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Development of novel therapies targeting biochemical mechanisms to halt progression of Alzheimer's and Parkinson's Disease

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By

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Development of novel therapies targeting biochemical mechanisms to halt progression of Alzheimer's and Parkinson's Disease

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DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

This thesis incorporates the outcome of joint research efforts undertaken in collaboration with Kyle Stokes, Dennis Ma, Darcy Wear, Simon Pupulin, Lauren Culmone, Rachel Huggard, Iva Okaj, Suzie Eren, Gabrielle Walach, Hasana Jayawardena, Subidsa Srikantha, Mathew Gagnon, Sidhartha Ray, Arun Rishi, and Jerome Cohen under the supervision of Dr. Siyaram Pandey. In all cases, experimental design, execution, data analysis, interpretation, and manuscript preparation were performed by the author. All authors have read and approved the final manuscript prior to submission.

Collaboration with Kyle Stokes, Dennis Ma, Darcy Wear, Jerome Cohen, and Sidhartha Ray is covered in Chapter 1 of this dissertation; there was partial contribution to information acquisition and interpretation.

Collaboration with Darcy Wear, Iva Okaj, Rachel Huggard, Lauren Culmone, Suzie Eren, Jerome Cohen, and Arun Rishi is covered in Chapter 2 of this dissertation; there was partial contribution to experimental execution and data interpretation.

Collaboration with Darcy Wear, Gabrielle Walach, Hasana Jayawardena, Suzie Eren, and Jerome Cohen is covered in Chapter 3 of this thesis; there was partial contribution to experimental execution.

Collaboration with Simon Pupulin, Darcy Wear, Lauren Culmone, Rachel Huggard, Kyle Stokes, and Dennis Ma is covered in Chapter and 4 of this dissertation; there was partial contribution to experimental execution, and data interpretation.

Collaboration with Darcy Wear, Lauren Culmone, Iva Okaj, Rachel Huggard, Hasana Jayawardena, Gabrielle Walach, Subidsa Srikantha, Mathew Gagnon, Suzie Eren, and

Jerome Cohen is covered in Chapter 5 of this dissertation; there was partial contribution to experimental execution.

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This thesis includes 3 original papers that have been previously submitted to journals for publication and two manuscripts prepared for submission, as follows:

Thesis Chapter	Publication title	Publication status
Chapter 1	*A Birds eye view of the multiple biochemical mechanisms that propel pathology of Alzheimer's Disease: Recent Advances and Mechanistic Perspectives on how to halt the disease progression targeting multiple pathways*	*Published*
Chapter 2	*Combined Ubisol-Q10 and Ashwagandha Root Extract Target Multiple Biochemical Mechanisms and Halt Neurodegeneration in a Paraquat Induced Rat Model of Parkinson's Disease*	*Published*
Chapter 3	*Anti-inflammatory and neuroprotective effects of a novel water-soluble formulation of coenzyme-Q10 and ashwagandha root extract in a paraquat induced rat model of Parkinson's Disease*	*Manuscript ready for submission*

Chapter 4	*Resumption of autophagy by Ubisol-Q10 in Presenilin-1 Mutated Fibroblasts and Transgenic AD Mice: Implications for Inhibition of Senescence and Neuroprotection*	*Published*
Chapter 5	*Investigation into efficacy and mechanisms of neuroprotection of ashwagandha root extract and Ubisol-Q10 in an aged transgenic mouse model of Alzheimer's Disease*	*Manuscript ready for submission*

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ABSTRACT

Alzheimer's Disease (AD) and Parkinson's Disease (PD) are the two most common and devastating neurodegenerative diseases in the world. There is no cure for either disease and current treatments provide only symptomatic relief. Furthermore, current treatments for AD and PD have shown to exhibit adverse side effects when taken over long periods of time. With age being the greatest risk factor for developing AD and PD, the increase in the aging population has the potential to lead to a serious medical/economic crisis due to the amount of time and care required for patients with AD and PD. The exact aetiologias of PD and AD is unknown but pathological features of these AD and PD respectively include: formation of neuritic plaques and neurofibrillary tangles, and neuron loss in the hippocampus leading to memory impairment; loss of dopaminergic neurons in the substantia nigra (SN) leading to movement impairment. While the targets of AD and PD are different, the end result of both lead to morbidity and eventually death. We have found from previous research, there are several biochemical mechanisms shared between AD and PD which include the following: 1) increased oxidative stress; 2) impaired autophagy/proteasome activity and accumulation of defective proteins; 3) mitochondrial dysfunction; 4) inflammatory modulation of microglia/astroglia; 5) accumulation of senescent cells. As a result of these multiple mechanisms, AD and PD should be considered a multifactorial disease and targeting only one of the mechanisms may prove unsuccessful in treating the diseases. Furthermore, many of the therapies used to treat symptoms of AD and PD are chemo-modulators and extended use has shown to have toxic and adverse psychological side effects. Therefore, in order to successfully treat these two neurodegenerative diseases, a multifaceted approach using natural health products that are well tolerated would be preferable. Prior research has shown the neuroprotective effects of Ubisol-Q10, a water-soluble formulation of coenzyme-Q10, and extracts of ashwagandha root, a plant used in Ayurveda (traditional Indian school of medicine). Previously, Ubisol-Q10 has been demonstrated to target oxidative stress, mitochondrial dysfunction, resume autophagy, and prevent cell senescence in in-vitro and in-vivo models of AD and PD. Ashwagandha extracts have shown to possess potent anti-inflammatory properties, prevent apoptosis of dopaminergic neurons in PD rodent models, and eliminated beta-amyloid plaques and improved memory in the brains of transgenic AD mice.

This work sought to investigate the therapeutic potential of combined Ubisol-Q10 and ashwagandha root extract to target the biochemical mechanisms of AD and PD in-vitro and in-vivo. Here Ubisol-Q10 and ashwagandha extract were combined for the first time to investigate whether these two well-tolerated natural health products are more efficacious when administered together compared to the agents alone at targeting AD and PD biochemical mechanisms. Dopaminergic neurons were better protected and motility improved with the combination of Ubisol-Q10 and ethanolic extract of ashwagandha root (compared to either agent alone) in a paraquat induced rat model of Parkinson's Disease. This was due to the agents working together to target all biochemical mechanism of PD Ubisol-Q10 was shown to reduce oxidative stress and enhance autophagy in the SN of paraquat injected rats. Meanwhile, ashwagandha extract acted as a potent anti-inflammatory by reducing activation of pro-inflammatory microglia and increasing activation of pro-survival/supporting astroglia. Increases in active astroglia coincided with an increased expression of pro-survival neurotrophic factors. Also, Ubisol-Q10 and ashwagandha prevented cell senescence in brains of paraquat injected rats. Efficacy of ashwagandha extract was further improved by using the same technology used in Ubisol-Q10 to increase solubility leading to better absorption and bioavailability. In Alzheimer's Diseased fibroblasts and double transgenic AD mice, Ubisol-Q10 reduced oxidative stress and enhanced expression of autophagy. Autophagy was shown to be essential at preventing stress induced premature senescence (SIPS) in these fibroblasts as SIPS returned when autophagy was inhibited using small molecule autophagy inhibitor SP600125. Ubisol-Q10 and ashwagandha extract were more effective at preventing the development of beta-amyloid plaques in the brains double transgenic mice compared to the agents alone. This was likely due to the combined agents targeting all biochemical mechanisms of AD. Ubisol-Q10 reduced oxidative stress and autophagy impairment in double transgenic AD mice brains. Ashwagandha extract reduced activation of pro-inflammatory microglia and increased activation of pro-survival astroglia which coincided with increased expression of pro-survival brain derived neurotrophic factor. Since Ubisol-Q10 and ashwagandha are simple nutraceuticals and GRAS-approved, they can also be taken over long periods of time without serious side effects and could prove to be a promising therapy for AD and PD that could halt neurodegeneration and improve quality of life.

DEDICATION

I dedicate this work to my grandparents Kaye and Ernie. You are the kindest and most honest people in the entire world.

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 2

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF APPENDICES

CHAPTER 1: GENERAL INTRODUCTION

List of Abbreviations

ETC	Electron Transport Chain
ATP	Adenosine Triphosphate
RNA	Ribonucleic Acid
MFF	Mitochondrial Fission Factor
MMP	Mitochondrial Membrane Potential
ER	Endoplasmic Reticulum
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Species
OXPHOS	Oxidative Phosphorylation
AD	Alzheimer's Disease
PD	Parkinson's Disease
DNA	Deoxyribonucleic Acid
mtDNA	Mitochondrial DNA
MOMP	Mitochondrial Membrane Permeabilization
SASP	Senescence Associated Secretory Phenotype
NFT	Neurofibrillary Tangle
APP	Amyloid Precursor Protein
PS-1	Presenilin-1

PS-2	Presenilin-2
AβPP	amyloid-Beta Protein Precursor
Aβ	Amyloid-Beta
DA	Dopaminergic
SN	Substantia Nigra
SNCA	Synuclein Alpha
PARK7	Parkinson Disease Protein 7
PRKN	Parkin
LRKK2	Leucine Rich Repeat Kinase 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDAR	N-methyl-d-aspartate Receptor
MAOI	Monoamine Oxidase Inhibitor
COMT	Catechol-O-methyltransferase
DBS	Deep Brain Stimulation
PTS	Polyoxyethanyl-α-tocopheryl Sebacate
LRP	Low Density Lipoprotein Receptor-related Protein
NEP	Neprilysin
SIPS	Stress Induced Premature Senescence
qPCR	Quantitative Polymerase Chain Reaction
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
H2DCFDA	2′-7′-Dichlorofluorescein Diacetate

Neurons and the importance of mitochondria

Neurons consume the highest amount of oxygen, depend on oxidative metabolism for energy, and survive for the lifetime of an individual. As a result, neurons are vulnerable to death caused by oxidative stress, accumulation of damaged/dysfunctional proteins and organelles. Therefore, it's imperative that we protect these neurons as we age. Due to neuronal reliance on oxidative phosphorylation for energy, they depend heavily on the mitochondrial electron transport chain (ETC) and require large amounts of adenosine triphosphate (ATP) for their survival [1,2].

While neuronal development is underway, mitochondria undergo maturation and morphological changes indicative of a change over from glycolysis to oxidative phosphorylation for energy production [3]. This is supported through RNA expression profiling which demonstrated a downregulation of glycolysis-related genes and the associated upregulation of oxidative-phosphorylation-related genes [4]. As previously mentioned, this switch to oxidative phosphorylation is crucial for neuronal survival due to their significant energy demands. Furthermore, the stimulation of neurogenesis from neural stem cells is significantly influenced by metabolic changes to the mitochondrion. Neuronal process outgrowth in later development through dendritogenesis and axonogenesis is heavily influenced by mitochondrial anchoring, fusion and fission, as well as their transport along microtubules [5]. Experiments have demonstrated that manipulation of mitochondrial anchoring protein syntaphilin or the LKB1-NUAK1 kinase pathways which regulate mitochondrial motility heavily influence axonal branching. Disruptions of syntaphilin or the kinase pathway lead to increased mitochondrial motility and an associated decrease in axonal branching both in-vitro and in-vivo [6].

Following their development, neurons require a continuous ATP supply to maintain functionality and plasticity. Cytosolic ion compositions can vary due to opening and closing of ion channels, particularly during neurotransmission. To maintain homeostasis, neurons utilize large amounts of ATP to pump these ions into intracellular organelles, such as the mitochondrion, or out of the cell completely [7]. The significant energy costs of neurotransmission and synaptic function further highlight mitochondrial importance in neurons. Additionally, synaptic plasticity requires local translation of essential proteins

which is fueled by the ATP provided from nearby dendritic mitochondrial compartments [8]. Experimentation involving the manipulation of Mitochondrial Fission Factor (MFF) demonstrated its importance in axonal mitochondria size and its lack of influence on dendritic mitochondrial size [10]. Although an increased mitochondrial size had no influence on the mitochondrial membrane potential (MMP) or ATP levels, it did result in enhanced calcium buffering in the presynaptic neuron via an inner mitochondria membrane calcium uniporter. Post-neuronal firing mitochondrial calcium entry (calcium buffering) reduced neurotransmitter release via inhibition of synaptic vesicle fusion at mitochondria-containing presynaptic regions [9,10]. These results signify the importance of axonal mitochondrial positioning on neurotransmission and plasticity.

Along with the endoplasmic reticulum (ER), the mitochondrion is a key factor in lipid biosynthesis for all cells, including neurons. Sphingolipids are largely localized to the plasma membrane playing both a structural and functional role through the potential formation of lipid rafts [11]. These rafts serve as major anchoring points for proteins to interact with the cell. Despite most sphingolipid synthesis occurring at the ER, a mitochondrial ceramide production pathway has been demonstrated in both yeast and mammalian cells suggesting the mitochondria may play an important role in both plasma membrane structure and lipid raft formation [12,13]. Various phospholipids including phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin undergo complete or partial-synthesis on the inner mitochondrial membrane [14,15]. PE is a polyunsaturated fatty acid (PUFA) that influences neuronal membrane fluidity, neurotransmission, neuroinflammation, and overall cellular survival [16,17]. PG and cardiolipin serve to maintain both plasma and mitochondrial membrane structure and curvature, aiding in the functionality of various enzymes and the ETC [18]. The significant contributions made by lipids to neuronal structure and function are made possible by mitochondrial modifications and processes which aid in lipid synthesis.

Despite the plethora of significant contributions that mitochondria provide for neuronal survival and functionality, they can also have detrimental effects when working inefficiently. Due to deficiencies in the mitochondrion, an increased turnover rate for the citric acid cycle and ETC can result in the production of reactive oxygen species (ROS)

and nitric oxide [19]. Nitric oxide proceeds to interfere with complex 4 of the ETC while the oxidative stress from accumulated ROS further damages mitochondria [19,20]. As defective mitochondria begin to accumulate, improper fission results in organelle enlargement and cellular hypertrophy leading to deficiencies in nutrient transport and potential cellular starvation [20].

The entanglement of mitochondrial dysfunction, oxidative stress, autophagy inhibition, senescence, and inflammation in neurodegeneration of Alzheimer's and Parkinson's Disease

As mentioned earlier, neurons almost exclusively rely on oxidative phosphorylation (OXPHOS) for energy production which is the important metabolic process in mitochondria that utilizes enzymes to oxidize nutrients to release energy which is used to drive ATP synthesis [21]. Therefore, neurons are especially susceptible to perturbations of mitochondria function [22]. Indeed, mitochondrial dysfunction is known to be involved in neurodegenerative diseases including Alzheimer's Disease (AD) and Parkinson's Disease (PD) [23,24]. Not only are mitochondria involved in producing energy, but they are also critically involved in apoptosis, senescence, and autophagy [25]. As a result, changes in mitochondria functionality can have dubious effects on the activity of apoptosis, senescence, and autophagy.

Mitochondria are major sources of reactive oxygen species (ROS) which are by-products of OXPHOS [26]. Increased oxidative stress can lead to significant DNA, protein, and lipid damage throughout the cell. Oxidative stress can also lead to mitochondria dysfunction as a results of ROS damage to mitochondrial OXPHOS components, membrane lipids, and mtDNA damage. This can result in a feedback loop of increased ROS and increased mitochondrial dysfunction [27]. There are several mechanisms cells can counteract aberrant mitochondrial function. In the case that too much damage has occurred within the cell, the cell can self-destruct via apoptosis. Apoptosis often begins at the mitochondrion involving Bax/Bak proteins or activation of caspases leading to mitochondrial outer membrane permeabilization (MOMP) [28]. MOMP is often the point

of no return for cells undergoing apoptosis as it damages mitochondria function and activates a proteolytic cascade of pro-apoptotic proteins [29].

Cells have developed important mechanisms to eliminate damaged mitochondria in a process known as mitophagy. Mitophagy is a type of macroautophagy in which cells use autophagy/proteasome degradation systems to remove dysfunctional mitochondria [30]. While in some situations autophagy is known to cause cell death when apoptosis is inhibited, lack of autophagic activity has been shown to activate apoptosis due to increased cell stress from damaged proteins and organelles [25]. While mitophagy seems like a full proof way to remove defective mitochondria, issues can still arise preventing degradation. It's been shown that elevated ROS can disrupt machinery required for regulating autophagy/mitophagy leading to accumulation of defective mitochondria [24,31]. Furthermore, mutations associated with AD and PD have also been observed to disrupt mitophagy/autophagy [23,24]. Another response to mitochondrial induced cell stress is cellular senescence. Senescence refers to a state of permanent cell division arrest characterized by insensitivity to growth factors and mitogens [32]. Impaired mitochondrial function stressors such as excessive ROS production and impaired mitochondrial dynamics can lead to activation of tumor suppressor pathways such as p53/21 leading to senescence [33,34,35]. While senescence can be beneficial in the case of preventing tumorigenesis, it can have adverse effects with regards to neurodegenerative diseases. Studies have shown that build-up of senescent cells can lead to increased inflammation due to the senescence associated secretory phenotype (SASP) which can result in excessive secretion of pro-inflammatory cytokines [36]. In AD and PD, accumulation of SASP expressing glial cells have shown to cause damage to neighbouring neurons leading to apoptosis [37,38].

Overall, mitochondria are critical organelles in maintaining neuronal health. In the case of AD and PD, perturbations to mitochondria leading to dysfunction can lead to detrimental effects on neurons such as apoptosis, impaired autophagy leading to accumulation of cytotoxic damaged proteins/organelles, and accumulation of damaging pro-inflammatory senescent cells. Furthermore, these cellular processes are intricately intertwined, and therefore any perturbations in one can affect the other.

Alzheimer's Disease overview

Alzheimer's Disease is a debilitating disease which can be characterized by severe memory loss and confusion and can lead to stark changes in mood and personality which may lead to depression [39]. This disease presumably makes up an estimated 75-90% of all dementia cases in Canada [39]. It has been projected that by the year 2030, the number of dementia patients is expected to double. With this in mind, it is imperative that effective agents are developed, and new therapeutic strategies are employed to combat the progression and devastating outcomes (or aftermath of) AD. The etiology of this disease is not well understood but is distinguished by the presence of neurofibrillary tangles (NFTs) and amyloid-beta plaques in the brain which lead to loss of synapses and death of neurons found in the hippocampus and cerebral cortex. Furthermore, the volume of these brain regions are reduced in size as seen post-mortem samples. The lesions seen in these areas seem to precede the clinical manifestations of the disease [40]. There are many risk factors associated with the progression of this disease, most being sporadic in origin and are related to both external factors and genetic predispositions [41]. It has been postulated that genetic factors may be contributing to familial type AD, but only make up approximately 1% of all cases. There are three autosomal dominant genes associated with familial forms of AD, including genes coding for: amyloid precursor protein (APP), presenilin-1 (PS-1), and presenilin-2 (PS-2) [42].

The APP gene codes for amyloid-beta protein precursor (AβPP), is proteolytically cleaved, it yields the amyloid-beta (Aβ) protein [43]. In the absence of any mutation in the APP gene, the amyloid-beta protein functions without adverse effects and may be a regulator in synapse formation [4]. Several other studies have suggested possible functions of Aβ including reduction of oxidative stress and a pro-inflammatory response during microbial invasion, although further validation is required [44]. The mutated protein ($A\beta_{40}$ and $A\beta_{42}$) arises through missense mutations, most of which are located in the secretase cleavage sites or the AβPP transmembrane domain [42]. Mutant Aβ is able to form aggregates/oligomers that are translocated inside the membrane of the mitochondria leading to mitochondrial dysfunction and oxidative stress (Figure 1) [45]. The 32 discovered mutations in these areas are responsible for 10–15% of early onset familial AD

[46]. PS-1-related AD is the most common form of familial AD and is responsible for 20–70% of cases [46]. The PS-1 gene produces a major component in atypical aspartyl protease complexes that forms the catalytic core of the membrane bound **γ**-secretase. This **γ**-secretase in turn cleaves AβPP [42, 47] and is found in small amounts in the endoplasmic reticulum, Golgi, and mitochondrial membranes [47]. Missense mutations found along one of the 12 exons on this gene were found to produce amino acid substitutions which translate to an increase in the $A\beta_{42}$ isoform and impaired autophagy [42,48].

