Title: The effects of Ashwaganda (WSP) on oxidative damage of drosophila exposed to rotenone, a Parkinson's Disease model.

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Introduction

Parkinson's disease (PD) is a chronic and progressive motor disorder effecting many people worldwide. It is the second most common neurodegenerative disorder affecting more than 1% of people over the Age of 55 (Han et al. 2012). Its symptoms include bradykinesia, rigidity, resting tremors, and postural instability, which are all motor control deficits or decreased voluntary movement control (Wang et al. 2018). The definitive cause for PD is unknown, there is also currently no accepted cure. One of the possible causes of these symptoms of PD is the loss of dopaminergic neurons in the Substantia Nigra, a region of the brain that is responsible for coordination and control of muscle activity (Hornykiewicz 2006). Today we test these possibilities in hopes of finding a cure or a way to reverse these ailments.

Researchers today use animal models to research ailments that are difficult to research in humans, such as PD. Many fundamental biological processes, such as those in dopaminergic neuron metabolism, are shared between people and Drosophila. Drosophila has similar genes to approximately 75% of all human genes that are disease related (Kasture 2018). So, the issue causing the progression of symptoms in humans could have a similar mechanism or be a similar issue in drosophila. Which is why we this animal as our model. Finding a cause in our animal model could lead to new ideas or breakthroughs in human medicine.

Rotenone has the ability to produce PD-like symptoms. It is naturally occurring pesticide that inhibits the activity of mitochondrial complex-1 (Manjunath 2013). Chronic exposure to rotenone can produce cell apoptosis, accumulation and aggregation of α-synuclein (a protein associated with PD), oxidative damage and endoplasmic reticulum stress (Ryu et al. 2002). In our animal model, drosophila, it has shown to produce dopaminergic neurodegeneration and PD-like motor deficit behaviors (Coulom and Birman, 2004).

According to the free radical theory of aging, oxidative reactions in many organisms generate free radicals, which can cause multiple lesions in macromolecules leading to their damage and aging (Harman 1994). This is thought to be the mechanism of pathology for rotenone (Ryu et al. 2002). *Withania Somnifera* (WSP), commonly known as Ashwagandha, is a plant in the Solanaceae family that is sometimes referred to as Indian ginseng because of its rejuvenating and tonic effects on the nervous system. It has been implicated in many therapeutic uses for over 5,000 years and has been widely used to treat diseases because of its anti-inflammatory, antitumor, antioxidant and immunomodulatory properties. The pharmalogical effects of WSP is attributed to withanolides the main active ingredient, which has a wide range of therapeutic applications. (Manjuath 2013). WSP root extracts and withanolides both are implicated in the ability to produce new neuron growth (Zhao et al. 2002) as well as inhibit the activity of acetylcholinesterase both in vitro and in vivo. (Choudhary et al. 2005)

In this experiment we want to test if WSP extract can be used as a tool to decrease oxidative damage and cell apoptosis that occurs because of rotenone exposure, which mimics the phenotypic expression of PD. We use rotenone on our animal model, Drosophila, to induce PD-like symptoms. A startle-induced negative geotaxis assay was used to determine the effects that rotenone and WSP have on motility. We expect flies that are exposed to rotenone to express decreased voluntary motor control and increased motor deficits compared to the control group because of the cellular and oxidative damage. We expect flies that are treated with WSP only to show similar voluntary motor control as the control group. We will use WSP in hopes in reverse these effects of rotenone on our animal model. We expect the flies exposed to rotenone and treated with WSP to behave like the control group and show increased voluntary motor control and decreased motor deficits compared to rotenone only exposed flies. With similar pathological genes, we hope that the success in treating PD-like symptoms in drosophila can translate to treating similar PD symptoms in humans.

Material and Methods

Subjects

This experiment studied how rotenone (Sigma-Aldrich, St. Louis, MO) and WSP (Sigma-Aldrich, St. Louis, MO) affects the motility of Drosophila. Drosophila were used to model Parkinsonian like symptoms. This was done by exposing Drosophila, to rotenone saturated food, Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply, Burlington, NC). Gender and age were not considered for this study

Material

In this experiment we studied the effects rotenone on Drosophila as well as WSP on Drosophila exposed to rotenone. To get the desired effect 4 vials of fly food were saturated with 4 different conditions, a control group, a .05% weight by volume WSP solution, a 62.5µM rotenone solution and a 62.5µM rotenone and .05% WSP solution, each vial contains 10 mL of the solution and 10µL DMSO (Sigma-Aldrich, St. Louis, MO) vehicle. DMSO is used to dissolve and carry rotenone which isn't soluble in water. DMSO was added to each vial so that it would not be a variable in the experiment. A tri-fold poster marked with lines every centimeter, a stopwatch and a camera phone were also used in this experiment. SPSS statistics software was used to for statistical analysis.

