 2021). The increase in cell death for the 0.1268 μM ROT sample may be attributed to the concentration of vitamin D3 it was combined with, 0.065 μM VD. While 0.065 μM VD is able to mitigate 0.0254 μM ROT, 0.065 μM VD is unable to mitigate 0.1268 μM ROT due to its low concentration.

From molecular docking, a link between mitochondrial complex I and rotenone and vitamin D3 was established. Concurring with previous studies, rotenone was able to bind well with mitochondrial complex I, inhibiting the protein and promoting ROS creation (Li et al., 2021). By increasing ROS production, rotenone can induce oxidative stress and cause mitochondrial toxicity, hindering cell proliferation. However, the molecular docking in this study found vitamin D3, on average, to have a higher binding affinity with mitochondrial complex I than rotenone. This indicates that vitamin D3 in vivo could potentially mitigate the effects of rotenone. Evidently, from the molecular docking of α-syn and vitamin D3, vitamin D3 binded well to the α-syn molecule, exhibiting vitamin D3's ability to suppress α-syn aggregation through a binding site with a similar polarity.

Despite many studies supporting the reversing effects of vitamin D3 in PD, they do not highlight the association between the pathology of PD and vitamin D3. This study indicates the function of vitamin D3 in mitochondrial complex I in mitigating rotenone. Previously, molecular docking has not been used to find the relationship between vitamin

D3, α-syn, rotenone, and mitochondrial complex I, so this study provides new evidence for the reversing effects of vitamin D3 on rotenone through mitochondrial complex I.

This study supports the theory of how vitamin D3 can help mitigate rotenone as a PD model. Through mitochondrial complex I, there is now new insight as to the relationship between vitamin D3 and the complex. While the relationship between mitochondrial complex I, rotenone, and, PD has been evaluated, this study introduces a method for vitamin D3 mitigating the action of rotenone. Past studies seldom use molecular docking to supplement their research. This study opens a new path to researching the relationship between vitamin D3, mitochondrial complex I, and rotenone with the use of molecular docking technology.

There are some limitations to this study that should be taken into consideration. Although rotenone has been proven to be a reliable substance to induce PD-like properties, it does not reflect all the hallmarks of PD. Currently, there is no chemical that can fully induce all the hallmarks of PD, so no model will be entirely accurate (Jackson-Lewis et al., (2012). Although very unlikely, microplate readings for assays conducted in this study could have been inaccurate for some wells due to miniscule bubbles or defective wells. Due to limited resources, samples with vitamin D3 and rotenone in combination in each test were only performed with one concentration of vitamin D3. If a variety of concentrations of vitamin D3 and rotenone in combination were tested, then the results may have yielded different results. In the assays, percentages exceeding 100% may have also been due to pipetting errors, but this conclusion is not fully supported.

Future tests using greater concentrations of vitamin D3 can be done to recognize what concentrations of vitamin D3 are necessary to mitigate the effects of rotenone. Additional molecular docking tests can be run to determine stronger potential binding sites, especially on α-syn.

Conclusion

Vitamin D3 can indirectly mitigate oxidative stress through the mitochondrial complex I by attenuating rotenone and α-syn. This is vital in understanding vitamin D3's role in diminishing PD as the main hallmarks of the condition are attributed to α-syn aggregation. Prevention of this protein aggregation could improve PD outcomes.

Acknowledgments

I would like to thank my advisor for helping me this research.

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