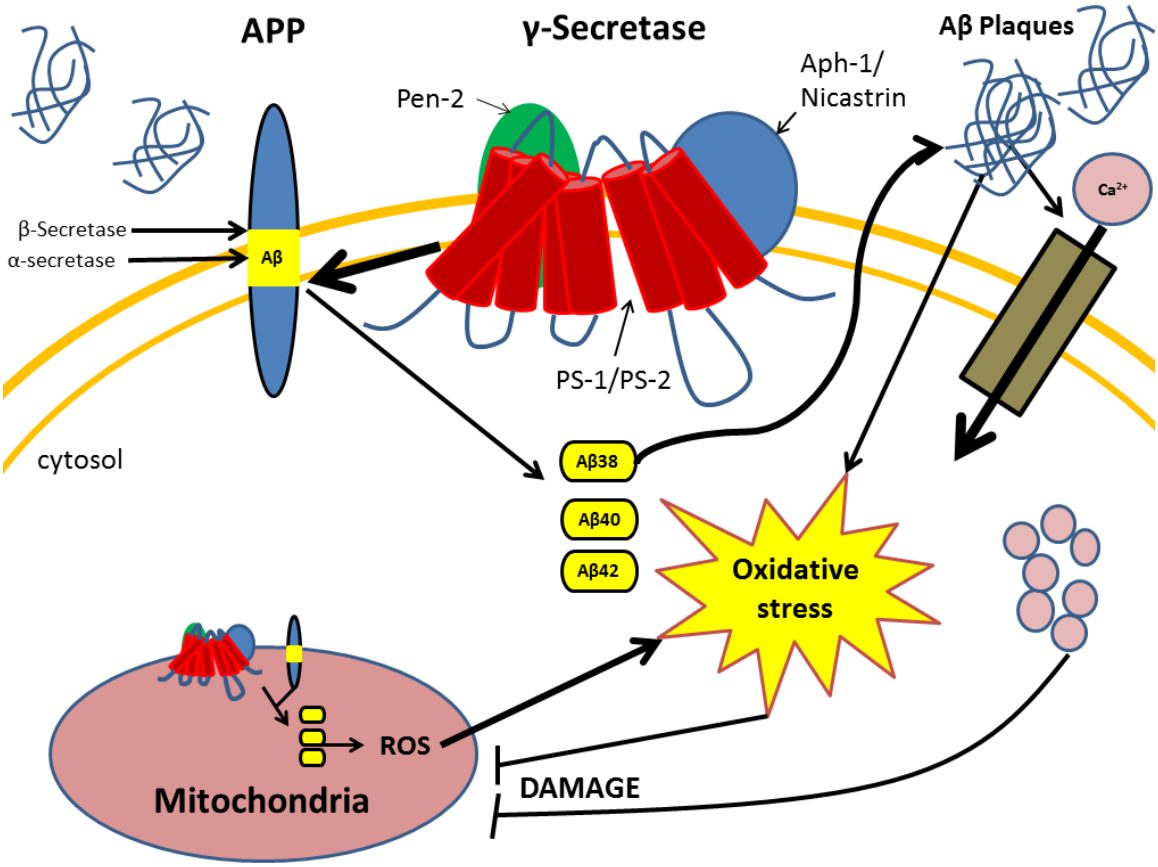

Figure 1. Presenilin-1 (PS-1) mutation leads to several changes in the cell due to improper cleavage of the amyloid precursor protein (APP) protein. PS-1 mutation causes the improper cleavage of APP leading to aberrant forms of amyloid beta (Aβ) which occurs in both the cell and mitochondrial membrane. Accumulation of aberrant Aβ proteins form Aβ plaques which cause increased intracellular oxidative stress and influx of calcium ion. Both events lead to mitochondrial dysfunction which in turn increases oxidative stress and cellular damage.

Parkinson's Disease overview

Parkinson's disease (PD) is a debilitating neurodegenerative disease characterized by loss of dopaminergic (DA) neurons in the substantia nigra (SN) region of the basal ganglia. The disease initially displays loss of movement coordination (resting tremors, postural instability, bradykinesia, and rigidity), which then progresses to cognitive impairments, psychiatric irregularity, and eventually morbidity and death [49,50]. While there are several genetic/familial factors (i.e., mutations in SNCA, PARK7, LRRK2, and PRKN genes) and environmental factors linked to PD, most PD cases are sporadic and caused by unknown factors [51,52]. SNCA (synuclein alpha) codes for the protein α-synuclein which regulates synaptic vesicle release and neurotransmitter release. When mutated, α-synuclein has been shown to accumulate into abnormal aggregate known as Lewy Bodies which have been shown to behave similar to cytotoxic amyloid-beta aggregates as mentioned prior [53]. PARK7 (Parkinson disease protein 7) codes for protein deglycase DJ-1 which is involved in protecting neurons from oxidative stress and aggregation α-synuclein [54]. PRKN (parkin) encodes an E3 ubiquitin ligase which plays a critical role in ubiquitination and when mutated in familial PD, results in accumulation of defective mitochondria and α-synuclein [55]. LRRK2 (leucine rich repeat kinase 2) encodes for multifunctional kinase dardarin which is involved in regulating intracellular signalling and cytoskeleton assembly, and when mutated in PD causes DA neuron apoptosis [56].

It has been demonstrated in various in-vitro and in-vivo models of PD that toxins structured similarly to dopamine such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (a synthetic heroin by-product) and paraquat/maneb/rotenone (various herbicides and pesticides) are preferentially taken up by DA neurons. These toxins then significantly elevate reactive oxygen species (ROS) when metabolized, leading to apoptosis. Furthermore, these toxins have also been implicated in the development of PD in humans [57–66]. As mentioned earlier, along with oxidative stress, there are several other biochemical mechanisms involved in PD, which include mitochondrial dysfunction, and inhibition of autophagy leading to the buildup of defective proteins and organelles, and neuroinflammation [27,52,67,68].

Importance of microglia and astroglia in Alzheimer's and Parkinson's Disease

Certain glial cells have been implicated with neuroinflammation. In particular astrocytes and microglia play a major role in inflammation with AD. Microglia become activated in the presence of β-amyloid plaques in a process known as microgliosis. During microgliosis, microglia change from their ramified (inactive) state to their ameboid (active) state [69]. In this active/ameboid state, microglia release various inflammatory cytokines which can cause the surrounding neurons around the β-amyloid plaques to become stressed and potentially undergo apoptosis [70].

Unlike microglia, astrocytes typically provide a supporting role to neurons. Astrocytes are involved in providing metabolic support, supporting synaptic connections, neurotrophic factor secretion, and even preventing microglia activation among other support roles [71-73]. In their inactive state, astrocytes have few processes extending from the cell body while active (reactive) astrocytes have a significant amount of branching of cytoplasmic processes extending from the cell body [74]. In transgenic AD mice (containing APP/PS-1 mutations), the amount of reactive (activated) astrocytes were reduced compared to wild-type mice. Furthermore, microglia and astrocytes are known communicate with each other and that they can either activate or inactivate each other [75].

Increasing evidence has also demonstrated the importance of neuroinflammatory responses in PD. Indeed, activated microglia were found in the brains of PD patients post-mortem [76–79]. It is thought that microglia are activated in earlier stages of PD alongside DA neuron loss, leading to apoptosis due to oxidative stress [77]. Contrary to microglia activation in PD, absence of astroglia activation/number has been implicated in PD pathology [78,80,81]. With reduced activation/numbers of protective astroglia and increased activation of microglia in PD, DA neurons are significantly more susceptible to neuroinflammation.

Therapies for Alzheimer's and Parkinson's Disease

Current treatments for AD, such as cholinesterase inhibitors, glutamate regulators, and Aβ-targeting monoclonal antibodies are designed to soften the symptoms of this age-related disease but do not halt its progression and prolonged used has shown to have negative side-effects [82]. Cholinesterase inhibitors work by preventing the breakdown of acetylcholine (a major neurotransmitter involved in the hippocampus) causing accumulation in synapses resulting in increased excitation and therefore reducing the memory impairing effects cholinergic neuron loss in the hippocampus [83]. Glutamate regulators functions by preventing glutamate excitotoxicity by inhibiting excessive extrasynaptic N-methyl-d-aspartate receptor (NMDAR) activity which has been implicated in AD and cause cell death [84]. While great strides have been made in understanding the pathologies of AD, there have been many failed attempts at developing effective disease modifying therapies for AD. A multitude of therapies have been developed that target various different aspects of AD such as immunotherapy, gamma/beta-secretase inhibitors, Aβ aggregation inhibitors, and alpha-secretase activators [85]. Many of these types of therapeutics are either undergoing clinical trials or have failed due to various reasons such as toxicity or lack of efficacy. Some drugs that target production of Aβ such as beta-secretase inhibitors were found to accelerate cognitive decline, possibly due to off-target effects [85]. Furthermore, non-mutated Aβ and enzymes associated with it still have a physiological role in facilitating neuronal function and therefore targeting them may have adverse side effects. Another possible reason that many clinical trials have failed may be due to many of them only targeting a single pathology such as Aβ. It is critical to remember that AD should be considered a multifactorial disease and targeting only a single pathology of AD may not be enough to halt the progression of the disease.

Conventional treatments for PD include dopamine replacement therapy, dopamine agonists, monoamine oxidase inhibitors (MAOIs), catechol-O-methyltransferase (COMT) inhibitors and deep brain stimulation (DBS) [86-89]. Dopamine replacement serves to increase overall amount of dopamine in the brain therefore increasing activation of dopaminergic neuron signal transduction [86]. MAOIs and COMT inhibitors prevent the metabolism of dopamine and are often administered in conjunction with dopamine

replacement therapy [87,88]. DBS involves the use of surgically implanted electrodes implanted deep in the brain at the subthalamic nucleus (a component of the basal ganglia). These electrodes send constant electrical impulses which modify subthalamic neuron activity and reduce involuntary Parkinsonian tremors [89]. Unfortunately, similar to AD, these therapies only provide symptomatic relieve and fail to halt progression of neurodegeneration and constant use has shown to have negative neurological side effects. There have been attempts at developing disease modifying drugs such as LRRK2 inhibitors, α-synuclein synthesis/aggregation inhibitors. Though similar to AD, issues have arisen due to off-target effects as well as affecting non-mutant versions of enzyme/proteins that still serve critical functions in neuron health [90].

With the above information in mind, and the increasing number of people entering their later years in life, it is imperative that precise therapeutics targeted at preventing onset (or even preventing further neurodegeneration at time of diagnosis to improve quality of life) of AD and PD are investigated and developed. AD and PD should be considered multifactorial diseases and therefore only targeting one or a few parts of the disease isn't enough to halt its progression. Along with the typical pathologies of AD and PD, there exist several shared biochemical mechanisms that are intricately intertwined as mentioned before which include: increased oxidative stress, mitochondrial dysfunction, autophagy inhibition, neuroinflammatory modulation of microglia and astroglia, and accumulation of senescence cells. If possible, it would be ideal if a well-tolerated treatment comprising of one or a combination of agents that work together to target most/all biochemical mechanisms of AD and PD could be used to prevent neurodegeneration in these diseases.

Therapeutic potential of Ubisol-Q10 and ashwagandha in Alzheimer's and Parkinson's Disease

Ubisol-Q10 is a water-soluble formulation of coenzyme-Q10 made using the amphipathic molecule polyoxyethanyl-α-tocopheryl sebacate (PTS) [91]. Ubisol-Q10 has been shown to display unprecedented neuroprotective properties in various models of PD. While previous preclinical studies with normal coenzyme-Q10 showed therapeutic efficacy at oral doses of 1600 mg/kg/day, a clinical trial [92] that had much lower required

doses failed to obtain effective results. When combined with PTS, doses of coenzyme-Q10 were able to be reduced significantly to 6 mg/kg/day. Ubisol-Q10 has been shown to target mitochondrial dysfunction and oxidative stress in in-vitro and in-vivo models of PD [66,93–96]. Furthermore, Ubisol-Q10 prevented oxidative-stress-induced premature senescence and prevented impaired autophagy in PS-1 mutated fibroblasts, while also improving memory and removing beta-amyloid plaques in a transgenic mouse model of AD (mice with APP/PS-1 mutations) [22,97,98].

Ashwagandha (*Withania somnifera*) is a plant of the nightshade family that has been used in Ayurveda (a traditional Indian school of medicine) as a nerve tonic for general debility, nervous exhaustion, insomnia, and memory impairment [99]. Past studies showed that various extracts of ashwagandha root were able to target oxidative stress and neuroinflammation in models of AD and PD [99–102]. Furthermore, a unique mechanism was observed with ashwagandha treatment in AD transgenic mice. In AD mice, low density lipoprotein receptor-related protein (LRP) expression and amyloid-beta degrading protease neprilysin (NEP) expression was enhanced in brain micro-vessels resulting in clearance of amyloid-beta plaques [102]. Liver LRP and NEP expression was also enhanced resulting in increases of plasma LRP which acted as a beta-amyloid sink when removed from the brain. The protective compounds of ashwagandha are thought to be steroidal lactones and saponins (withanolides and sitoinosides, respectively). These key components have been shown to act as antioxidants, aid in axonal regeneration, and target certain activators of inflammation [99]. Though it should be noted that studies measuring ashwagandha's efficacy alone using orally administered doses were too high for human therapeutic development.

While Ubisol-Q10 or ashwagandha seem like promising therapeutics, they do not target all biochemical mechanisms of AD and PD when used alone. Discussed prior, AD and PD should be considered multifactorial diseases, and targeting only one or a few of the biochemical mechanisms will not be enough to stop the progression of neurodegeneration. By combining Ubisol-Q10 with ashwagandha, a therapeutic more effective at preventing AD/PD neurodegeneration could be developed as they could target all proposed biochemical etiologies of the diseases (mitochondrial dysfunction, oxidative stress,

autophagy inhibition, inflammatory modulation of glial cells, and senescent cell accumulation). Furthermore, ashwagandha might exercise a synergistically enhanced efficacy at a much-lowered dose when combined with an agent such as Ubisol-Q10 that could permit further human clinical trials.

Objectives

Parkinson's Disease

Project 1: Investigation of the Neuroprotective Efficacy and Mechanism of Ubisol-Q10 and Ethanolic Ashwagandha Root Extract (E-ASH) in a Paraquat Induced Rat Model of Parkinson's Disease: The neuroprotective efficacy of Ubisol-Q10 and ashwagandha combined will be investigated using a PQ induced rat model of PD. Ubisol-Q10 will be sourced from Next™ Remedies Inc. While E-ASH will be made in-house using a standardized protocol. Previous work has been done with Ubisol-Q10 or E-ASH separately but for the first time here, the agents will be combined in order to target all biochemical pathologies of PD and further reduce DA neuron loss. Efficacy of neuroprotection will be assessed via immunocytochemical staining for tyrosine hydroxylase, a marker or DA neurons in the SN. Ability of the treatments' abilities to target biochemical PD pathologies will be assessed via fluorescent immunohistochemistry.

Project 2: Creation of a water-soluble E-ASH (WS-ASH) using PTS: While E-ASH was effective at protecting DA neurons in Project 1, there was the economic issue of using E-ASH as a PD therapy due to small yields of extract and overall effective dose required due to the extract hydrophobicity leading to poor absorption/bioavailability. Therefore, E-ASH will be water solubilized using the same PTS technology (in collaboration with Next™ Remedies) used in Ubisol-Q10. Once made, the neuroprotective efficacy of the water-soluble E-ASH, WS-ASH at various doses versus the original E-ASH will be assessed via immunocytochemical staining for tyrosine hydroxylase, a marker or DA neurons in a PQ induced model of PD.

Project 3: Water-Soluble Ashwagandha Extract (WS-ASH) Synthesis/Dosing and investigation of neuroprotective efficacy of WS-ASH and Ubisol-Q10 in a Paraquat Induced Rat Model of Parkinson's Disease: Following dosing of WS-ASH, the neuroprotective efficacy of WS-ASH combined with Ubisol-Q10 versus the agents alone will be assessed in a PQ induced model of PD. While similar to Project 1, it's possible PTS could alter the properties of E-ASH. Efficacy of neuroprotection will be assessed via immunocytochemical staining for tyrosine hydroxylase, a marker or DA neurons. Ability

of the treatments' abilities to target biochemical PD mechanisms will be assessed via fluorescent immunohistochemistry.

Alzheimer's Disease

Project 4: Mechanism of induction of autophagy and inhibition of SIPS in AD fibroblasts: Previously, Ubisol-Q10 was shown to prevent oxidative stress induced premature senescence (SIPS) in AD mutated fibroblast. Pilot data using quantitative polymerase chain reaction (qPCR) to probe for genes related to apoptosis and autophagy showed enhanced expression of genes associated with autophagy in cells treated with Ubisol-Q10. AD mutated fibroblasts will be treated with an inhibitor targeting the autophagy proteins shown to be upregulated with Ubisol-Q10 to assess their role in protecting these cells and preventing SIPS. Assessment of SIPS will be assessed via senescence-associated beta-galactosidase and immunofluorescence probing for p21. Autophagic flux/activity will be assessed via staining for autophagic vacuoles using monodansylcadaverine and staining for LC3B puncta via immunofluorescence. Effect of autophagy inhibition on status of oxidative stress will also be assessed via immunofluorescence probing for 4-hydroxynonenal and H_2DCFDA staining.

Project 5: Investigation of the Neuroprotective Efficacy and Mechanism of Ubisol-Q10 and E-ASH in a Double Transgenic Mouse Model of Alzheimer's Disease: The neuroprotective efficacy of Ubisol-Q10 and ashwagandha combined will be investigated using a double transgenic mouse model of AD. Ubisol-Q10 will be sourced from Next™ Remedies Inc. While E-ASH will be made in-house using a standardized protocol. Previous work has been done with Ubisol-Q10 or E-ASH separately but for the first time here, the agents will be combined in order to target all biochemical pathologies of AD. Efficacy of neuroprotection will be assessed via immunocytochemical staining for beta-amyloid plaques as well as neuronal nuclei of neurons in the hippocampus. Ability of the treatments' abilities to target AD biochemical mechanisms will be assessed via fluorescent immunohistochemistry.

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CHAPTER 2: Combined Ubisol-Q10 and Ashwagandha Root Extract Target Multiple Biochemical Mechanisms and Reduces Neurodegeneration in a Paraquat-Induced Rat Model of Parkinson's Disease

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List of Abbreviations

PD	Parkinson's disease
DA	Dopaminergic
SN	Substantia Nigra
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
ROS	Reactive Oxygen Species
ETC	Electron Transport Chain
CoQ10	Coenzyme-Q10
PTS	Polyoxyethanyl-α-Tocopheryl Sebacate
AD	Alzheimer's Disease
PQ	Paraquat
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
TH	Tyrosine Hydroxylase
DAB	3,3′-diaminobenzidine
GFAP	Glial Fibrillary Acidic Protein
Iba-1	Ionized Calcium-Binding Adapter Molecule 1
BDNF	Brain Derived Neurotrophic Factor
GDNF	Glial Derived Neurotrophic Factor
4-HNE	4-Hydroxynonenal
CARP1	Cell Division Cycle Apoptosis Regulator 1

Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative disease characterized by loss of dopaminergic (DA) neurons in the substantia nigra (SN) region of the brain. The disease initially displays loss of movement coordination (resting tremors, postural instability, bradykinesia, and rigidity), which then progresses to cognitive impairments, psychiatric irregularity, and eventually morbidity [1,2]. While there are several genetic/familial factors and environmental factors linked to PD, most PD cases are sporadic and caused by unknown factors [3,4]. It is known that several similarly structured toxins such as MPTP (a synthetic heroin by-product) and paraquat/maneb/rotenone (various herbicides and pesticides) are preferentially taken up by DA neurons. These toxins then significantly elevate reactive oxygen species (ROS) when metabolized, leading to apoptosis [5–14]. Along with oxidative stress, there are several other biochemical mechanisms involved in PD, which include mitochondrial dysfunction, inhibition of autophagy leading to the buildup of defective proteins and organelles, and neuroinflammation.

Neurons are almost entirely dependent on oxidative phosphorylation to produce energy with the central nervous system requiring 20% of the total oxygen input [15,16]. As a result of high oxygen demand and since the electron transport chain (ETC) is not a perfect system, neurons have an increased tendency to produce ROS. Consistent evidence exists that links deficits of ETC complex I to excessive ROS production in both familial and sporadic forms of PD [15–18]. Furthermore, DA neurons have been shown to have reduced levels of antioxidant enzymes, further increasing their susceptibility to ROS [16]. Increased oxidative stress can eventually lead to damage to nucleic acids, proteins, and lipids. As these three constituents comprise mitochondria, ROS damage to them can lead to mitochondria dysfunction, which can then lead to further production of ROS, creating a feedback loop of increased oxidative stress and mitochondrial dysfunction [19].

As mentioned earlier, increased oxidative stress can lead to damage and the buildup of defective proteins and organelles. Cells have mechanisms to clear these defective by-products via autophagy and proteosome degradation systems. Both of these systems are involved in the degradation of defective proteins, while organelle clearing is mainly

achieved via autophagy [4]. It has been shown that these systems are compromised in PD, resulting in the buildup of defective proteins and organelles leading to neurotoxicity [20].

Increasing evidence has also demonstrated the importance of neuroinflammatory responses in PD. Indeed, activated microglia were found in the brains of PD patients post-mortem [21–24]. Microglia are involved in cell debris clearance and immune response regulation due to injury/infection. This immune response can lead to apoptosis as these microglia release pro-inflammatory cytokines and ROS. It is thought that microglia are activated in earlier stages of PD alongside DA neuron loss, leading to apoptosis due to oxidative stress and inflammation [22]. Contrary to microglia activation in PD, absence of astroglia activation/number has been implicated in PD pathology [23,25,26]. Astroglia serve a variety of functions including nutrient supplementation, structural support, and secretion of pro-survival neurotrophic factors [27–29]. With reduced activation/numbers of protective astroglia and increased activation of microglia in PD, DA neurons are significantly more susceptible to neuroinflammation.