Methods

The four vials of fly food each with a different composition, as mention earlier, were created one week before the experiment. The purpose of the four groups are to show the effects rotenone exposure to flies compared to the control group, as well as the effects WSP on drosophila exposed to rotenone compared to flies only effected with rotenone, and the control group. The flies were left in the vials for the entire week where they were exposed to the test variables.

This was a conductor blind experiment. Groups blindly transferred the flies from their original housing vials, marked A, B, C & D, into testing vials. Groups carefully transferred flies into an open vial and covered it with another empty vial and taped them together with parafilm. The minimum number of

flies in one testing vial was 15. The dependent variable in the experiment was the Drosophila's motility after being exposed to the specific conditions. This was recorded using the startle-induced negative geotaxis assay. What this observes is the natural behavior of Drosophila to move upward after being startled. To record the motility of the flies a tri-fold poster marked with lines every centimeter was used and a threshold value that the fly must climb to "pass" the test was determined. The threshold value was 10cm in a 25 second time limit.

Each condition went through 3 trials. To begin one trial, groups took one testing vial (A) and gently tapped flies to the bottom of the vial. Once all flies reached the bottom time started, after 25 seconds a picture was taken and the flies above threshold were counted and recorded. In the next trial, flies from the previous vial (A) rested while another vial's test began (B, C or D). We gave flies time to rest to minimize confusion and discombobulation of the flies in our results. This was continued until each vial had 3 trials.

Statistical Analysis

For the statistical analysis we calculated a percentage of flies that cross threshold instead of an amount to account for variation in mortality between the different vials. A one-way ANOVA test was used to determine if there were any significant differences between any of the four conditions. Once it was determined that there was a significant difference between at least one condition we used an LSD post-hoc test to determine between which conditions there was a significant difference. An α level of 0.05 was used to determine significance.

Narrative

In this experiment Drosophila were used to make an animal model of Parkinson's disease. Flies were exposed to rotenone to attempt to obtain Parkinsonian like symptoms. We then used WSP to examine its effect on flies exposed to rotenone. To do this test four different conditions were used: a

control, WSP only group (0.05%), rotenone only group (62.5µM) and rotenone (62.5µM) and WSP group (0.05%)

A one-way ANOVA was used to see if there were any significant differences between the groups of different conditions (Figure 1). It determined that there is a significant decrease of motility between at least one of the four groups ($F(3,8) = 5.914$, $p < 0.05$). We then used LSD post-hoc test to decide between which conditions were significant. The LSD post-hoc test determined that there was a significant decrease in motility in the rotenone group compared to control ($p<0.05$) as well as rotenone + WSP group compared to control ($p<0.05$). On average, less than half of the number of flies that crossed the threshold in the control group, also crossed the threshold in the rotenone group. There was no significant difference between the rotenone + WSP group compared to the rotenone only group ($p=.645$). There was also no significant difference between the WSP only and control group ($p=.405$)