Although good progress has been made in providing symptomatic relief with dopamine supplements and deep brain stimulation, there is no known available remedy to stop the progression of the disease. Recently, we have observed that Ubisol-Q10, a water-soluble formulation of coenzyme-Q10 (CoQ10) made using polyoxyethanyl-α-tocopheryl sebacate (PTS) [30], displays unprecedented neuroprotective properties in various models of PD. While previous preclinical studies with normal coenzyme-Q10 showed therapeutic efficacy at oral doses of 1600 mg/kg/day, a clinical trial [31] that had much lower required doses failed to obtain effective results. When combined with PTS, doses of coenzyme-Q10 were able to be reduced significantly to 6 mg/kg/day. Ubisol-Q10 has been shown to target mitochondrial dysfunction and oxidative stress in in vitro and in vivo models of PD [14,32–35]. Furthermore, Ubisol-Q10 prevented oxidative-stress-induced premature senescence and resumed impaired autophagy in Alzheimer's Disease (AD) fibroblasts, while also improving memory and removing beta-amyloid plaques in a transgenic mouse model of AD [36–38]. Although not directly pertaining to PD, AD shares the same biochemical mechanisms (oxidative stress, mitochondrial dysfunction, impaired autophagy, and neuroinflammation) [39]. While Ubisol-Q10 seems like a promising therapeutic, it does

not target all biochemical mechanisms of PD. PD should be considered a multifactorial disease and targeting only one or a few of the biochemical mechanisms will not be enough to stop the progression of neurodegeneration.

While Ubisol-Q10 is able to target oxidative stress, mitochondrial dysfunction, and impaired autophagy, there is still the issue of neuroinflammation that could still be occurring. Therefore, it would be better to combine Ubisol-Q10 with another well-tolerated natural product that could target neuroinflammation. Recent research interest has increased with using ashwagandha root extracts to treat neurological diseases/disorders. Ashwagandha (Withania somnifera) is a plant of the nightshade family that has been used in Ayurveda (a traditional Indian school of medicine) as a nerve tonic for general debility, nervous exhaustion, insomnia, and memory impairment [40]. Past studies showed that various root extracts of ashwagandha were able to target oxidative stress and neuroinflammation [40–43]. The protective compounds of ashwagandha are thought to be steroidal lactones and saponins (withanolides and sitoinosides, respectively). These key components have been shown to act as antioxidants, aid in axonal regeneration, and target certain activators of inflammation [40]. We note that studies measuring ashwagandha's efficacy alone orally administered doses that were too high for human therapeutic development. However, ashwagandha might exercise a synergistically enhanced efficacy at a much-lowered dose when combined with a water-soluble CoQ10 agent that could permit further human clinical trials.

In this study, based on these exciting findings, we combined Ubisol-Q10 and ashwagandha extract for the first time to investigate whether these two well-tolerated natural health products are more efficacious when administered together compared to the agents alone in a paraquat (PQ)-induced rat model of Parkinson's disease. Since these treatments target different biochemical mechanisms of PD, we hypothesize that the combined formulation of Ubisol-Q10 and ashwagandha extract will be more effective, compared to their separate use, in protecting the brains of PQ-treated rats.

Materials and Methods

Ethanolic Extraction of Ashwagandha Root and Phytochemical Assessment

Ashwagandha root powder (Premier Herbal Inc., North York, ON, Canada) was soaked/stirred in anhydrous ethanol at a ratio of 1:10 (w/v) at ~70 ◦C for 24 h. Following 24 h, the crude extract was filtered through a P8 paper filter, and ethanol was removed using a rotary evaporator. The solid extract was then resuspended with anhydrous ethanol to a concentration of 200 mg/mL. The final suspended extract was analyzed for several phytochemicals including withanolides and flavonoid content. Ultra-performance liquid chromatography coupled with ultraviolet spectroscopy (UPLC-UV) was used to analyze withanolide content, and colorimetric analysis based on Dowd's reagent was used for flavonoids. Phytochemical analyses were performed by Laboratoire PhytoChemia (Chicoutimi, QC, Canada).

Animal Care

All animal care, treatments, and procedures were approved by the University of Windsor's Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines (Animal Utilization Project Protocol #17–04). Experiments were conducted on male Long-Evans hooded rats (Charles River Laboratories, Wilmington, MA, USA). Rats arrived at 2.5 months of age and were habituated to the basic handling, feeding, transportation, and rotarod task until the age of 5 months and then underwent experimental behavioral testing following the injection procedure. Rats were housed in groups of 3–4 animals per cage for convenience and to prevent hierarchies that could arise due to the extent of neurodegeneration. Rats were individually fed outside their group cages a daily amount of 25–35 g of Purina LabDiet Rodent 5001 Chow (purchased from North American Lab Supply, Fort Worth, TX, USA) to prevent competition among animals. Animals were housed at 20°C under a 12-hr light–dark cycle to ensure they were awake during the day for behavioral assessments. The overall schedule of the experiment, including training, injection, and treatment regimens, is summarized in Figure 1 below.

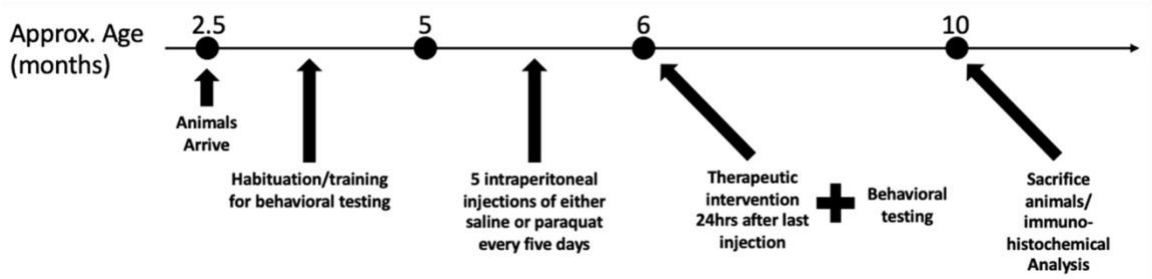

Figure 1. Experimental plan demonstrating the training, injection, and treatment regimen for rats.

Injection Regimen

Rats underwent the injection regimen at 5 months of age. Rats received 5 intraperitoneal injections of PQ dissolved in 1× phosphate-buffered saline (PBS) at 10 mg/kg body weight per injection. One injection occurred every 5 days over 20 days. Control rats received only saline injections according to the same schedule as PQ-injected rats. Animal health following injections was monitored daily by the University of Windsor's Animal House veterinarians.

Drinking Water Treatment

Animals were provided the following treatments in their drinking water 24 h after the last day of injections: saline-injected rats were given plain drinking water (n = 7); saline-injected rats were given the tonic (combination of 50 μg/mL Ubisol-Q10 (provided by Next™ Remedies, Toronto, ON, Canada) and 2 mg/mL ethanolic ashwagandha extract (ASH)(n = 5); PQ-injected rats were given plain drinking water (n = 9); PQ-injected were rats given PTS carrier (n = 6); PQ-injected were rats given 50 μg/mL Ubisol-Q10 (n = 7); PQ-injected rats were given 2 mg/mL ASH (n = 8); PQ-injected rats were given the tonic (n = 8). Groups in which ASH was not provided had 1% anhydrous ethanol added to the drinking water to account for 1% ethanol present in the water when ASH was added. Fresh drinking solutions were provided every 3–4 days. Treatment continued for 4 months during which behavioral assessments were conducted. Following 4 months, animals were sacrificed, and their brains were extracted for biochemical analysis.

Behavioral Assessments

Motor/balance coordination was measured on the rotarod. The rats' ability to maintain balance on a slowly rotating cylinder was measured with a rotarod apparatus similar to that previously described in [44]. Our modified rotarod apparatus is shown in Figure 2 It consisted of either a 9-cm diam (wide) or a 5-cm diam (thin), 15-cm long black polyethylene dowel with metal strips embedded lengthwise in its surface to prevent rats from slipping off the rotating rod. Either rod could be attached to a variable speed motor

hidden behind a vertical 30 × 48 cm flat grey plastic panel. During the course of the experiment, we ran different 2-min sessions with either rod that rotated counterclockwise at 6, 9, or 12 rpm during a session. A digital video camera was positioned 1 m in front of and in level with the rod. Regular fluorescent ceiling lighting and a 60-W lamp approximately 3 m in front of the apparatus illuminated it. The MPEG recordings of each rat's rotarod performance were converted to JPEG images at a rate of 5 frames per second. We analyzed each animal's movements over its last 600 frames (120 s). The position of the tip of the nose was tracked on a 450 × 450-pixel Cartesian system of coordinates with tracking software (Seven Software, Inc., Rockaway, NJ, USA). The grid was divided by a horizontal line above the rotarod and by two vertical lines, one to the right and the other to the left. The proportion of frames in which the animal's nose is beyond the right and beyond the left of these vertical lines was our measure of the proportion of time the animal spent walking forward and backward, respectively. We note from past experience that the time the animal's nose is between the two vertical lines, as it is when turning around, does not account for more than 10% of its total time on the rotarod. Statistical Product and Service Solutions (SPSS; IBM, Armonk, NY, USA) was used to calculate statistical significance using one-way ANOVA.

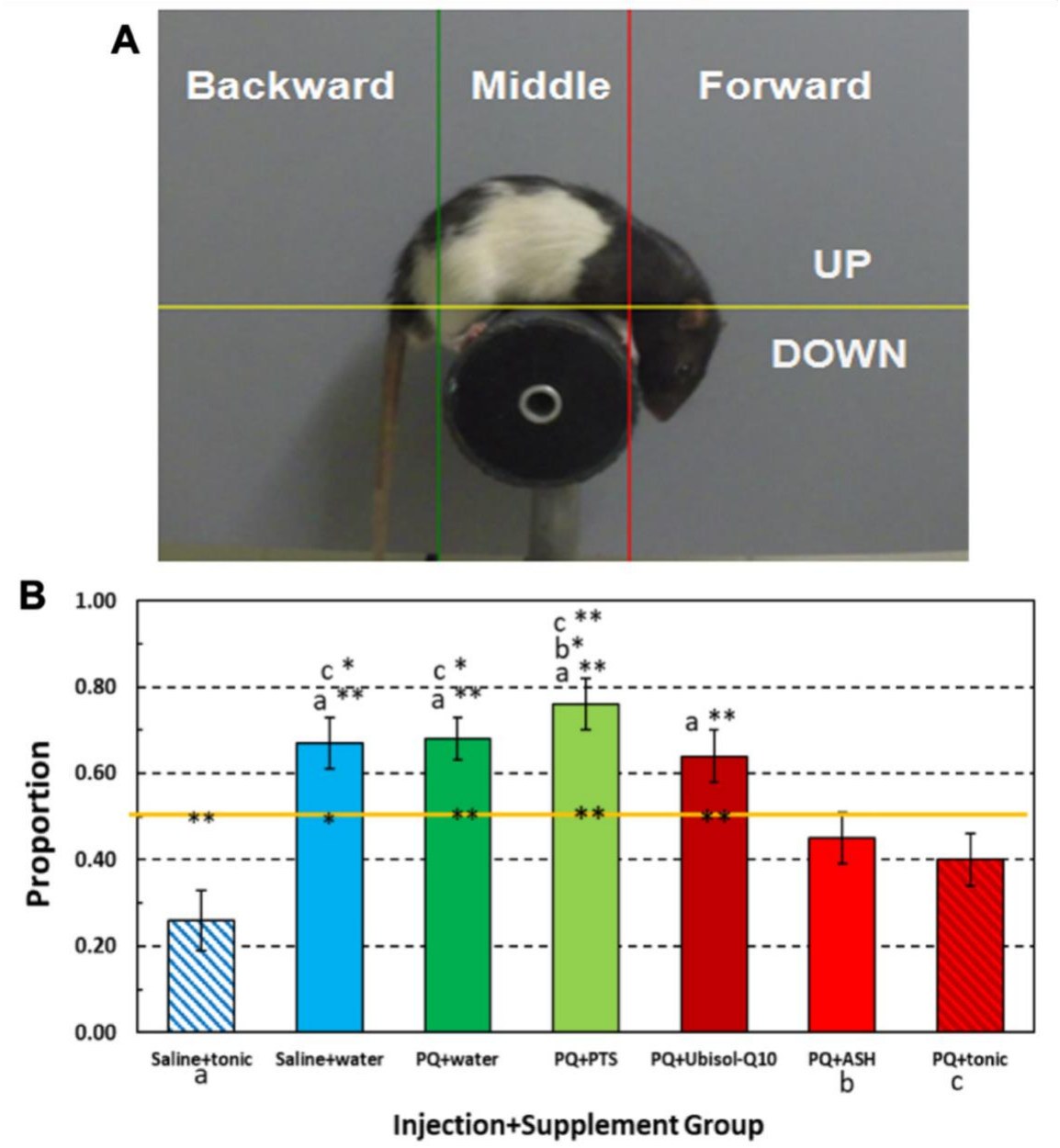

Figure 2. Ubisol-Q10 and ashwagandha prevent PQ induced motor deficits. (A) JPEG image from a digital 2-min video of a rat on a thin rotarod with vertical and horizontal guideline lines for determining body orientation (forward, backward) and head position based on nose location (see text for further details). **(B)** Mean proportion of time rats in each group spent walking in a head-down position on a 6-rpm rotating rod as determined by nose location above and below the horizontal orange line. Three groups marked along the X-axis are: a = Saline+tonic (one of two saline-injected control groups), b-PQ+ASH (the second of three neuro-protected treatment groups), and c- PQ+ tonic (the third of three neuro-protected treatment groups). Only these three groups were so marked because each of them spent significantly less time walking in the head-down position than any of the other four unmarked groups as indicated by its mark on top of any of four unmarked

groups' mean proportion bars. Vertical error lines = ±1 SE (standard error of the mean). * $p < 0.05$; ** $p < 0.01$. The p values that appear at the top of any of the four unmarked group's bar assess which a, b, or c group significantly differs that unmarked groups mean value as determined from supplementary pair-wise comparisons between groups. Those p values occurring on the horizontal line within or near each group's mean data bar indicates which group's rats spend significantly more or less time in a head-down walking position.

Tissue Preparation for Immunohistochemistry

Following the experimental period, rats were euthanized while under anaesthetization via 3% isoflurane at a flow of 2 L oxygen/min. Once the animal showed a lack of withdrawal reflex (indicating stage 3 anesthesia/lack of pain), the entire animal body was perfused with ice-cold PBS containing 28 ug/mL heparin (Sigma-Aldrich, Oakville, ON, Canada, Cat. No. H3393), followed by fixation with ice-cold 10% formaldehyde made in PBS. Following perfusion, brains were dissected and stored in 10% formalin at 4°C. To prepare for sectioning, brains were incubated in 30% sucrose (w/v in PBS) until brains sank in the solution. Following sucrose incubation, brains were cryosectioned at 30 μm thickness with Shandon™ M-1 embedding matrix (Thermo Scientific, Mississauga, ON, Canada, Cat. No. 1310 TS) onto glass microscope slides.

Immunohistochemistry (Colorimetric)

Sections were washed for 5 min twice with tris-buffered saline (TBS), followed by incubation with 0.3% H_2O_2 to block endogenous peroxidase activity. Sections were rinsed for 5 min twice with TBS, followed by a 30-min block with Dako serum-free protein block (Agilent Technologies Canada Inc., Mississauga, ON, Canada, Cat. No. X0909) and normal serum according to instructions of the Vector Laboratories VECTASTAIN Elite ABC-HRP kit, Peroxidase (rabbit IgG; MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. VECTPK4001). Tissue sections were incubated overnight at 4°C, with tyrosine hydroxylase (TH) primary antibody (rabbit IgG; 1:1000; Cat. No. P40101–150) (Pel-Freez Biologicals, Rogers, AR, USA). Tissue sections were washed for 5 min twice with TBS, followed by incubation with secondary biotinylated antibody according to instructions from the VECTASTAIN Elite ABC-HRP Peroxidase kit. Sections were washed twice with TBS for 5 min, then incubated with avidin-conjugated horseradish peroxidase from the VECTASTAIN Elite ABC-HRP Peroxidase kit for 45 min. Sections were washed twice with TBS for 5 min and incubated with 3,3′-diaminobenzidine (DAB) stain solution according to the Vector Laboratories DAB Peroxidase Substrate kit (MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. SK-4100). Sections were dehydrated with two 5-min washes in anhydrous ethanol then a 7-min xylenes wash, followed by coverslipping using

Permount® mounting medium (Fisher Scientific Canada, Ottawa, ON, Canada, Cat. No. SP15-500). Cells were imaged using bright-field microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada).

Immunohistochemistry (Fluorescent)

Sections were washed for 5 min twice with TBS, followed by incubation with Dako serum-free protein block (Agilent Technologies Canada Inc., Mississauga, ON, Canada, Cat. No. X0909). Tissue sections were then incubated overnight at 4°C in the following primary antibodies: glial fibrillary acidic protein (GFAP) (rabbit IgG, 1:500; Novus Biologicals, Centennial, CO, USA, Cat. No. NB300-141), ionized calcium-binding adapter molecule 1 (Iba-1) (rabbit IgG, 1:300; Novus Biologicals, Cat. No. NB100-1028), tyrosine hydroxylase (rabbit IgG, 1:1000; Pel-Freeze Biologicals, Cat. No. P40101-150), beclin-1 (mouse IgG, 1:500; Santa Cruz Biotechnology, Dallas, TX, USA, Cat. No. sc-48342), pro-brain-derived neurotrophic factor (pro-BDNF) (mouse IgG, 1:500; Santa Cruz Biotechnology, Cat. No. sc-65513), glial-derived neurotrophic factor (GDNF) (mouse IgG, 1:500; Santa Cruz Biotechnology, Cat. No. sc-13147), 4-hydroxynonenal (4-HNE) (rabbit IgG, 1:500; Abcam Inc., Cambridge, UK, Cat. No. ab46545), and cell division cycle and apoptosis regulator 1 (CARP1) (rabbit IgG, 1:1000; provided by Dr. Arun Rishi of Wayne State University). The following day, tissue sections were washed for 5 min twice with TBS and incubated at room temperature for 2 h in the following secondary antibodies: Vector Laboratories fluorescein horse anti-mouse IgG (1:500; MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. FI-2000) and Alexa Fluor™ 568 goat anti-rabbit IgG (Thermo Scientific Canada, Brockville, ON, Canada, Cat. No. A11011). Sections were then washed twice for 5 min in TBS followed by coverslipping with VECTASHIELD® Vibrance® antifade mounting medium with DAPI (4′,6-diamidino-2-phenylindole) (MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. VECTH18002). Tissue sections were imaged using epifluorescence microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada). Fluorescence was quantified in images captured using ImageJ software. For each group, the fluorescence was quantified for each

specified protein per overall SN image captured. Fluorescent analyses were performed on 3 sections per animal.

Results

Phytochemical Content of Ashwagandha Extract

Following extraction of the ashwagandha root powder, we sought to determine the concentration of various withanolides and a flavonoid of the extract. Phytochemical analysis was performed by Laboratoire PhytoChemia. A summary of the phytochemical analysis of the extract is provided below in Table 1. Withanolide content was determined via UPLC-UV. Withaferin A concentration was 13.6 mg/mL, 12-Deoxy-withastramonolide was 3.8 mg/mL, withanolide A was 5.5 mg/mL, and withanolide B was 1.9 mg/mL. Combined together, total withanolides account for approximately 12.4% of the extract composition (24.8 mg/mL of total withanolides in the 200 mg/mL extract). Flavonoid content was determined via colorimetric analysis based on Dowd's reagent. Quercetin concentration was determined to be 1.63 mg/mL per 100 mL of extract.

	Withanolides				Flavonoids
	Content (mg/mL)				Content (mg/100 mL)
	Withaferin A	12-Deoxy-withastramonolide	Withanolide A	Withanolide B	Quercetin
Assay 1	13.0	3.7	5.3	1.8	Mean 1.63 ± 0.07
Assay 2	13.9	4.0	5.6	2.0	
Assay 3	13.8	3.8	5.6	1.7	
Assay 4	13.6	3.9	5.5	1.8	
Mean ± standard deviation	13.6 ± 0.4	3.8 ± 0.1	5.5 ± 0.1	1.9 ± 0.1	

Table 1. Withanolide and flavonoid content of ethanolic ashwagandha extract.

Effect of Ubisol-Q10 and Ashwagandha Combined Compared to Agents Alone in Protecting DA Neurons from PQ-Induced Neurotoxicity

Here, we combined Ubisol-Q10 and ASH for the first time to examine if the agents combined are more effective compared to their individual use. We observed that rats exposed to PQ and given only plain drinking water or the PTS vehicle had significant neurodegeneration in the SN, as indicated by reduced immunoreactivity for tyrosine hydroxylase (TH) (a marker of DA neurons [34]) (Figure 3). Rats exposed to PQ and given either Ubisol-Q10 or ASH alone had significant protection for their DA neurons (Figure 3). Interestingly, while ASH protected fewer AD neurons than Ubisol-Q10 (reduced tyrosine hydroxylase immunoreactivity), it appeared to better maintain the morphology of protected neuron as shown by apparently greater abundance of fibers extending from the cell bodies, appearing more similar to neurons in the animals injected with only saline and given plain drinking water (Figure 3). When PQ-treated rats were given the tonic treatment (combination of Ubisol-Q10 and ASH), we saw the benefits of both treatments in that Ubisol-Q10 maintained the number of DA neurons while ASH maintained their morphology. Indeed, the PQ + tonic rats' SNs appeared almost identical to the control (saline + water) group (Figure 3). Furthermore, we also wanted to confirm that the tonic treatment of Ubisol-Q10 and ashwagandha was not toxic to healthy animal brains. Indeed, the tonic treatment did not result in any observable neurodegeneration of the SN in animals injected with saline (Figure 3). Along with colorimetric immunohistochemistry to detect TH, we also performed immunofluorescent staining (Figure 4). While immunofluorescent images were taken towards the tip of the SN instead of towards the overall/center of the SN, as in Figure 3, similar observations were made in the rats' brains. PQ-treated animals given plain water or PTS exhibited reduced levels of fluorescence for TH compared to the two saline-injected groups. Treatment with Ubisol-Q10, ASH, or the tonic had higher amounts of TH fluorescence compared to PQ animals fed plain water or PTS.

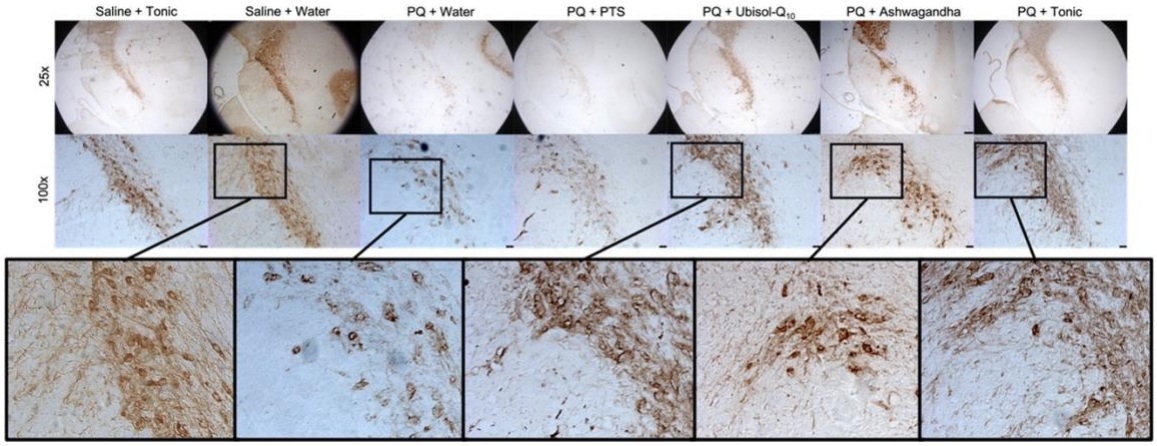

Figure 3. Effect of Ubisol-Q10 and ashwagandha on neurodegeneration of substantia nigra neurons in saline/paraquat (PQ)-injected rats. Light micrographs of midbrain sections showing tyrosine hydroxylase (TH) positive neurons at 25× (scale bar = 250 microns) and 100× (scale bar = 50 microns) magnification in the substantia nigra (SN). Images are representative of 3 independent experiments with similar trends.

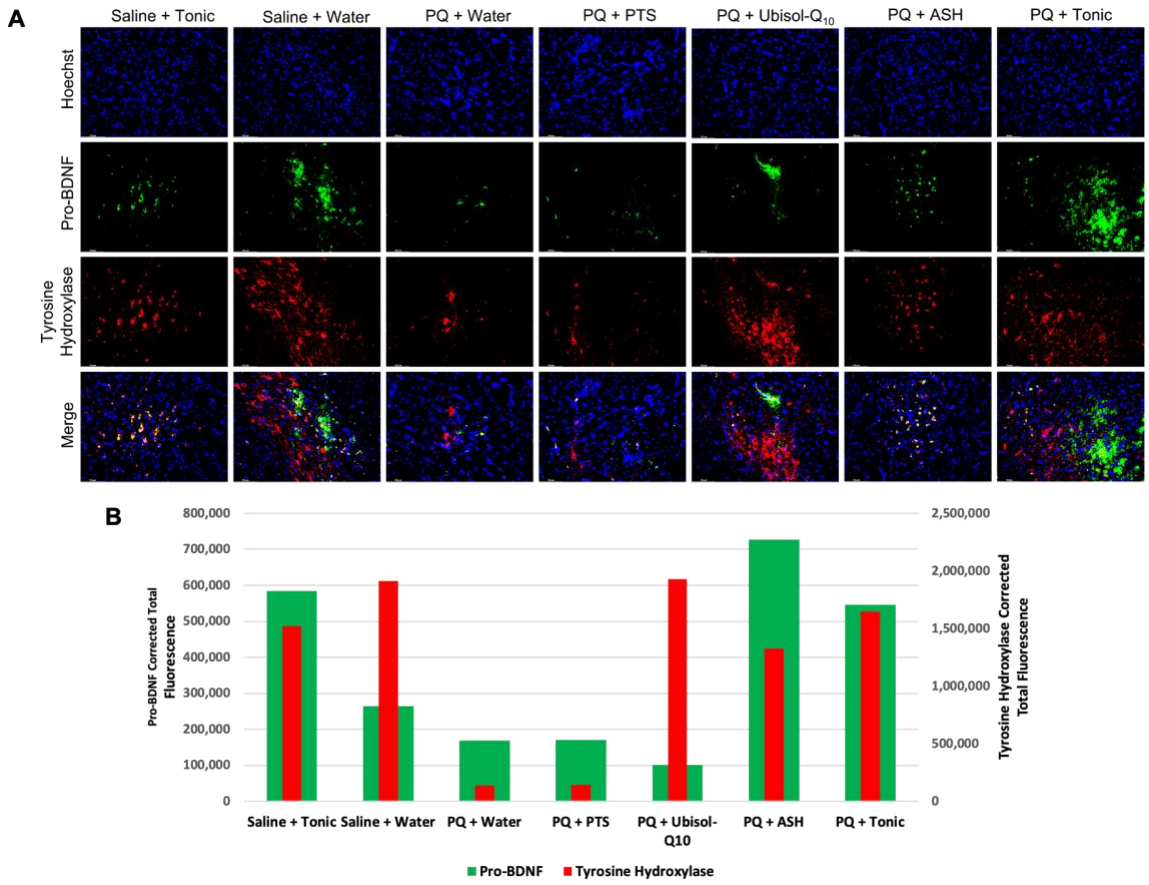

Figure 4. Effect of Ubisol-Q10 and ashwagandha on dopaminergic (DA) neuron apoptosis and pro-brain-derived neurotrophic factor (pro-BDNF) secretion in saline/PQ-injected rats. (A) Immunofluorescent staining at the tip of SN in midbrain sections probing for pro-survival neurotrophic factor, pro-BDNF, and tyrosine hydroxylase (TH) and **(B)** Quantification of fluorescent of pro-BDNF and TH. Nuclei were counterstained with DAPI. Micrographs were taken at 100× magnification. Scale bar = 100 microns. Saline injected animals given plain water quantitation is lower due to background staining being accounted for in ImageJ. Images are representative of 3 independent experiments with similar trends.

Ubisol-Q10 and ASH Antioxidant Effects against PQ-Induced Neurotoxicity

It is well known that exposure to PQ results in increased production of ROS [10,11,14]. We observed increases in levels of the lipid peroxidation product 4-hydroxynonenal (4-HNE) in the brains of PQ-treated rats given plain or PTS-supplemented drinking water (Figure 5A,B). When these animals were given Ubisol-Q10, ASH, or the tonic, 4-HNE levels were reduced, appearing similar to the saline-injected rats given plain water or the tonic as indicated by reduced immunoreactivity for 4-HNE (Figure 5A,B).

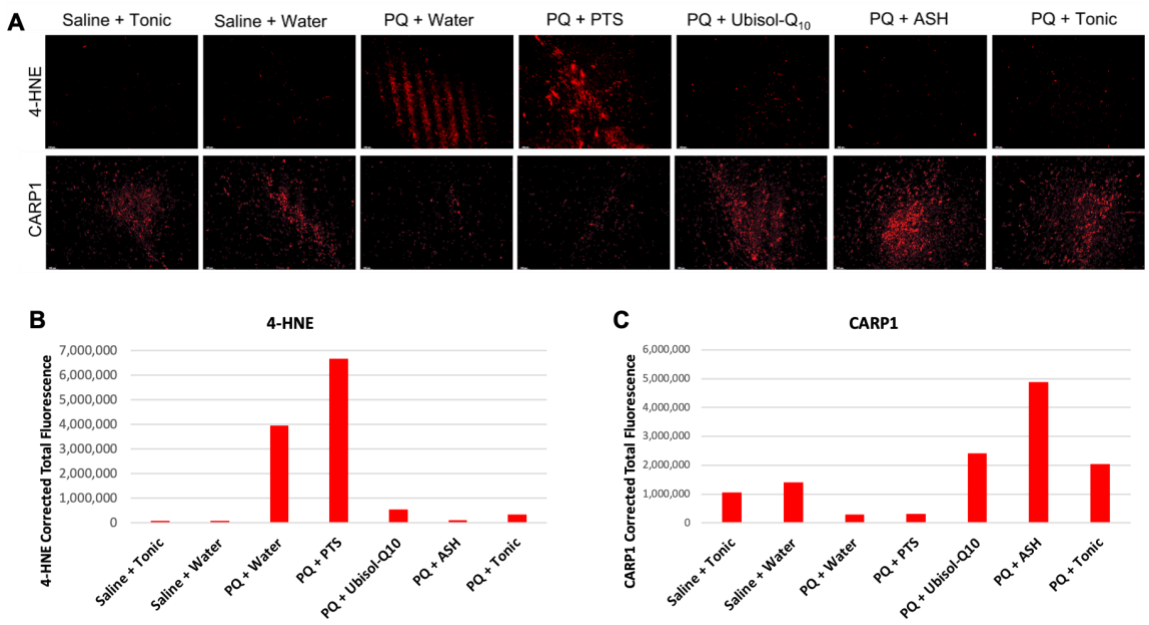

Figure 5. Effect of Ubisol-Q10 and ashwagandha on oxidative stress and apoptosis regulation. (A) Immunofluorescent staining of the SN in midbrain sections probing for oxidative stress marker and lipid peroxidation by-product 4-hydroxynonenal (4-HNE) and cell division cycle and apoptosis regulator 1 (CARP1). **(B,C)** Fluorescent quantification of 4-HNE and CARP1, respectively. Micrographs were taken at 100× magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

CARP1 Expression in Response to PQ Insult and Treatment with Ubisol-Q10 and Ashwagandha Extract

Cell division cycle and apoptosis regulator 1 (CARP1) is involved with regulating cell death and is known to be a positive regulator of apoptosis [45]. Here, we wanted to observe the status of CARP1 and determine its role in PQ-mediated neurotoxicity. Interestingly, CARP1 levels were reduced in the SN of PQ-injected rats fed plain water or PTS compared to the saline groups (Figure 5A–C). Furthermore, PQ-injected rats fed Ubisol-Q10, ASH, or the tonic had higher levels of CARP1 compared to the saline groups (Figure 5A–C).

Effect of Ubisol-Q10 and Ashwagandha Treatment on Beclin-1 Expression

We have shown that when AD fibroblast and transgenic AD mice were treated with Ubisol-Q10, the major autophagy regulator Beclin 1 was upregulated compared to untreated groups [36,37,38]. As mentioned earlier, PD and AD share several biochemical mechanisms leading to neurodegeneration including impaired autophagy. We wanted to investigate whether this same mechanism was activated in the rats of this study. Indeed, we observed that animals injected with PQ and fed plain or PTS-supplemented water had decreased levels of Beclin 1 compared to saline-injected animals (Figure 6). We saw Beclin 1 expression was increased in rats given Ubisol-Q10 and to a lesser extent with the tonic (Figure 6). While not as effective as Ubisol-Q10, PQ-injected rats fed ASH also had elevated levels of Beclin 1 compared to PQ rats fed plain water or PTS (Figure 6).

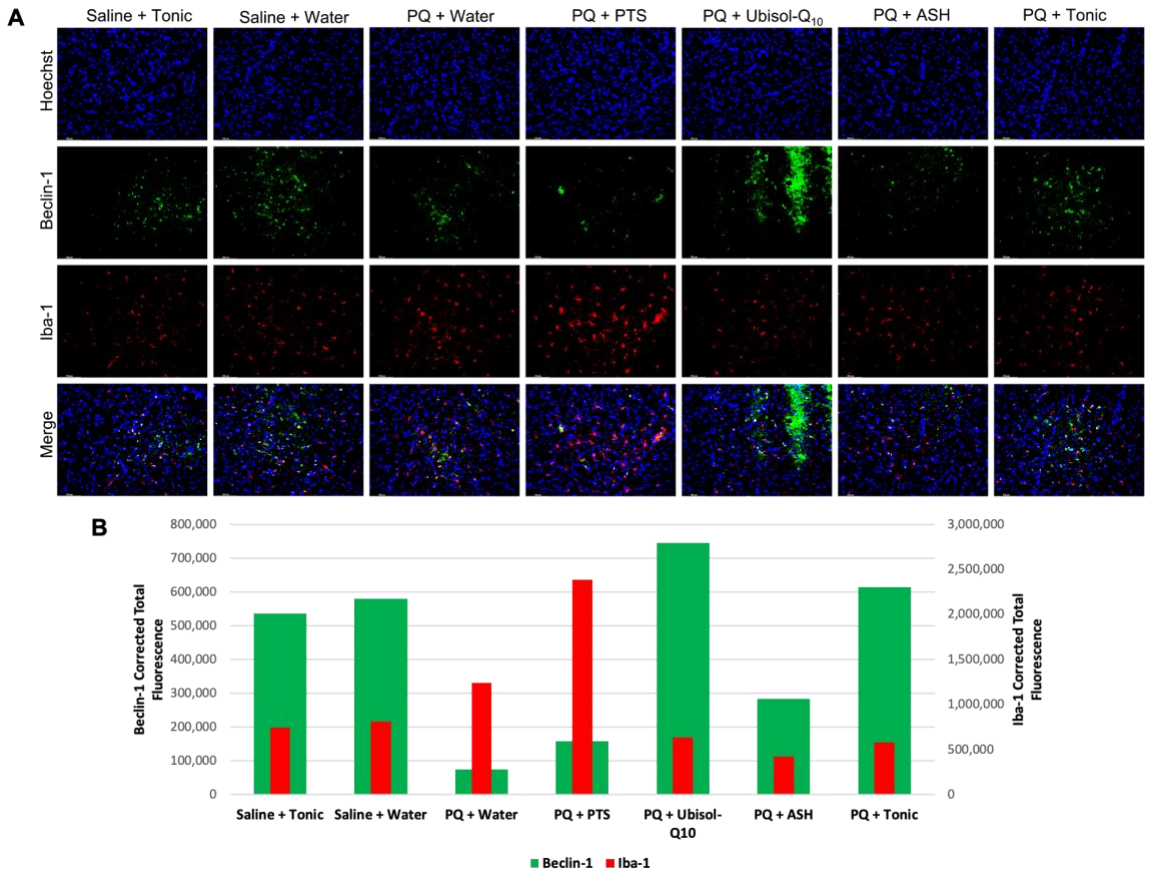

Figure 6. Effect of Ubisol-Q10 and ashwagandha on activation of pro-inflammatory microglia and autophagy. (A) Immunofluorescent staining of the SN in midbrain sections probing for beclin-1, a major regulator of autophagy, and ionized calcium-binding adapter molecule 1(Iba-1), a marker of microglia activation. **(B)** quantification of fluorescence of beclin-1 and Iba-1. Nuclei were counterstained with DAPI. Micrographs were taken at 100× magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Inflammatory Status in the Brains of Rats in Response to PQ Neurotoxicity and Treatment with Ubisol-Q10 and Ashwagandha Extract

We examined the status of both microglia and astroglia in response to treatment with Ubisol-Q10 and ashwagandha (Figure 6 and Figure 7). We saw that in rats injected with PQ and only fed plain water or PTS, levels of pro-inflammatory microglia were elevated compared to saline-injected animals as indicated by elevated immunoreactivity for Iba-1 (Figure 6). We saw that presence of active pro-survival astroglia was reduced in PQ-treated rats fed plain water or PTS, as indicated by measured levels of GFAP. With either treatment of Ubisol-Q10, ASH, or the tonic, microglia activation was reduced compared to PQ-treated rats given plain water or PTS. Furthermore, microglial activation was even reduced slightly in saline-injected animals given the tonic compared to the saline animals given water. Compared to microglia (Figure 6), an opposite outcome was observed with astroglia activation, as shown in Figure 7. Animals injected with PQ and fed plain water or PTS had reduced activation of astroglia compared to saline-injected animals. PQ-injected animals given Ubisol-Q10 or ASH showed significant increases in activation of astroglia compared to the PQ animals fed plain water or PTS. Furthermore, when combined, Ubisol-Q10 and ASH had an even greater effect on astroglial activation compared to the agents alone (Figure 7). Interestingly, we observed that the saline-injected animals given the tonic had around double the amount of astroglia activation compared to saline animals given plain water.

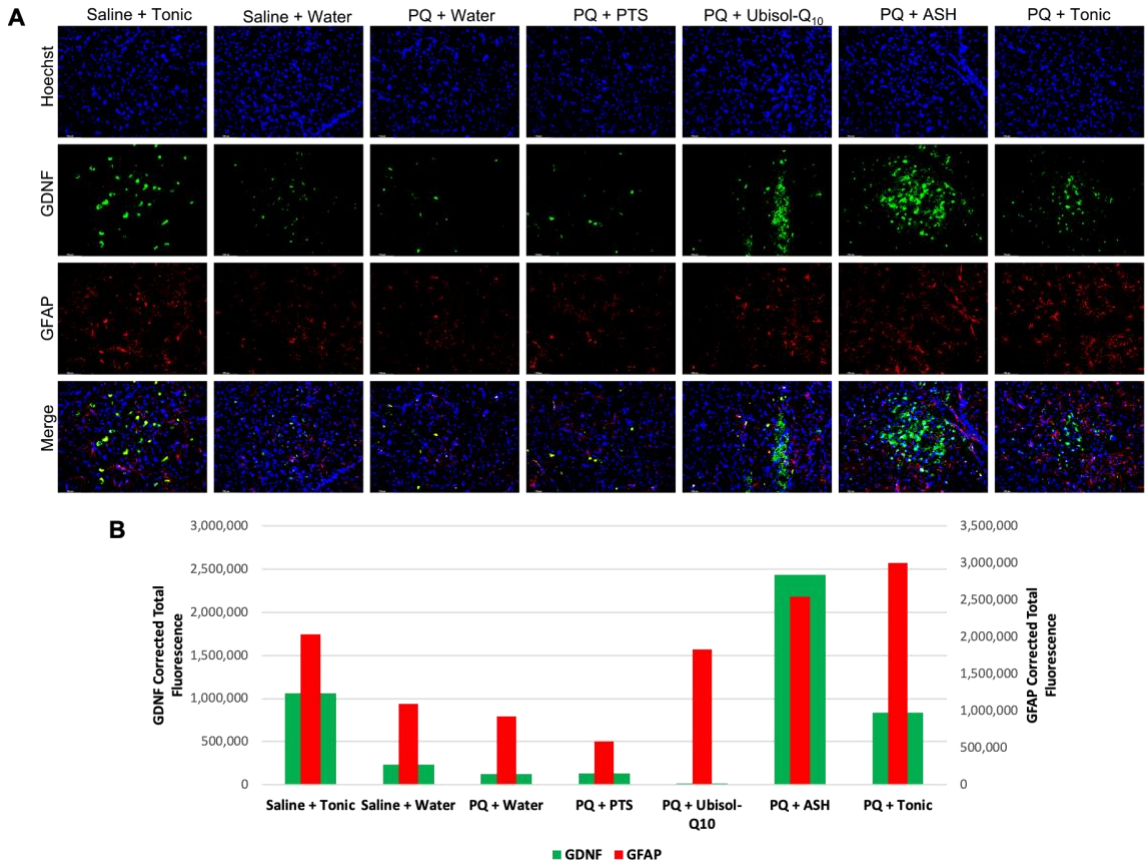

Figure 7. Effect of Ubisol-Q10 and ashwagandha extract on activation of pro-survival astroglia and secretion of glial-derived neurotrophic factor. (A) Immunofluorescent staining of the SN in midbrain sections probing for pro-survival neurotrophic factor glial-derived neurotrophic factor (GDNF) and glial fibrillary acidic protein (GFAP), a marker of astroglia activation. **(B)** Quantification of fluorescence of GDNF and GFAP. Nuclei were counterstained with DAPI. Micrographs were taken at 100 × magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Modulation of Levels of Neurotrophic Factors, GDNF and Pro-BDNF in Response to PQ Insult and Treatment with Ubisol-Q10 and Ashwagandha Extract

Along with looking at the status of astrocytes, we also probed for both GDNF and pro-BDNF to examine whether neurotrophic factor levels are affected by astrocyte activity. Interestingly, all animal groups that had ASH in their drinking water exhibited elevated levels of GDNF compared to all other groups (Figure 7). Similarly, pro-BDNF was also elevated in all groups with drinking water containing ASH compared to groups without ASH (Figure 4).