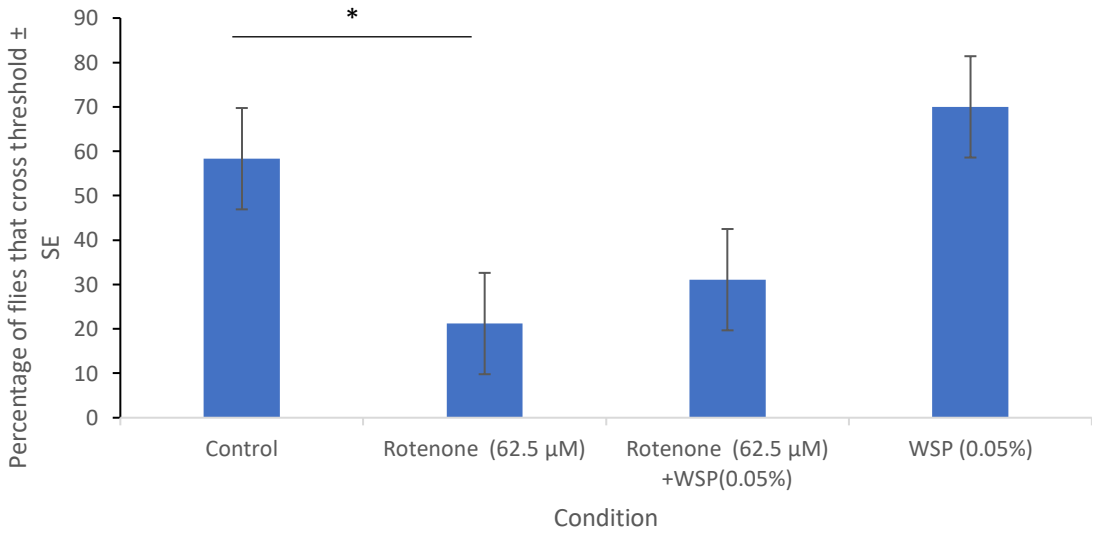

Figure Legend

Figure 1. This graph shows the effects of WSP (0.05%) on motility of drosophila exposed to rotenone (62.5µM). A one-way ANOVA revealed that there was a significant main effect of food condition on motility ($F(3,8)=5.914$, $p< 0.05$). An LSD post-hoc test indicated that significantly

fewer flies crossed the threshold in the rotenone condition compared to the control condition ($p<0.05$) Compared to flies exposed to rotenone alone, there was no significant changes in motility of flies that were exposed to rotenone that were co-exposed to WSP ($p=.645$). The motility of the flies that were co-exposed to rotenone and WSP was significantly less than the than the control group ($p<0.05$). WSP only exposure did not affect motility of flies compared to control.

Discussion

PD is a common, chronic and progressive motor disorder effecting millions of people worldwide (Han et al. 2012). Its symptoms are characteristic of motor control deficits or decreased voluntary movement control (Wang et al. 2018). There is currently no definitive cause and no accepted cure for PD. This study and studies like it attempt to find a definitive cause or methods to reverse the symptoms of PD. In this experiment drosophila were used to model PD by using rotenone to induce Parkinsonian disease like symptoms. Rotenone is a naturally occurring pesticide that negatively effects the activity of mitochondrial complex-1 causing oxidative damage and endoplasmic reticulum stress that is phenotypically similar to Parkinson's Disease in drosophila (Manjunath 2013). We then attempted to use WSP, an Indian herb implicated for its therapeutic properties (Manjuath 2013, as a remedy to the progression of the disease. In the experiment we tested for four things: rotenone's ability to produce PD like symptoms in drosophila compared to normal drosophila; WSP effect on normal drosophila compared to normal drosophila; WSP effect on flies exposed to rotenone compared rotenone only exposed flies; and WSP effect on flies exposed to rotenone compared to normal flies. We hypothesized rotenone would produce PD like symptoms and decrease voluntary movement control compared to normal flies. We hypothesized flies treated with WSP only to behave similar to normal flies. Lastly, we hypothesized that flies exposed to rotenone and treated with WSP would show increased voluntary movement control compared to flies exposed to rotenone only and similar voluntary movement control as the control flies.

Our result showed a significant decrease in motor control of the flies that were exposed to rotenone compared to the control group, a much lower percentage of flies crossed the threshold in the

rotenone exposed group compared to the control group. This supports our hypothesis and suggests that rotenone does cause neuronal cell damage and can induce phenotypically similar symptoms to PD. They show a non-significant increase in flies that cross the threshold in the WSP only group compared to control. This also supported our hypothesis and suggest that there is no significant, and more importantly no negative, impact on motility of flies due to WSP. The results did not support our final hypotheses because flies exposed to rotenone and treated with WSP did not show a significant increase in voluntary movement control compared to flies exposed to rotenone only and did not show motility similar to the flies in the control group. This suggest that WSP is not an acceptable remedy for the symptoms of PD like symptoms in drosophila and cannot reverse the effects of the damages of rotenone. These results are consistent with other research in this field by using oxidative damage as a mechanism for inducing PD like symptoms as well as remedies being insignificant in solving the problem.