Effect of Ubisol-Q10 and Ashwagandha Extract on PQ-Induced Motor Deficits

Chronic PQ or MPTP exposure in rats or mice is known to cause impaired motor performance on the rotarod task [14,35,36,46]. As already discussed, we focused our analysis on comparing the groups' proportion of head-down positions as they walked on a slowly rotating (6 rpm), 5-cm cylinder (rotarod) (Figure 2). A one-way ANOVA for groups uncovered a significant effect for this factor, F6, 43 = 8.29, p = < 0.001, $\acute{\eta}_p^2$ = 0.54, observed Power = 1. Post-hoc pair-wise comparisons between groups revealed that animals given a tonic supplement after receiving either saline or PQ injections (a- or c-labeled groups) spent significantly less time walking with their heads in a downward position than rats given only water or PTS after receiving PQ injections and, unexpectedly, than rats that continued to receive only water after being injected with saline or that received Ubisol-Q10 after being injected with PQ. Although PQ-injected rats given ashwagandha (b-labeled group) spent significantly less time walking on the rotarod in a head-down position than the solely PQ-injected rats given PTS, they, along with PQ or saline-injected rats given the tonic, did not significantly exceed more than 50% of their time doing so, compared to PQ-injected rats given PTS, water, or Ubisol-Q10, or compared to saline-injected rats given water, who spent significantly more than 50% of their time in a head-down position. Indeed, only saline-injected rats given the tonic solution spent significantly less than 50% of the time in a head-down position. We must point out that we had two groups of healthy saline-injected animals (i.e., tonic fed or plain water). We were not expecting any motor deficits in the saline-injected animals, and this is true for the saline-injected, tonic-fed

animals. However, saline-injected, water-fed animals did show some deficits, which was unexpected.

Discussion

In this study, we found that Ubisol-Q10 and ethanolic ashwagandha extract (ASH) combined, two simple and well-tolerated nutraceuticals, contributed in a complementary way to target the multiple biochemical mechanisms implicated in PD. With PD being a multifactorial disease, the two agents combined were more effective at halting the progressive neurodegeneration of PD compared to the agents administered alone. Furthermore, by combining the agents, each targeting different mechanisms of PD, we were able to use a lower dose of one known neuroprotectant, ASH, compared to those in other studies that used ASH alone.

Paraquat is an herbicide and environmental toxin well known to cause in people exposed to it the development of Parkinson's disease [10,11,12,13]. Other mammals such as rats are also known to develop similar neurodegeneration in the SN when exposed to PQ. The toxic effects of PQ have been studied in rats, and a well-established model has been developed [34]. After chronic exposure to low doses of PQ, progressive neurodegeneration of DA neurons occurs in the brains of rats. While Ubisol-Q10 did target some mechanisms of PD, it did not target all. With PD being a multifactorial disease, targeting only one or a few mechanisms is not enough to halt neurodegeneration. We sought to combine Ubisol-Q10 with another reagent, one that may target the other mechanisms of PD that Ubisol-Q10 did not as effectively such as inflammation. As a result, we combined Ubisol-Q10 with ethanolic extract of ashwagandha, and, indeed, we found that DA neurons were better protected by the tonic compared to the agents alone (Figure 3). While Ubisol-Q10 was able to maintain the overall amount of DA neurons in the substantia nigra of PQ-treated rats, their morphology was damaged compared to saline-injected animals, as there were very few nerve fibers extending from their cell bodies. ASH, while not as effective at maintaining the same amount of DA neurons, better protected the overall morphology of the neurons as there were more fibers that extended off the cell

bodies similar to that in the saline-injected animals. When combined, the benefits of Ubisol-Q10 and ASH were seen in the brains of PQ-treated rats (neuron numbers maintained by Ubisol-Q10 and cell morphology protected by ASH) (Figure 3). Furthermore, we found that the tonic of Ubisol-Q10 and ASH had no ill effects on DA neurons in saline-injected rats.

4-HNE, a lipid peroxidation by-product and oxidative stress marker was almost completely eliminated in the SN of PQ-treated rats fed Ubisol-Q10 (Figure 5). Similarly, ASH also reduced levels of 4-HNE, as seen in Figure 5, confirming the previously reported antioxidant effects of ashwagandha extracts [41]. Furthermore, Ubisol-Q10-fed animals (either given saline or PQ injections) showed elevated levels of Beclin 1, a major autophagy regulator, indicating increased levels of autophagy (Figure 6). These are very exciting results, as upregulation of Beclin 1 by Ubisol-Q10 was only previously reported in AD fibroblasts and transgenic AD mice [38]. Thus, our results further confirm the autophagy activating mechanism of Ubisol-Q10 via upregulation of Beclin 1. Activation of autophagy is especially important in PD as defective mitochondria are known to accumulate, which can result in further production of ROS leading to apoptosis [15,19,20].

In this study, we sought to determine the status of microglia and astroglia in response to PQ insult and Ubisol-Q10 and ASH treatment. We observed enhanced microglial activation in PQ-treated rats fed plain water or PTS compared to saline-injected animals (Figure 6). Treatment with either Ubisol-Q10, ASH, or the tonic resulted in drastic inhibition of active microglia. Furthermore, microglial activation levels were similar to levels of 4-HNE. It is possible that microglia could be involved, along with PQ, in inducing oxidative stress. Along with microglia, we also investigated the status of astroglia. Opposite to our observations of microglia, levels of active astroglia were reduced in PQ-treated rats fed plain water or PTS compared to rats injected with saline (Figure 7). We saw an increase in astroglia activation in both Ubisol-Q10 and more so in ASH (Figure 7). When combined together, the tonic showed increased astrogliosis compared to the agents alone. Saline-injected animals given the tonic had elevated levels of astroglia compared to the saline-injected animals fed plain water. Astroglia are also known to secrete several pro-survival neurotrophic factors including GDNF and BDNF [27,28,29]. While both Ubisol-

Q10 and ASH resulted in increased astrogliosis, only groups where ASH was given showed increased levels of GDNF and pro-BDNF (precursor to BDNF, which eventually gets cleaved to form BDNF) (Figure 4 and Figure 7). This could indicate that ASH not only acts as an anti-inflammatory by inhibiting pro-inflammatory microglia and activating pro-survival astroglia but also stimulates another pro-survival response.

Previously, CARP1 was considered to be mainly involved in mediating apoptosis [45]. Here, we observed that CARP1 was down-regulated in PQ-treated rats given plain water or PTS compared to all other groups (Figure 5A–C), suggesting that CARP1 is acting as a pro-survival regulator in our model of PD. While surprising, this observation is not totally unexpected. CARP1 has been shown to be involved in NR3A (NMDA-type glutamate receptor subunit) synaptic signaling, along with regulating β-catenin in colon cancer metastasis, co-activating GR (glucocorticoid receptor) signaling during adipogenesis, or neurogenin3-mediated pancreatic endocrine differentiation [47,48,49,50]. CARP1 also interacts with Necdin to regulate myoblast survival [51]. Furthermore, CARP1 interaction with NEMO (NF-kappa-B essential modulator) is involved in the regulation of pro-inflammatory NF-KB survival signaling [45]. CARP1-NEMO signaling is proposed to be involved in the regulation of DNA-damage-induced survival signaling. CARP1 is also shown to be a co-activator of the cell cycle regulatory APC/C E3 ligase [52]. APC/C E3 ligase is a critical regulator of G2/M transition, where APC/CCDC20 E3 ligase regulates cyclin B degradation to manage G2 exit. It could be possible that elevated levels of CARP1 in our study helped sustain optimal APC/C CDC20 activity for enhanced G2/M exit and cell cycle progression and survival signaling.

One of the most striking and commonly observed symptoms in humans with PD is their tendency to form a forward lean in their gait [53]. A similar head-down symptom was first observed in PQ-injected rats on the rotarod [46]. Although in a later study from our laboratory that employed a more standardized rotarod test, this symptom was not replicated in PQ-exposed rats, but rather the animals not given any neuroprotection from Ubisol-Q10 displayed less time walking backward than their neuroprotected counterparts or saline-injected control rats [14]. Despite the fact that the present study did not replicate these body orientation effects; it did reveal head-down position differences as a function of rats'

injection/water supplement condition, as we already described. It seems that the tonic composed of Ubisol-Q10 and ashwagandha was more potent in limiting PQ-injected rats' time spent walking in the head-down position than when ashwagandha or Ubisol-Q10 were administered separately. Of these two separately administered neuro-protectants, only ashwagandha produced any statistically significant head-down limiting effects. Thus Ubisol-Q10 alone may be a less effective behavioral treatment for PD if not combined with a greater anti-inflammatory agent. The question remains whether such an anti-inflammatory agent must also have unrelated neurotrophic properties to provide a more potent treatment with another non-anti-inflammatory neuroprotectant such as Ubisol-Q10. The saline-injected rats (non-lesioned) given the tonic or water should not show any deficits in motor balance. Indeed, the saline-injected, tonic-fed rats showed better motor balance performance than PQ-injected rats. However, the saline-injected rats given only water showed deficiency in motor balance, which was an unexpected anomaly and difficult to explain. This raises the question of whether either of the tonic's components given separately offer equally potent effects in rats with unaffected brains.

Following extraction, the ashwagandha extract was subjected to phytochemical analysis. It was determined that the total extract comprised 12.4% withanolides. The extract also was determined to contain 1.63 mg/mL quercetin equivalents per 100 mL of the total extract. Both flavonoids and withanolides are known to have potent anti-inflammatory properties [40,54], and we reported similar observations with our extract, as mentioned above. Previous studies used ashwagandha extract concentrations that are too high for human consumption/clinical development [40,41,42,43]. In this study, we were able to use significantly lower doses of ethanolic ashwagandha extract that still had neuroprotective effects against PQ toxicity. While the extract was not as effective by itself in protecting DA neurons, the extract combined with Ubisol-Q10 was more effective at protecting DA neurons compared to either agent alone. Additionally, while our extract did not have as high a withanolide content compared to other groups [43], our extract did not require the use of toxic solvents, and by combining the extract with Ubisol-Q10, the dose of the extract was able to be drastically reduced to a concentration (2 mg/mL) suitable for human clinical development.

Conclusions

In this study, we combined Ubisol-Q10 and ashwagandha root extract for the first time as a potential therapeutic for PD so as to determine whether the agents combined are more effective compared to the agents alone. We found the tonic of Ubisol-Q10 with ethanolic extract of ashwagandha root extract combined was more efficacious in protecting DA neurons in a PQ-induced rat model of PD compared to the therapeutic agents alone. Ubisol-Q10, ashwagandha, or the tonic were able to reduce oxidative stress as well as stimulate the activation of the apoptosis regulator CARP1 in the brains of the rats. Furthermore, we found that animals given Ubisol-Q10 or the tonic showed increased expression of the autophagy regulator Beclin-1. This is the first time Ubisol-Q10 was shown to enhance autophagy activation in the brains of PQ rat models of PD. Activation of pro-survival astroglia and inhibition of pro-inflammatory microglia was observed in the brains of PQ-exposed rats given ashwagandha or the tonic, which also coincided with the expression of pro-survival neurotrophic factors GDNF and pro-BDNF only in the brains of rats given ashwagandha or the tonic. Thus, the tonic of Ubisol-Q10 and ashwagandha was more efficacious at protecting PQ-treated rats from the biochemical mechanisms of PD compared to the agents alone. The reason for the better neuroprotective results with the tonic is likely due to the fact that the two agents are targeting different biochemical etiologies of PD. With both Ubisol-Q10 and ASH being simple nutraceutical compounds and GRAS (generally regarded as safe)-approved, they can also be taken over long periods of time without serious side effects. Thus, Ubisol-Q10 and ashwagandha root extract could prove to be a promising therapy for PD that could halt neurodegeneration and improve quality of life.

Patents

Ubisol-Q10 is a patented formula.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 3: Water-solubilization of ashwagandha root extract: Anti-inflammatory and neuroprotective effects of a novel water-soluble formulation of coenzyme-Q10 and ashwagandha root extract in a paraquat induced rat model of Parkinson's Disease

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List of Abbreviations

PD	Parkinson's disease
DA	Dopaminergic
SN	Substantia Nigra
PQ	Paraquat
ROS	Reactive Oxygen Species
PTS	Polyoxyethanyl-α-Tocopheryl Sebacate
E-ASH	Ethanolic Ashwagandha Extract
WS-ASH	Water-Soluble Ethanolic Ashwagandha Extract
TH	Tyrosine Hydroxylase
GFAP	Glial Fibrillary Acidic Protein
Iba-1	Ionized Calcium-Binding Adapter Molecule 1
BDNF	Brain Derived Neurotrophic Factor
GDNF	Glial Derived Neurotrophic Factor
4-HNE	4-Hydroxynonenal
NGF	Nerve Growth Factor
LC3B	Microtubule-associated proteins 1A/1B light chain 3B

Introduction

Parkinson's Disease (PD) is a neurodegenerative disorder caused by loss of dopaminergic (DA) neurons in the substantia nigra (SN). Symptoms include motor deficits such as resting tremors, bradykinesia, postural instability, and rigidity. Eventually, PD progresses to impaired cognitive functions and eventual morbidity. Furthermore, those living with PD also experience loss of balance which may be implicated in their impaired movement and their fear of falling [1-5]. While most PD cases are sporadic and caused by unknown factors, there are several environmental risk factors implicated in PD. Examples include herbicides and pesticides such as maneb, paraquat (PQ), and rotenone. These toxins are preferentially taken up by DA neurons which are then metabolized and produce elevated levels of reactive oxygen species (ROS) leading to apoptosis [6-16]. Along with ROS production leading to oxidative stress, there are also several other biochemical mechanisms involved in PD including mitochondrial dysfunction [17-21], inhibition of autophagy leading to buildup of defective proteins/organelles [22,23], activation of proinflammatory microglia [24-27], and reduced presence of pro-survival astroglia [26,28,29].

While there are therapies that provide symptomatic relief such as dopamine replacement or deep brain stimulation, no therapy exists that can stop the progression of neurodegeneration or PD. Recently, we have shown that Ubisol-Q10, a water-soluble formulation of coenzyme-Q10 created using polyoxyethanyl-α-tocopheryl sebacate (PTS) [30] displayed neuroprotective properties in different models of PD. While normal coenzyme-Q10 (oil-soluble/lipophilic) taken orally showed therapeutic efficacy in pre-clinical trials, its oral dose was too high (1600mg/kg/day) to be used in clinical trials. When used in clinical trials, regular coenzyme-Q10 failed to show similar results due to its required lower oral dose [31]. When combined with PTS to make Ubisol-Q10, oral doses were drastically reduced (6mg/kg/day). Ubisol-Q10 has been shown to target PD related mitochondrial dysfunction, oxidative stress, and autophagy impairment in-vitro and in-vivo [15,32-34].

Even though Ubisol-Q10 targets oxidative stress, mitochondrial, dysfunction, and autophagy impairment, its use alone is still not enough to protect from PD neurodegeneration as inflammation still poses a serious problem. PD should be considered a multifactorial disease due to more than one biochemical mechanism being implicated in the disease (oxidative stress, mitochondrial dysfunction, autophagy impairment, and inflammation). Therefore, targeting only one or several mechanisms isn't enough and all need to be considered with PD treatment. Recent research interest has grown and shown the potential anti-inflammatory properties of extracts of ashwagandha root (Withania somnfiera) which is a plant of the nightshade family used in Ayurveda (traditional Indian medicine) [35]. Various extracts of ashwagandha root have been shown to act as an antioxidant and anti-inflammatory agent in rodent models of PD [34-37]. Ashwagandha is known to contain several phytochemicals that act as antioxidants and anti-inflammatories such as withanolides and sitoinosides [35]. Unfortunately, oral doses of ashwagandha alone in some of these studies were too high to be translated to human use. Fortunately, by combining an ethanolic extract of ashwagandha (E-ASH) with Ubisol-Q10, we were able to reduce the dose of ashwagandha and make an even more effective neuroprotective cocktail that better protected DA neurons in a PQ rat model of PD compared to the agents when used alone [34].

While the therapeutic potential of ashwagandha combined with Ubisol-Q10 seems promising, there was still an economic issue associated with being able to use ashwagandha. The creation of our extract involved an extraction ratio of 10:1 (10 parts ethanol to 1-part ashwagandha root) which only resulted in a 10-20% yield of extract [34]. Furthermore, with the ethanolic extract comprising of mostly hydrophobic components, the bioavailability is poor when administered orally in an aqueous solution as the extract would precipitate out of solution when added in water. To remedy this, we combined E-ASH with PTS similar to Ubisol-Q10 to create a water-soluble ethanolic extract of ashwagandha root (WS-ASH). In this study we hypothesize that WS-ASH will be more effective compared to E-ASH in protecting DA neurons due to increased solubility leading to better bioavailability of the hydrophobic ashwagandha phytochemicals. Furthermore, by combing with Ubisol-Q10, we can create an even more effective therapy that can protect

the brains of PQ treated rats compared to the agents alone as they will target all biochemical mechanism rather than just a few.

Materials and Methods

Ubisol-Q10 and preparation of water-soluble ashwagandha root extract

E-ASH was prepared as described by Vegh et al. [34]. WS-ASH was prepared from E-ASH and PTS using a patented protocol (similar to how Ubisol-Q10 is produced) Next™ Remedies, Toronto, ON, Canada. Ubisol-Q10 was provided by Next™ Remedies, Toronto, ON, Canada.

Animal care

All animal care, treatments, and procedures were approved by the University of Windsor's Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines (Animal Utilization Project Protocol #17–04). Experiments were conducted on male Long-Evans hooded rats (Charles River Laboratories, Wilmington, MA, USA). Rats arrived at 2.5 months of age and were habituated to the basic handling, feeding, transportation, and rotarod task until the age of 5 months and then underwent experimental behavioral testing following the injection procedure. Rats were housed in groups of 3–4 animals per cage for convenience and to prevent hierarchies that could arise due to the extent of neurodegeneration. Rats were individually fed outside their group cages a daily amount of 25–35 g of Purina LabDiet Rodent 5001 Chow (purchased from North American Lab Supply, Fort Worth, TX, USA) to prevent competition among animals. Animals were housed at 20°C under a 12-hr light–dark cycle to ensure they were awake during the day for behavioral assessments.

Injection regimen

At 5 months of age, rats received 5 intraperitoneal injections of 1x phosphate buffer saline (PBS) as a control or PQ dissolved PBS at 10mg/kg body weight per injection. Rats

were injected every 5 days over 20 days. Animal health was monitored daily during the injection regimen by the University of Windsor's Animal House veterinarians.

Drinking water treatment

The experiment was divided into two separate parts. Part 1 involved dosing of water-soluble ashwagandha extract (WS-ASH) and Part 2 involved the combining of Ubisol-Q10 and WS-ASH. For Part 1, animals were provided the following treatments in their drinking water 24 h after the last day of injections: PQ-injected rats given PTS carrier (n=5); PQ-injected rats given 2mg/mL ethanolic ashwagandha extract (E-ASH) (n=5); PQ-injected rats given 2mg/mL water-soluble ashwagandha extract (WS-ASH) (n=5); PQ-injected rats given 1mg/mL water-soluble ashwagandha extract (WS-ASH) (n=5); PQ-injected rats given 0.2mg/mL water-soluble ashwagandha extract (WS-ASH) (n=5). For Part 2, animals were provided the following treatments in their drinking water 24 h after the last day of injections: Saline-inject rats given plain drinking water (n=6); PQ-injected rats given PTS carrier (n=6); PQ-injected rats given 50ug/mL Ubisol-Q10 (n=6); PQ-injected rats given 1mg/mL WS-ASH (n=6); PQ-injected rats given the tonic (combination of 50ug/mL Ubisol-Q10 and 1mg/mL WS-ASH).

Tissue Preparation for Immunohistochemistry

Following the experimental period, rats were euthanized while under anaesthetization via 3% isoflurane at a flow of 2 L oxygen/min. Once the animal showed a lack of withdrawal reflex (indicating stage 3 anesthesia/lack of pain), the entire animal body was perfused with ice-cold PBS containing 28 μg/mL heparin (Sigma-Aldrich, Oakville, ON, Canada, Cat. No. H3393), followed by fixation with ice-cold 10% formaldehyde made in PBS. Following perfusion, brains were dissected and stored in 10% formalin at 4°C. To prepare for sectioning, brains were incubated in 30% sucrose (w/v in PBS) until brains sank in the solution. Following sucrose cryoprotection, brains were cryosectioned at 30μm thickness with Shandon™ M-1 embedding matrix (Thermo Scientific, Mississauga, ON, Canada, Cat. No. 1310 TS) onto glass microscope slides.

Immunohistochemistry (Colorimetric)

Sections were washed for 5 min twice with tris-buffered saline (TBS), followed by incubation with 0.3% H_2O_2 to block endogenous peroxidase activity. Sections were rinsed for 5 min twice with TBS, followed by a 30-min block with Dako serum-free protein block (Agilent Technologies Canada Inc., Mississauga, ON, Canada, Cat. No. X0909) and normal serum according to instructions of the Vector Laboratories VECTASTAIN Elite ABC-HRP kit, Peroxidase (rabbit IgG; MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. VECTPK4001). Tissue sections were incubated overnight at 4°C, with tyrosine hydroxylase (TH) primary antibody (rabbit IgG; 1:1000; Cat. No. P40101–150) (Pel-Freez Biologicals, Rogers, AR, USA). Tissue sections were washed for 5 min twice with TBS, followed by incubation with secondary biotinylated antibody according to instructions from the VECTASTAIN Elite ABC-HRP Peroxidase kit. Sections were washed twice with TBS for 5 min, then incubated with avidin-conjugated horseradish peroxidase from the VECTASTAIN Elite ABC-HRP Peroxidase kit for 45 min. Sections were washed twice with TBS for 5 min and incubated with 3,3′-diaminobenzidine (DAB) stain solution according to the Vector Laboratories DAB Peroxidase Substrate kit (MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. SK-4100). Sections were dehydrated with two 5-min washes in anhydrous ethanol then a 7-min xylenes wash, followed by coverslipping using Permount® mounting medium (Fisher Scientific Canada, Ottawa, ON, Canada, Cat. No. SP15-500). Cells were imaged using bright-field microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada).

Immunohistochemistry (Fluorescent)

Sections were washed for 5 min twice with TBS, followed by incubation with Dako serum-free protein block (Agilent Technologies Canada Inc., Mississauga, ON, Canada, Cat. No. X0909). Tissue sections were then incubated overnight at 4°C in the following primary antibodies: glial fibrillary acidic protein (GFAP) (rabbit IgG, 1:500; Novus Biologicals, Centennial, CO, USA, Cat. No. NB300-141), ionized calcium-binding adapter molecule 1 (Iba-1/IBA1) (rabbit IgG, 1:300; Novus Biologicals, Cat. No. NB100-1028),

tyrosine hydroxylase (TH) (rabbit IgG, 1:1000; Pel-Freeze Biologicals, Cat. No. P40101-150), beclin-1 (mouse IgG, 1:500; Santa Cruz Biotechnology, Dallas, TX, USA, Cat. No. sc-48342), pro-brain-derived neurotrophic factor (pro-BDNF) (mouse IgG, 1:500; Santa Cruz Biotechnology, Cat. No. sc-65513), glial-derived neurotrophic factor (GDNF) (mouse IgG, 1:250; Santa Cruz Biotechnology, Cat. No. sc-13147), 4-hydroxynonenal (4-HNE) (rabbit IgG, 1:500; Abcam Inc., Cambridge, UK, Cat. No. ab46545), microtubule-associated proteins 1A/1B light chain 3B (LC3B) (rabbit IgG, 1:500, Abcam Inc., Cambridge, UK, Cat. No. ab192890), nerve growth factor (NGF) (mouse IgG, 1:250; Santa Cruz Biotechnology, Cat. No. sc-365944), and p21 (mouse IgG, 1:250; Santa Cruz Biotechnology, Cat. No. sc-817). The following day, tissue sections were washed for 5 min twice with TBS and incubated at room temperature for 2 h in the following secondary antibodies: Vector Laboratories fluorescein horse anti-mouse IgG (1:500; MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. FI-2000) and Alexa Fluor™ 568 goat anti-rabbit IgG (Thermo Scientific Canada, Brockville, ON, Canada, Cat. No. A11011). Sections were then washed twice for 5 min in TBS followed by incubation with 10μM Hoechst 33342 (Molecular Probes, Eugene, OR, USA Cat. No. H3570). Sections were washed twice for 5 min in TBS followed by coverslipping with VECTASHIELD® Vibrance® antifade mounting medium (MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. VECTH18002). Tissue sections were imaged using epifluorescence microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada). Fluorescence was quantified by determining corrected total fluorescence (CTF) in images captured using ImageJ software. For each group, the fluorescence was quantified for each specified protein per overall SN image captured.

Results

Comparison of E-ASH and WS-ASH treatment in protecting DA neurons from PQ-induced neurotoxicity and dose dependency of WS-ASH

PQ injected rats given the PTS carrier showed drastic neurodegeneration in the SN as indicated by minimal immunoreactivity for tyrosine hydroxylase (TH) a marker of DA neurons (Figure 1). Rats given E-ASH still showed reduced immunoreactivity for TH indicating DA neuron loss but neurons that did remain showed better morphological protection compared to PTS treated rats as indicated by the greater abundance of fibers extending from the cell bodies. While there was not a positive control group (i.e. saline injected animals fed plain drinking water), this observation of reduced neurodegeneration and protected neuron morphology supports previously published observations [34]. Animal fed WS-ASH at the same dose as E-ASH (2mg/mL in water) showed better neuroprotection as indicated by increased TH immunoreactivity and maintained neuron morphology (increased fibers as mentioned prior). Furthermore, WS-ASH was still as effective at protecting DA neurons at half the dose of 1mg/mL as indicated by increased TH immunoreactivity and protected morphology compared to PTS fed animals. Finally, while we were only able to use one additional dose in this experiment, we did observe a dose dependency with WS-ASH as 0.2mg/mL of WS-ASH was not very effective at protecting SN DA neurons compared to 2 or 1mg/mL WS-ASH as indicated by similar immunoreactivity to 2mg/mL E-ASH fed animals.

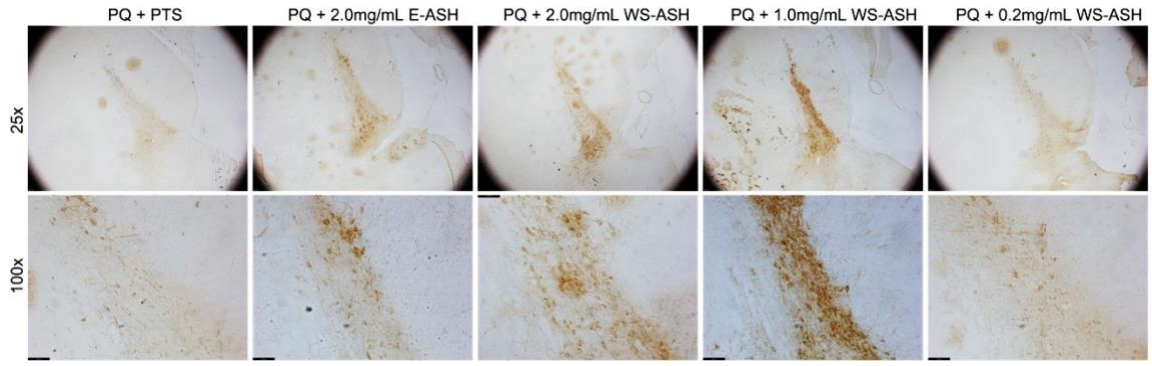

Figure 1. Effect of ethanolic ashwagandha extract (E-ASH) and water-soluble ashwagandha extract (WS-ASH) on neurodegeneration of substantia nigra neurons in saline/paraquat (PQ) injected rats. Light micrographs of midbrain sections showing tyrosine hydroxylase (TH) positive neurons at 25x (scale bar = 100 microns) and 100x (scale bar = 100 microns) in the substantia nigra. Images are representative of 3 independent experiments with similar trends.

Effect of Ubisol-Q10 and WS-ASH treatment in protecting DA neurons from PQ-induced neurotoxicity

Following dosing determination for WS-ASH as seen in figure 1, we used a drinking water concentration of 1mg/mL WS-ASH for the experiment involving the combination of WS-ASH and Ubisol-Q10. Following PQ injections, we observed neurodegeneration of DA neurons in rats fed water supplemented with PTS compared to the saline injected/plain water control as indicated by reduced TH CTF (Figures 2 and 3). PQ injected animals fed Ubisol-Q10 showed increased protection of DA neurons as indicated by increased TH CTF compared to PTS fed animals. PQ injected animals fed WS-ASH showed greater DA neuron protection compared to Ubisol-Q10 fed animals indicated by greater TH CTF. PQ injected rats fed the tonic of both Ubisol-Q10 and WS-ASH showed the greatest protection of DA neurons as indicated by TH CTF most similar to saline injected animals.

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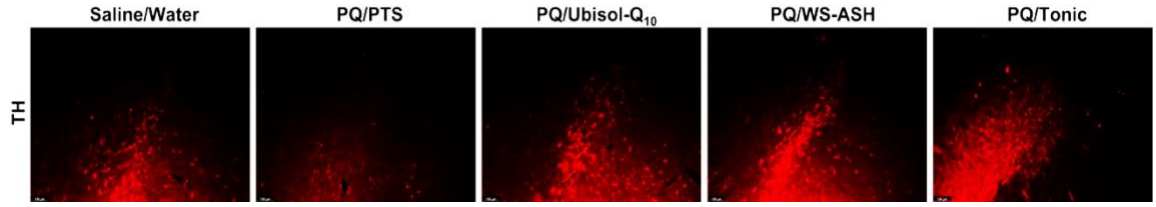

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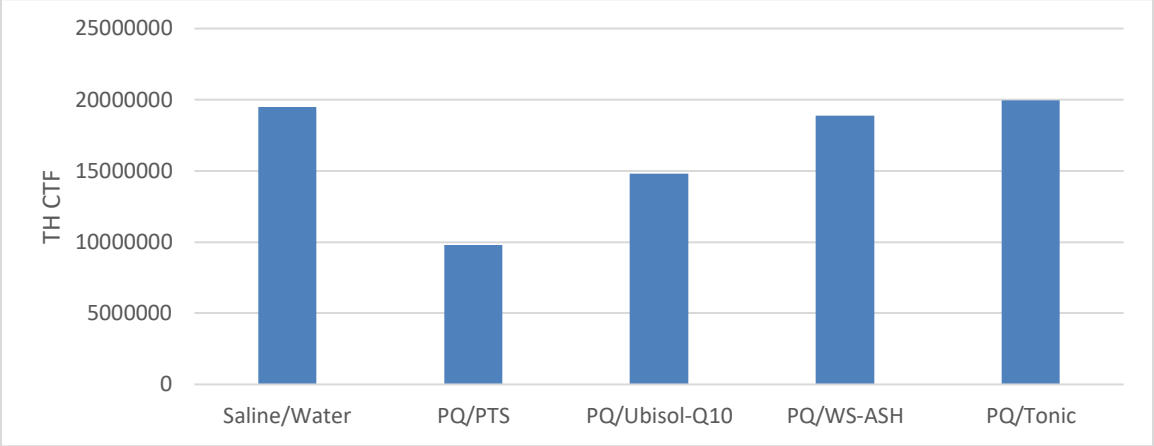

Figure 2. Effect of Ubisol-Q10 and water-soluble ashwagandha extract on neurodegeneration of substantia nigra neurons in saline/paraquat (PQ) injected rats. **(A)** Immunofluorescent staining of the tyrosine hydroxylase (TH) neurons at 100x (scale bar = 100 microns) magnification in the substantia nigra (SN). **(B)** Fluorescent quantification of fluorescence of TH. Images are representative of 3 independent experiments with similar trends.

Effect of Ubsiol-Q10 and WS-ASH treatment on microglia, astroglia, and neurotrophic factors

Following staining for DA neurons, we wanted to investigate the status of microglia and astroglia in response to WS-ASH. Previously, we reported the Ubisol-Q10 and E-ASH were effective at reducing pro-inflammatory microglia activity and enhance activation of pro-survival astroglia [34]. Increased IBA1 CTF was observed in PQ injected rats fed PTS compared to saline injected animals (Figure 4). PQ injected rats given Ubisol-Q10, WS-ASH, or the tonic all showed reduced IBA1 CTF compared to PTS-fed animals appearing similar to saline injected animals (Figure 4). Following microglia staining, we probed for the status of pro-survival astroglia. Following PQ injections, PTS fed rats showed reduced GFAP CTF compared to control animals (Figure 5). PQ injected animals fed Ubisol-Q10 showed greater GFAP CTF compared to PTS fed animals, but activation was still reduced compared to control animals. PQ injected animals given treatment with WS-ASH or the tonic showed greater GFAP CTF compared to the saline injected animals. Along with providing direct support to neurons, astroglia are also known to support neurons via affecting neurotrophic factor expression/secretion [38-40]. Here we probed for the neurotrophic factors NGF, pro-BDNF, and GDNF. PQ injected animals given Ubisol-Q10, WS-ASH, or the tonic all showed increased presence of all three neurotrophic factors compared to PQ injected animals given PTS appearing similar to the control (Figures 3-5).

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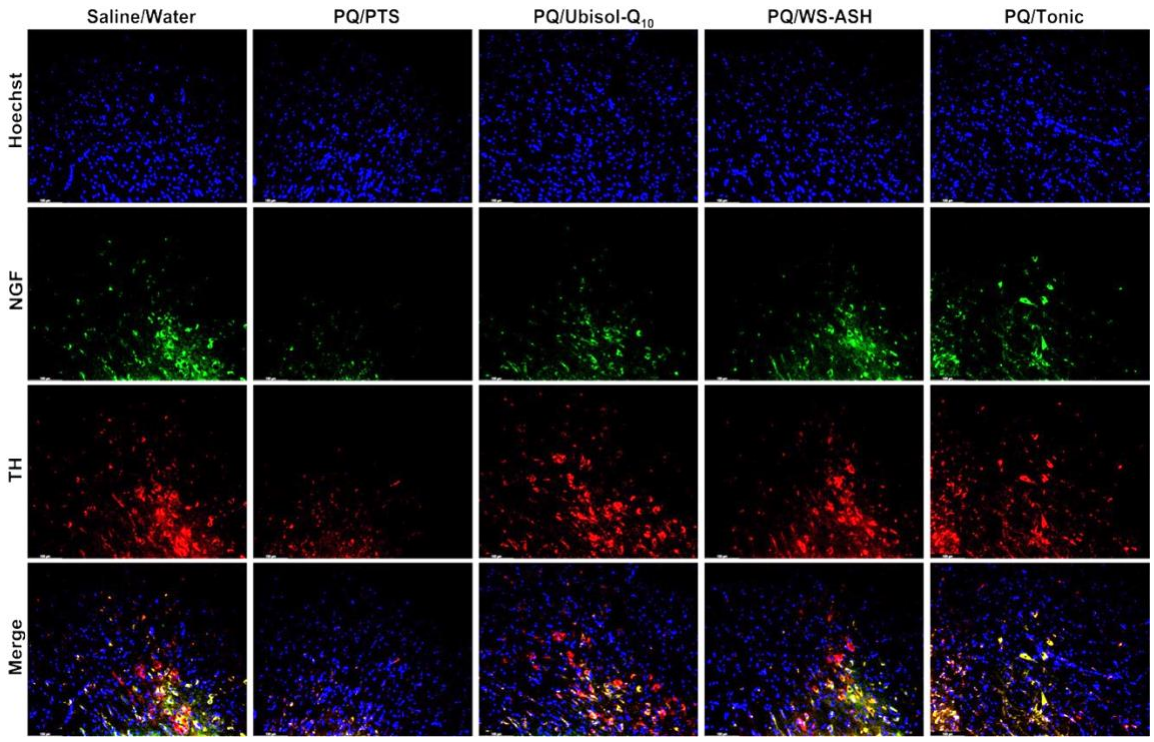

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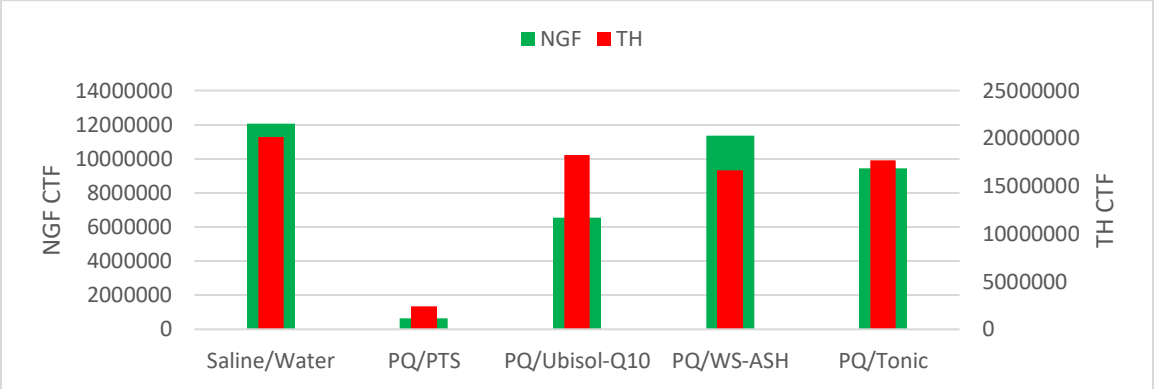

Figure 3. Effect of Ubisol-Q10 and water-soluble ashwagandha extract on neurodegeneration of substantia nigra neurons and nerve growth factor (NGF) secretion in saline/paraquat (PQ) injected rats. (A) Immunofluorescent staining of the tip of the SN in midbrain sections probing for pro-survival factor NGF, and tyrosine hydroxylase (TH). **(B)** Quantification of fluorescence of NGF and TH. Nuclei were counterstained with Hoechst. Micrographs were taken at 200x magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

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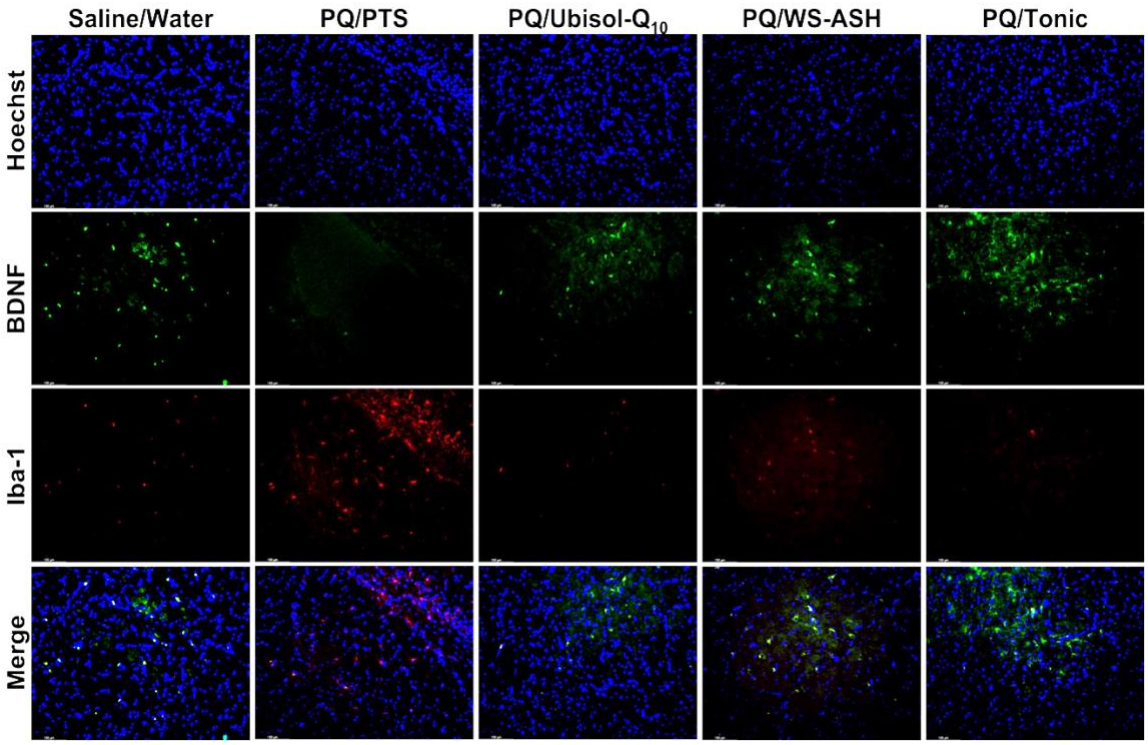

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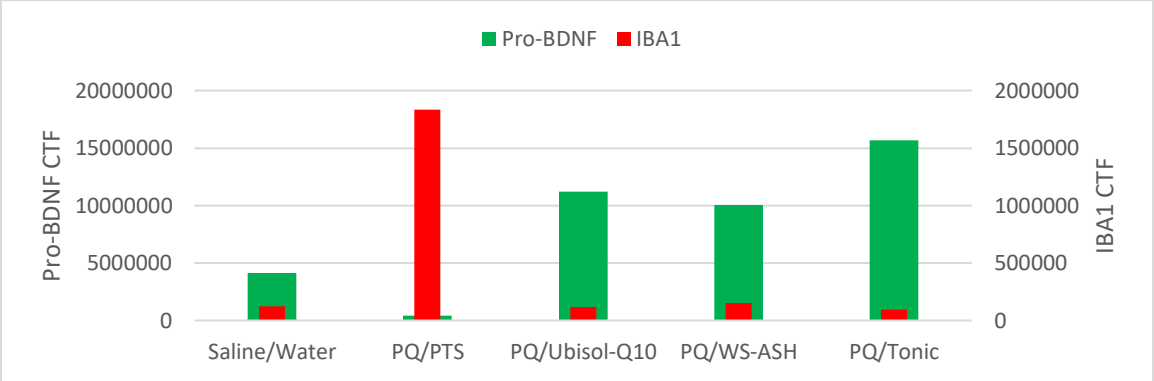

Figure 4. Effect of Ubisol-Q10 and water-soluble ashwagandha extract on pro-brain derived neurotrophic factor (pro-BNDF) and proinflammatory microglia. (A) Immunofluorescent staining of the tip of the SN in midbrain sections probing for pro-survival factor pro-BNDF, and ionized calcium-binding adapter molecule (IBA1), a marker of microglia activation. **(B)** Quantification of fluorescence of NGF and IBA1. Nuclei were counterstained with Hoechst. Micrographs were taken at 200x magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