Although our results did not show the significant differences that we wanted in the experimental groups, they did show insignificant differences that could be the correct path to follow for future experimenters. Flies exposed to rotenone and treated WSP showed a minor, insignificant increased in percentage of flies that crossed the threshold compared to flies exposed to rotenone only. This could mean that WSP is helping the symptoms of PD but at a small insignificant rate. One of the limitations of this experiment is that we only used one consistent amount of WSP (0.05%). It could be possible that WSP does help battle the progression of PD but that it is dosage dependent, meaning that if there was more WSP that it could decrease the progression or reverse the effects of PD. Another limitation is that WSP was only administered to the flies one time in one week, instead of multiple times over a long period of time. The WSP could have been helping battle the progression of PD but was depleted and just needed another dose of the drug. These are experiments that can be done in the future that will help understand the progression and mechanism of Parkinson's disease.

In this study our goal of finding an acceptable cure or the definitive mechanism to PD was not achieved, the gap in knowledge of this field is still wide open. But the path to follow and targets to study

to achieve that goal could be known. Oxidative damage which can cause multiple lesions in macromolecules leading to their damage and aging is believed to be the mechanism for rotenone which phenotypically mimics PD (Ryu et al. 2002). Lewy bodies, insoluble aggregations which mainly consist of α-synuclein, is a major hallmark of this disease (Bellucci 2016). This means oxidative damage and Lewy bodies could be related when it come to the progression of PD. Reactive oxygen species (ROS) are normally produced in the cell during the mitochondrial electron transfer chain or redox reactions (Puspita 2017). This suggests that ROS from the ETC could be the cause of the oxidative damage that presents itself as PD. With knowing these things along with the findings from this study there are many ways future experimenters can follow up this experiment to further our understanding of the mechanism of Parkinson's disease or finding a cure. One is replicating this experiment and changing this dosage of the WSP, rather it is changing the amount or the frequency. Another is determining the effects of WSP or any antioxidizing agent on the amount of Lewy bodies or ROS found in the body of a model with PD like symptoms. Or lastly the effects of ROS created in the ETC on the amount of Lewy bodies or α-synuclein found in the body of a model of PD. We currently have implicated a system, cell type, organelle and a protein in the progression of PD, although this study did not completely close that gap in knowledge needed to find a cure or reverse this ailment it did possibly shed some light on the mechanism which could potentially lead to the correct experiment to find the actual cure.

Work Cited

Bellucci A, Mercuri N, Venneri, et al.(2016) Parkinson's disease: from synaptic loss to connectome dysfunction. Neuropathol Appl Neurobiol. 42:77–94

Coulom H, Birman S, (2004). Chronic Exposure to Rotenone Models Sporadic Parkinson's Disease in Drosophila melanogaster. The Journal of Neuroscience 24(48):10993–10998

Han M, Nagele E, DeMarshall C, et al., (2012) "Diagnosis of Parkinson's disease based on disease-specific autoantibody profiles in human sera,". PLoS One, vol. 7, article e32383.

Harman D, (1994) Free-radical theory of aging: increasing the functional life span, Ann. N. Y. Acad. Sci. 717 (1994) 257–266.

Hornykiewicz O (2006) The discovery of dopamine deficiency in the parkinsonian brain. J Neural Transm Suppl. pp 9–15.

Kasture A, Hummel T, Sucic S, et al., (2018) Big Lessons from Tiny Flies: Drosophila melanogaster as a Model to Explore Dysfunction of Dopaminergic and Serotonergic Neurotransmitter Systems. Int. J. Mol. Sci. 19,1788

Manjunath M, Muralidhara, (2013) Standardized extract of Withanina somnifera (Ashwagandha) markedly offsets rotenone-induced locomotor deficits, oxidative impairments and neurotoxicity in Drosophila melanogaster. J Food Sci Technol 52(4):1971-1981

Puspita L, Chung S, Shim J (2017) Oxidative stress and cellular pathologies in Parkinson's disease. Molecular Brain 10:53

Ryu E, Harding H, Angelastro J, et al. (2002) Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci 22:10690 –10698

Wang Y, Li Y, Zhang X, et al. (2018) Apathy following Bilateral Deep Brain Stimulation of Subthalamic Nucleus in Parkinson's Disease: A Meta-Analysis. Hindawi 2018:9756468

Zhao J, Nakamura N, Hattori M, et al. (2002) Withanolide Derivatives from the Roots of Withania somnifera and Their Neurite Outgrowth Activities. Chem. Pharm. Bull. 50(6) 760—765 (2002)