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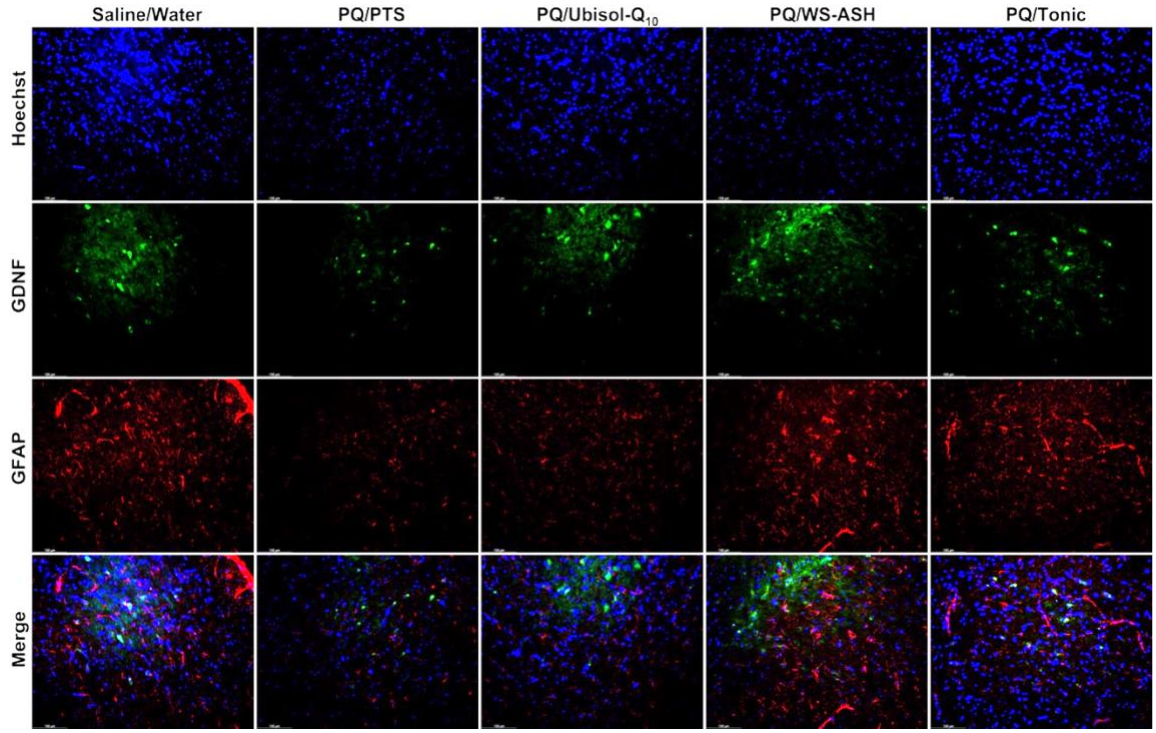

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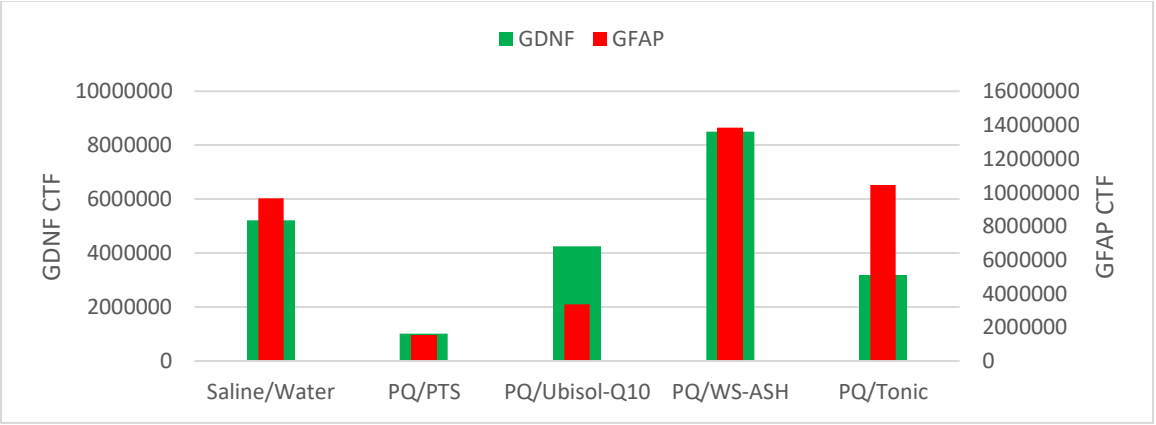

Figure 5. Effect of Ubisol-Q10 and water-soluble ashwagandha extract on glial derived neurotrophic factor expression (GDNF) and pro-survival astroglia. (A) Immunofluorescent staining of the tip of the SN in midbrain sections probing for pro-survival factor GDNF, and glial fibrillary acidic protein (GFAP), a marker of astroglia. **(B)** Quantification of fluorescence of GFAP and GDNF. Nuclei were counterstained with Hoechst. Micrographs were taken at 200x magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Antioxidant and senolytic effects of Ubisol-Q10 and WS-ASH treatment against PQ-induced neurotoxicity

It's been previously reported that Ubisol-Q10 and ashwagandha in WS-ASH have both antioxidant and senolytic properties. Indeed, we have previously seen that Ubisol-Q10 was a potent antioxidant and prevented premature senescence in Alzheimer's Diseased fibroblasts via the resumption of autophagy [41,42]. Also, our extract was shown to contain the senolytic flavonoid, quercetin [34,43]. Following PQ injections, animals fed PTS in their drinking solutions showed increased CTF compared to saline injected animals of both senescence associated protein p21 and lipid peroxidation product and marker of oxidative stress 4-HNE (Figure 6). PQ animals given Ubisol-Q10 alone, WS-ASH alone, or the tonic all had reduced CTF for p21 and 4-HNE appearing similar to saline injected animals.

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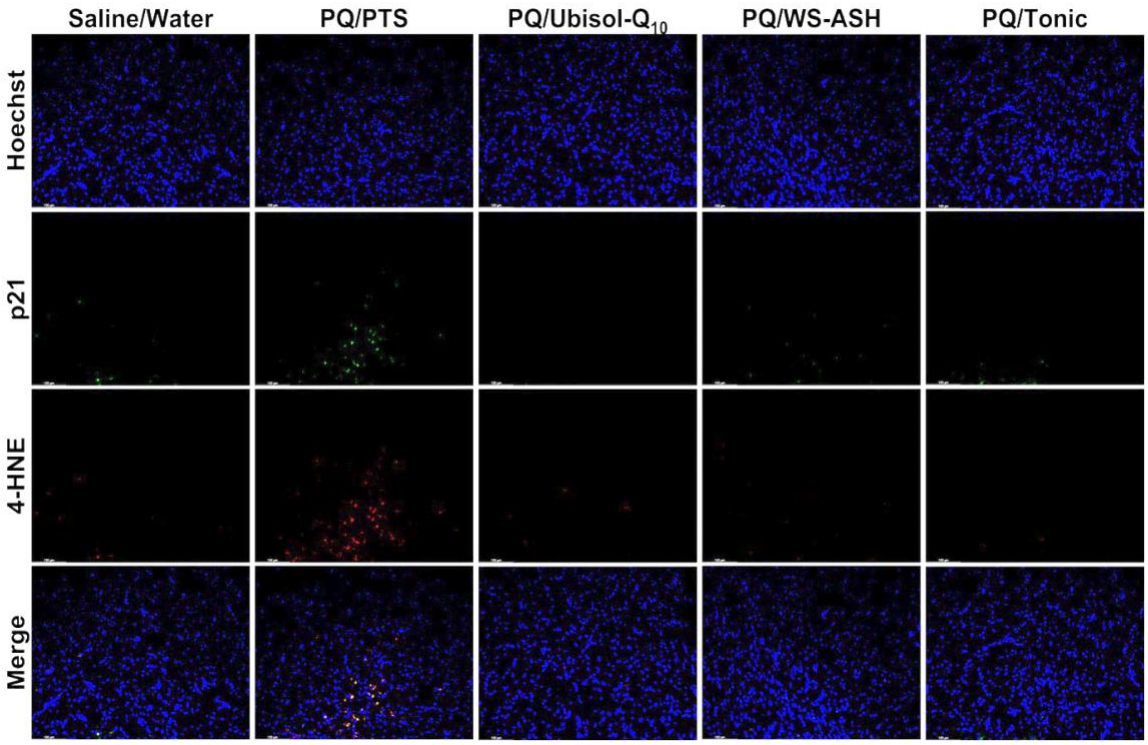

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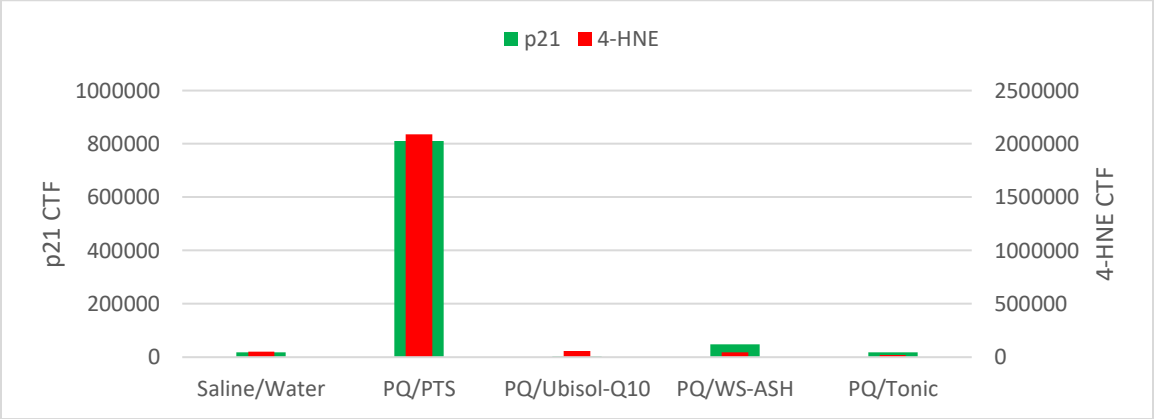

Figure 6. Effect of Ubisol-Q10 and water-soluble ashwagandha extract on p21 expression and levels of 4-hydrononenal (4-HNE). (A) Immunofluorescent staining of the tip of the SN in midbrain sections probing for lipid peroxidation by-product/oxidative stress marker 4-HNE, senescence marker p21. **(B)** Quantification of fluorescence of 4-HNE and p21. Nuclei were counterstained with Hoechst. Micrographs were taken at 200x magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Effect of Ubisol-Q10 and WS-ASH treatment on beclin-1 and LC3B expression

Previously we have observed that Alzheimer's Diseased fibroblast containing a presenilin-1 and double transgenic Alzheimer's Diseased (AD) mice treated with Ubisol-Q10 had increased expression for major autophagy regulator beclin-1 [41,42]. While this observation was made in an AD model, PD and AD share similar biochemical aetiologies. We also have reported that the same observation was made in a previous experiment involving E-ASH in a PQ induced model of PD [34]. Here we again wanted to see the status of beclin-1 as well as another autophagy related protein LC3B in the brains of PQ injected rats in response to WS-ASH. PQ injected rats fed PTS showed reduced CTF for beclin-1 and LC3B compared to saline injected animals (Figure 7). When given Ubisol-Q10, PQ injected animals showed increased CTF for belcin-1 and LC3B similar to the control. These observations or consistent what we have previously reported [34,41,42]. Unlike our previous experiment involving E-ASH which showed E-ASH to have minimal effect on expression of these two autophagy proteins, beclin-1 and LC3B expression are both enhanced with WS-ASH, similar to saline injected rats or PQ injected rats given Ubisol-Q10. Animals given the tonic showed increased CTF for beclin-1 and LC3B similar to saline injected animals.

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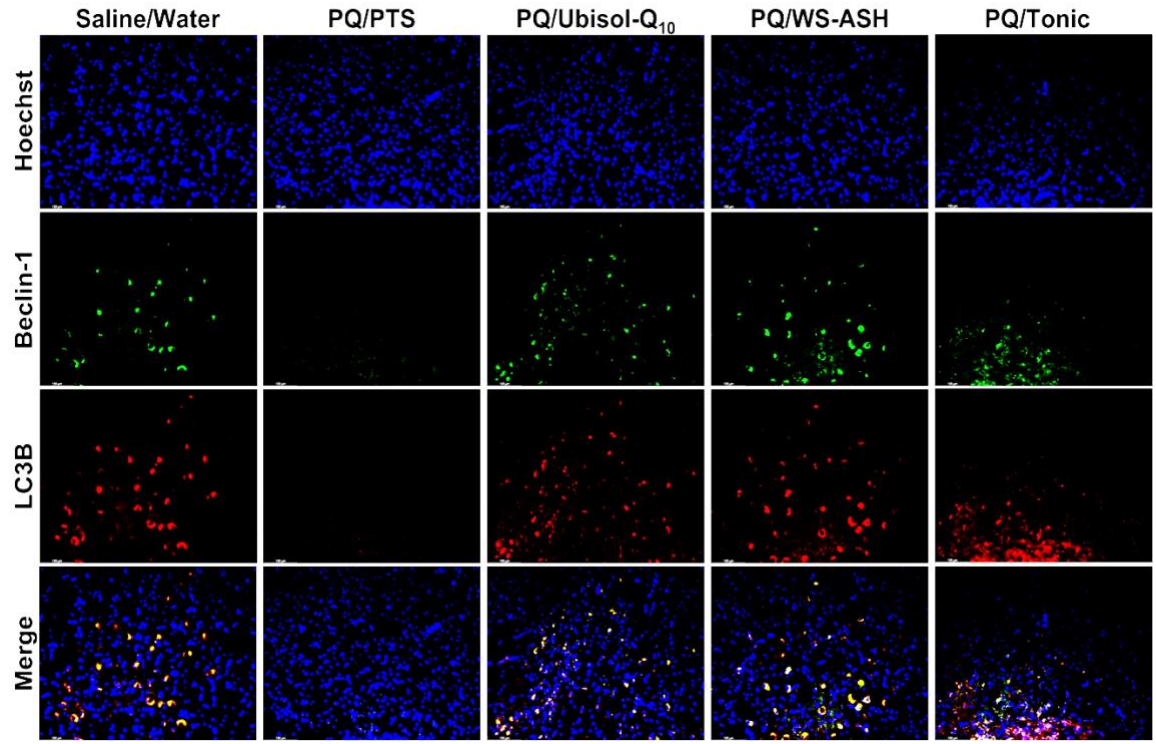

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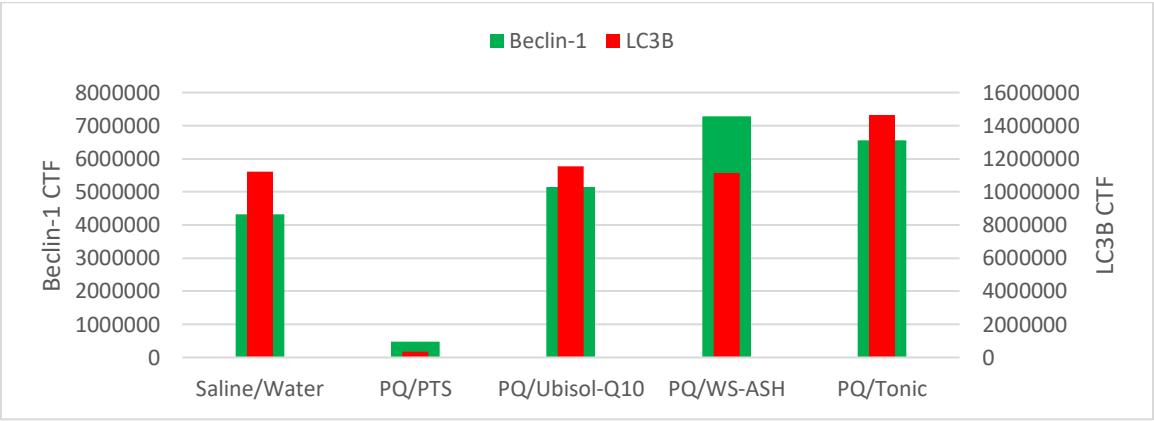

Figure 7. Effect of Ubisol-Q10 and water-soluble ashwagandha extract on beclin-1 expression and levels of LC3B. (A) Immunofluorescent staining of the tip of the SN in midbrain sections probing for major autophagy regulator, beclin-1, and autophagy protein LC3B. **(B)** Quantification of fluorescence of 4-HNE and p21. Nuclei were counterstained with Hoechst. Micrographs were taken at 200x magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Discussion

In this study we created a water-soluble formulation of an ethanolic extract of ashwagandha root using PTS technology previously used in Ubisol-Q10, a water-soluble formulation of coenzyme-Q10. WS-ASH was found to be more efficacious in protecting DA neurons in a PQ rat model of PD compared to our E-ASH used previously at half the dose used for E-ASH. Also, we combined WS-ASH with Ubisol-Q10 and determined that the two reagents combined were more effective at protecting DA neurons than the agents alone which was consistent with our previous work with E-ASH. Here, we report these exciting observations of the neuroprotective effects of our newly developed WS-ASH alone as well as in combination with another known neuroprotective nutraceutical, Ubisol-Q10.

We employed a well-established rodent model of PD using rats treated with a series of intraperitoneal injections of PQ, which leads to neurodegeneration of DA neurons in the SN [33,34], to test the efficacy of our newly developed water-soluble formulation of ethanolic ashwagandha extract (WS-ASH). Following treatment with PQ, rats given the PTS carrier displayed reduction in number of dopaminergic neurons (Figure 1). PQ inject rats given E-ASH at a dose of 2mg/mL displayed some protection of DA neurons compared to the PTS carrier as indicated by greater TH immunoreactivity. This observation is consistent with previous work with E-ASH as reported by Vegh et al. [34]. As mentioned before, while E-ASH was effective at protecting DA neurons either alone or in combination with Ubisol-Q10, there was the economic issue of using this extract due to the time and cost to obtain small yields of ashwagandha root extract. Therefore, we sought to use the same PTS technology used in Ubisol-Q10 to enhance solubility of ashwagandha and therefore increase bioavailability and be able to reduce the dose of extract required without impacting protective efficacy. WS-ASH was made by a patented process in collaboration with Next™ Remedies. WS-ASH at 2mg/mL, was more effective than the 2mg/mL E-ASH at protecting DA neurons as indicated by the increased TH immunoreactivity (Figure 1). Furthermore, 1mg/mL WS-ASH was as effective as 2mg/mL WS-ASH indicating the neuroprotective effectiveness of WS-ASH plateaus beyond 1mg/mL. While 1mg/mL WS-ASH does stain darker than 2mg/mL WS-ASH, the overall number of neurons are the same

with the discrepancy due to slight differences in background than occur during immunohistochemistry. A dose dependency was observed with WS-ASH as 0.2mg/mL WS-ASH was not as effective at preventing DA neuron death compared to other doses appearing similarly to 2mg/mL E-ASH treated animals (Figure 1). Along with protecting neuron count, we observed that WS-ASH protected neuron morphology (abundance of fibers extending from cell body) similar to E-ASH as previously reported [34]. From these results, we decided to use 1mg/mL WS-ASH in the next part of this study.

As mentioned before, PD is a multifactorial disease and therefore targeting only one or a few of the biochemical mechanisms of PD with one agent (i.e., Ubisol-Q10, E-ASH, OR WS-ASH) is not enough to halt/reduce DA neuron depletion in the SN. Therefore, we combined Ubisol-Q10 with WS-ASH to observe if this combination (Tonic) is more affective at protecting DA neurons compared to them alone. Again, we used a well-established method to induce Parkinsonism in rats using PQ [33,34]. Similar to what was observed in Figure 1, PQ-injected rats fed drinking water containing PTS showed marked reduction of DA neurons compared to the saline-injected rats fed plain drinking water (Figure 2,3). Treatment with either Ubisol-Q10 or WS-ASH alone protected some DA neurons, there was still an overall reduction in the amount of neurons compared to the saline control animals. The tonic containing both Ubisol-Q10 and WS-ASH was most effective at protecting DA neurons as the amount of DA neurons staining positive for TH was nearly identical to that of the saline control group.

The number of active pro-inflammatory microglia and pro-survival astroglia are reported to affected in PD [24-29]. To investigate the status of microglia and astroglia, we probed for IBA1 and GFAP which are markers of microglia and astroglia respectively. Following treatment with PQ, the amount of active microglia was increased in the brains of rats fed PTS-supplemented drinking water as indicated by greater IBA1 CTF seen in Figure 4. PQ-injected rats treated with Ubisol-Q10, WS-ASH or the tonic showed decreased fluorescence/reduced CTF for IBA1 indicating reduced amount of active pro-inflammatory microglia compared to PQ-injected, PTS-fed rats (Figure 4). Similar to the TH probing in Figure 2, these observations of reduced microglia following tonic treatment are consistent with previous work done with E-ASH [34]. Following probing for the status

for pro-inflammatory microglia, we wanted to see the status of astroglia in response to the tonic treatment. PQ-injected rats fed PTS showed a decrease in amount of active pro-survival astroglia as indicated by reduced GFAP CTF compared to the saline-injected control rats (Figure 5). PQ-injected rats fed Ubisol-Q10-supplemented water had elevated levels of astroglia compared to PTS fed rats but amounts of active astroglia were still reduced compared to the saline control animals (Figure 5). Following treatment with either WS-ASH or the tonic, rats injected with PQ showed a greater increase in levels of pro survival astroglia compared to all other groups (Figure 5).

Along with providing direct support to neurons, astroglia are also known to support neurons via affecting pro-survival neurotrophic factor expression/secretion [38-40]. Nerve growth factor (NGF) is considered an essential neurotrophic factor and its absence results in the apoptosis of certain neurons [44]. Following treatment with PQ, rats given PTS showed a decrease in NGF compared to control animals (Figure 3). PQ-injected rats given Ubisol-Q10, WS-ASH, or the tonic all showed an increase in levels of NGF compared to PTS-fed rats but were still reduced compared to saline control rats. Along with NGF, astroglia are known to modulate pro-survival neurotrophic factors GDNF and BDNF [38-40]. PTS-fed rats that were injected with PQ showed a reduction in the amount of pro-BDNF compared to the control animals (Figure 4). Following treatment with Ubsiol-Q10, WS-ASH, or the tonic, PQ-injected rats showed a greater increase in pro-BDNF expression compared to the saline control animals and PQ-injected, PTS-fed animals. GDNF was decreased in PQ-injected rats fed PTS compared to the control rats (Figure 5). PQ-injected rats treated with Ubisol-Q10 showed an increased in GDNF compared to the PTS treated animals but was GDNF levels were still lower compared to the control. WS-ASH fed animals showed the highest-level expression which also coincided with the greatest levels of astroglia activation compared to all other animal groups. Animals fed the tonic did show an elevated amount of GDNF compared to PQ/PTS animals, but expression was reduced compared to the saline control or PQ-injected rats fed Ubisol-Q10 or WS-ASH. It's possible the reason for this reduction could be that less of a dependency is required on the protective properties of GDNF in the tonic due to the presence of two potent neuroprotective nutraceuticals. With these two agents combined they could be having a more direct protective effect (i.e., directly acting as two antioxidants rather than one, or

greater presence of pro-BDNF saving neurons, therefore resulting in less dependency on GDNF).

Following investigation of glia and neurotrophic factor status, we wanted to investigate the status of oxidative stress and cellular senescence in response to the treatment of Ubisol-Q10, WS-ASH, or the tonic. 4-HNE, a lipid peroxidation by-product due to elevated ROS and marker of oxidative stress was increased in PQ-injected rats given PTS-supplemented water compared to saline control animals (Figure 6). Treatment with Ubisol-Q10, WS-ASH, or the tonic resulted in almost complete elimination of 4-HNE presence in PQ-injected animals compared to PTS-fed animals. Along with oxidative stress, interest in cellular senescence and its roll in PD pathogenesis has grown recently. Previously we have reported that oxidative stress in Alzheimer's Diseased fibroblasts leads to premature senescence which was negated by Ubisol-Q10 [41,42]. As mentioned before, AD and PD share similar biochemical aetiologies (oxidative stress, mitochondrial dysfunction, autophagy inhibition, and inflammation), therefore its possible that senescence is playing a similar role in PD. Indeed, senescent glia have been reported to contribute to PD development [45,46]. As cells become senescent, they can secrete senescence associated phenotype factors such as IL6/7, IL-1β, HGF, etc. which can lead to inflammation and oxidative stress [43]. In this study we investigated the status of cellular senescence by probing for p21, a marker of cellular senescence [41,42,45,46]. Similar to 4-HNE, p21 expression was only observed in the SN of PQ-injected rats fed PTS. It's possible that the same mechanism observed by Ma et al. or Vegh et al. [41,42] is happening here in that the tonic (or even the agents alone) is acting as an antioxidant and preventing premature senescence of glia cells though more work is needed to confirm this hypothesis.

Previously, it's been shown that autophagy is impaired in the SN of the brains of PQ treated rats [34]. Autophagy inhibition is an especially important cellular mechanism involved in PD development/progression as it is involved in the clearance of defective mitochondria are known to accumulate in PD. These defective mitochondria produce ROS leading to apoptosis [16,21,23]. The expression of beclin-1, a major autophagy regulator, and LC3B, a protein involved in autophagy were both reduced in PQ-injected rats fed PTS compared to the saline control animals (Figure 7). Animals fed Ubisol-Q10 both showed

resumed autophagy appearing similar to the control as indicated by increased expression of beclin-1 and LC3B. These results are consistent with previous observations made with Ubisol-Q10 in various AD and PD models [34,42]. Surprisingly, PQ-injected rats fed WS-ASH also showed increased expression of beclin-1 and LC3B which was not previously observed with E-ASH [42]. Rats given the tonic showed increased resumption of autophagy similar to the other groups except for PTS-fed rats.

Conclusions

Here we report that a newly developed water-soluble formulation of an ashwagandha root extract (WS-ASH) was more effective compared to its ethanolic counterpart at protecting DA neurons in a PQ induced rat model of PD. Furthermore, with the tonic of WS-ASH combined with Ubisol-Q10, there was a greater amount of neuroprotection compared to the agents alone as there are more than one biochemical mechanism of PD (oxidative stress, mitochondrial dysfunction, autophagy impairment, neuroinflammation (activation of pro-inflammatory microglia and suppression of pro-survival astroglia), and senescence). Both Ubisol-Q10 and WS-ASH are nutraceuticals and had no negative impact on animals' health during the experiments and therefore can be taken over a long period of time. Furthermore, the tonic halted the progressive neurodegeneration that is not prevented with conventional PD treatments. This formulation has the potential to be developed into a therapeutic that can improve the quality of life of PD patients.

Patents

Ubisol-Q10 and WS-ASH is a patented formula.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 4: Resumption of Autophagy by Ubisol-Q10 in Presenilin-1 Mutated Fibroblasts and Transgenic AD Mice: Implications for Inhibition of Senescence and Neuroprotection

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List of Abbreviations

AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
PS-1	Presenilin-1
PS-2	Presenilin-2
ROS	Reactive Oxygen Species
MCI	Mild Cognitive Impairment
TOC	Total Antioxidant Activity
ETC	Electron Transport Chain
SIPS	Stress Induce Premature Senescence
NHF	Normal Human Fibroblast
PSAF	PS-1 Mutated AD Familial Type 3 Fibroblast
PTS	Polyoxyethanyl-α-Tocopheryl Sebacate
MAPK8	Mitogen Activated Protein Kinase 8
JNK1	C-Jun N-terminal Kinase 1
H_2DCFDA	2′-7′-Dichlorofluorescein Diacetate
SA-β-gal	Senescence-Associated Beta-Galactosidase
MDC	Monodansylcadaverine
4-HNE	4-Hydroxynonenal
DAB	3,3′-diaminobenzidine

LC3B	Microtubule-associated proteins 1A/1B light chain 3B
GRAS	Generally Regarded as Safe
CTSB	Cathepsin B

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and leading form of dementia across the globe. AD is characterized by decline in neurocognitive function leading to severe morbidity and eventually death [1]. While the majority of cases of AD are sporadic, mutations in the genes coding for amyloid precursor protein (APP) and presenilin-1 and presenilin-2 (PS-1 and PS-2) have been linked to familial and early-onset AD [2–4]. The exact etiology of AD is unknown, but some pathological features include the formation of toxic β-amyloid plaques and neurofibrillary tangles and neuron loss in the hippocampus [5, 6]. Furthermore, there are a number of mechanisms associated with AD which include the following: (1) increased oxidative stress [7], (2) mitochondrial dysfunction, and (3) impaired autophagy activity and accumulation of defective proteins/organelles [8]. Furthermore, it is hypothesized that these brain lesions/biochemical mechanisms occur before the symptoms of AD show implying that neuronal death is happening before disease diagnosis [9]. Current therapies only reduce the symptoms of AD, and there are unfortunately no treatments available that can prevent the progression of the disease. Many of the therapies used to treat symptoms of AD are chemomodulators, and extended use has shown to have toxic and adverse psychological side effects.

Neurons are virtually entirely dependent on oxidative phosphorylation for energy production, and as a result, mitochondrial dysfunction and oxidative stress are integral players in the development of AD pathology. Oxidative stress is a phenomenon where the amount of reactive oxygen species (ROS) in a cell increases as a result of reduced detoxification ability within the cell. It has been observed in patients with AD or mild cognitive impairment (MCI), where total antioxidant capacity (TOC) was reduced [10]. Importantly, it is proposed that increased ROS production precedes any other hallmarks of AD. The exact source of ROS in AD is unknown, but in some cases, it is known to originate from dysfunctional mitochondria [11–13]. Mutations in genes coding for components of cytochrome-c oxidase and presenilin-1 (PS-1) were found to be responsible for mitochondrial dysfunction in AD patients [13–15]. Increased mitochondrial dysfunction results in ineffective electron flow in the electron transport chain (ETC) leading to elevated

ROS production. Neuronal cells are dependent on oxidative phosphorylation for energy production and thus are prone to produce more ROS. If the ROS quenching capabilities are reduced, then serious adverse effects occur due to ROS reacting with nucleic acids, proteins, and lipids [16]. Furthermore, increased oxidative stress also causes mitochondrial dysfunction that in turn produces more ROS thus creating a vicious cycle of increasing ROS and mitochondrial dysfunction [17]. This dysfunction has also been observed in peripheral tissue of AD patients including fibroblasts, making them suitable models for observing the biochemical pathology of AD [18]. Prolonged exposure of sublethal doses of ROS was shown to cause stress-induced premature senescence (SIPS) in AD fibroblasts. This phenomenon has been well characterized in fibroblasts with senescence-associated betagalactosidase staining in fibroblasts from AD patients [8, 19].

As a consequence of increased oxidative stress, cells accumulate dysfunctional organelles and proteins that can lead to cellular dysfunction and apoptosis [16]. Cells have evolved efficient mechanisms to eliminate these damaged proteins/organelles using autophagy/proteasome degradation systems. Autophagy is the cell's mechanism for recycling old or damaged cytoplasmic constituents such as organelles or misfolded proteins [20]. There are multiple forms of autophagy, but the main form implicated in AD is macroautophagy. Interestingly, it has been shown that autophagy is either impaired or inhibited in AD patients [20–23]. Under these circumstances, defective/misfolded toxic proteins such as β-amyloid and dysfunctional organelles such as mitochondria can accumulate causing stress on cells leading to eventual cell death. Excessive oxidative stress can also affect various autophagy regulators such as beclin-1 (a major regulator of autophagosome maturation) [24]. Furthermore, PS-1 mutations have been shown to inhibit autophagy progression via blocking autophagosome maturation [25].

Based on these findings, oxidative stress, mitochondrial dysfunction, and autophagy could provide novel therapeutic targets for AD. By targeting these mechanisms, it would be possible to halt neurodegeneration in AD. Previously, we have observed that a water-soluble formulation of coenzyme-Q10 (Ubisol-Q10) has great potential to halt progression of neurodegenerative diseases including Parkinson's and Alzheimer's diseases [26]. Indeed, Ubisol-Q10 stabilized mitochondria and inhibited oxidative stress in vitro

[27, 28]. Ubisol-Q10 also prevented oxidative SIPS and enhanced activation of autophagy via upregulation of beclin-1 (a major regulator of autophagy) in PS-1 mutated fibroblasts [8]. Additionally, Ubisol-Q10 at low doses (orally delivered) reduced circulating Aβ peptide, reduced oxidative stress, had positive effects on long-term and working memory, and drastically inhibited β-amyloid plaque formation in 16-month-old transgenic AD mouse brains [7].

Ubisol-Q10 seems to be a promising therapeutic for targeting AD pathology. Ubisol-Q10 not only is acting as a potent antioxidant but also could act as an activator of autophagy. PS-1 mutated fibroblasts could act as a good model for studying the induction of senescence and autophagy (under increased oxidative stress). Furthermore, the relationship between senescence and autophagy is not well understood. In this paper, we have shown that inhibition of oxidative stress by Ubisol-Q10 could not only inhibit SIPS but also activate autophagy. Here, we measured the differential gene expression profile of oxidative stress/autophagy genes in NHF and PSAF fibroblasts. Results indicated that Ubisol-Q10-treated PSAF cells display gene expression profiles similar to healthy NHF. In particular, there is upregulation of autophagy-related genes which was also confirmed at the protein level. Interestingly, inhibition of autophagy in Ubisol-Q10-treated PSAF cells leads to return of their SIPS phenotype. Furthermore, we demonstrated that autophagy is inhibited in the brains of transgenic AD mice and that it was activated with Ubisol-Q10 treatment. Thus, activation of autophagy is critical for the neuroprotective effect of Ubisol-Q10 in PSAF as well as in transgenic AD mice.

Materials and Methods

Cell Culture

Healthy non-fetal human skin fibroblasts (NHF) and PS-1 mutated AD familial type 3 fibroblasts (PSAF) from healthy and AD patients, respectively (Coriell Institute for Medical Research, Cat. Nos. AG09309 and AG04159, Camden, NJ, USA), were used throughout this study. NHF were derived from the skin of the toe, and PSAF were derived from the skin of the forearm. All fibroblasts were cultured in Eagle's minimum essential

medium with Earle's salts and nonessential amino acids supplemented with 15% (v/v) fetal bovine serum (Thermo Scientific, Waltham, MA, USA) and 10mg/mL gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada). PSAF were grown in medium with or without supplementation with 50 µg/mL Ubisol-Q10 (provided by Next™ Remedies, Toronto, ON, Canada) or with the PTS carrier. During the autophagy inhibition experiments mentioned below, another treatment group included PSAF that had Ubisol-Q10 withdrawn during the 48hr period. All fibroblasts were grown at 37˚C and 5% CO_2.

Autophagy Inhibition

PSAF in the above-mentioned growth conditions were also subjected to autophagy inhibition via incubation with JNK1 inhibitor SP600125. SP600125 is a well-known inhibitor of autophagy via beclin-1 inhibition as JNK1 is a major regulator of beclin-1 activation. PSAF were incubated for 48hrs in media containing 10µM SP600125 (Sigma-Aldrich, Oakville, ON, Canada, Cat. No. S5567) and 0.1% DMSO to maintain solubility.

Quantitative Polymerase Chain Reaction (qPCR) of Autophagy and Oxidative Stress-Related Genes

A RT^2 profile PCR assay was performed in order to measure the relative gene expression of autophagy and oxidative stress-related genes. RNA from cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc., Toronto, ON, Canada, Cat. No. 74106). RNA quality and quantity were determined my measuring the A280:A260 (Nanodrop 200). cDNA was produced from RNA extracts using a RT^2 First Strand Kit (Qiagen Inc., Toronto, ON, Canada, Cat. No. 330401). Following cDNA synthesis, qPCR was performed on samples using the RT^2 Profiler PCR Array Human Oxidative Stress Plus Array (Qiagen Inc., Toronto, ON, Canada, Cat. No. PAHS-065Y). The array containing 84 primers that probe for autophagy and oxidative stress-related genes was performed following the manufacturer's protocol using SYBR Green. Real-time amplification data was acquired using the ABI ViiATM 7 real-time PCR system with a 384-well block and respective ABI ViiATM 7 software. Amplification occurred for 40 cycles for 15 s at 95˚C and 1 min at

60˚C. A melting curve of each sample confirmed specificity of amplification, and gene expression was normalized to housekeeping genes. Results were obtained as fold changes in gene expression between the controls and treated groups using the ΔΔCT method.

Measurement of Reactive Oxygen Species (ROS)

ROS production was measured by membrane permeable 2′-7′-dichlorofluorescein diacetate (H_2DCFDA) (Life Technologies Inc., Cat. No. D-399, Burlington, ON, Canada) which is oxidized by ROS to fluorescent 2′,7′-dichlorofluorescein (DCF) following cleavage of acetate groups by intracellular esterases. Cells were incubated in 10μM H_2DCFDA dissolved in DMSO for 30 min at 37˚C. DCF fluorescence was detected using epifluorescence microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada). Fluorescence was quantified in images captured using ImageJ software.

Senescence-Associated Beta-Galactosidase (SA-β-gal) Staining

SA-β-gal stain was used to detect prematurely senescent fibroblasts. Cells were washed in 1x PBS, fixed for 4 min at room temperature in 3% formaldehyde, washed with 1x phosphate-buffered saline (PBS) again, and incubated at 37˚C with no CO_2 with fresh SA-β-gal staining solution (1 mg/mL X-Gal, 20 mg/mL dimethylformamide, 40 mM citric acid, 40 mM sodium phosphate, 5 mM potassium ferrocyanide, 5mM potassium ferricyanide, 150mM NaCl, and 2 mM $MgCl_2$, pH 6.0) for 16 hrs. Senescent cells were detected using phase contrast microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada). The proportion of cells staining positive for SA-β-gal activity was counted manually.

Monodansylcadaverine (MDC) Staining for Autophagic Vacuoles

Cells were seeded on 4 chamber slides (Bio Basic Inc., Markham, ON, Canada, Cat. No. SP41215) 24 hrs prior to experimentation. Cells were incubated with 0.1 mM

MDC (Sigma-Aldrich, Canada, Cat. No. 30432, Mississauga, ON, Canada) dissolved in DMSO for 15 min. Cells were washed with PBS, and cells containing autophagic vacuoles tagged with MDC were detected using epifluorescence microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada). Fluorescence was quantified in images captured using ImageJ software.

Immunofluorescence Staining

Cells were seeded on 4 chamber slides (Bio Basic Inc., Markham, ON, Canada, Cat. No. SP41215) 24hrs prior to experimentation. Cells were fixed with 3.7% formaldehyde at room temperature, followed by permeabilization with 0.15% Triton X-100 for 2 minutes, and then blocked with 5% bovine serum albumin (BSA) for 1hr at room temperature. Cells were incubated for 1hr at room temperature in the following primary antibodies: beclin-1 (mouse IgG, 1:500, Cat. No. sc-48342), C-Jun terminal kinase 1 (JNK1) (mouse IgG, 1:500, Cat. No. sc-137018), p21 (mouse IgG, 1:250, Cat. No. sc-817) (Santa Cruz Biotechnologies), 4-hydroxynonenal (rabbit IgG, 1:500, Cat. No. ab46545), and LC3B (rabbit IgG, 1:500, Cat No. ab192890) (Abcam Inc.). Cells were washed with PBS and incubated with horse anti-mouse FITC (1:500, MJS BioLynx Inc., Cat. No. Fl-2000) and/or a goat anti-rabbit Alexa Fluor™ 568 (1:500, Thermo Scientific Canada, Cat. No. A11011) secondary antibody for 1 hr at room temperature. Cells were washed again with PBS and incubated with 10μM Hoechst 33342 (Molecular Probes, Eugene, OR, USA Cat. No. H3570). Cells were imaged using epifluorescence microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada). Fluorescence was quantified in images captured using ImageJ software.

Western Blot Analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on protein samples from fibroblasts and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% w/v milk TBST (tris-buffered saline Tween-20) solution for 1 hr. Membranes were incubated in mouse anti-

beclin-1 IgG (1:1000, Santa Cruz Biotechnology Inc., Mississauga, ON, Canada, Cat. No. sc-48342) overnight at 4˚C. Membranes were washed with TBST, incubated in goat anti-mouse horseradish peroxidase-conjugated secondary IgG (1:2000, Novus Biologicals, Oakville, ON, Canada, Cat. No. NBP2-30347H) for 1hr at room temperature, washed with TBST, and imaged for bands with a chemiluminescence reagent (Thermo Scientific Canada, Cat. No. 34095). Densitometric analysis was performed using ImageJ software.

Animal Care

The same protocol was performed according to Muthukumaran et al. 2018. Experiments performed on animals were approved by the University of Windsor's Animal Care Committee in accordance with the Canadian Council of Animal Care guidelines. Twelve male double transgenic APP/PS-1 mice (Jackson Laboratory; strain: B6C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax) and six male C57BL/6 wild-type counterpart mice (Charles River Laboratories) were housed in groups of three or four. Transgenic mice were housed separately to avoid any social hierarchies due to functional neurological changes. The home cages contained baby-food jars, overturned cardboard cup holders, and cardboard tubes to provide environmental enrichment. Mice had continuous access to food and water, and their weight was measured once a week. The colony room was maintained at 20˚C, and mice were under a controlled 12 hr : 12 hr dark-light cycle. Following the experimental period, the mice (approximately 18 months old) were euthanized and perfused using ice-cold PBS containing 28 μg/mL heparin (Sigma-Aldrich, Canada, Cat. No. H3393) followed by tissue fixation with ice-cold 10% formaldehyde made in PBS.

Animal Treatment Regimen

The treatment group consisted of Ubisol-Q10 (Zymes LLC, Hasbrouck, NJ, USA)-supplemented drinking water at a concentration of 200 μg/mL which contained an equivalent of 50 μg/mL. The control groups consisted of either water supplemented with the PTS carrier molecule (Zymes LLC, Hasbrouck, NJ, USA) or regular drinking water. Fresh water was provided weekly, and the treatment period lasted 18 months.

Immunohistochemistry

Following perfusion, brains were extracted and stored in 10% formalin at 4˚C. Brains were transferred to 30% (w/v) sucrose (made in 1x PBS) prior to sectioning. Once brains sank in 30% sucrose, they were cryosectioned at 30µm thickness with Shandon™ M-1 embedding matrix (Thermo Scientific Canada, Cat. No. 1310TS) onto glass microscope slides. Slides were washed twice with tris-buffered saline (TBS) for 5 min each followed by incubation with 1% H_2O_2 to block endogenous peroxidases. Slides were rinsed twice with TBS for 5min each followed by a 30 min block using a DAKO serum-free protein block (Agilent Technologies Canada Inc., Cat. No. X0909) and normal serum according to instructions of the Vector Laboratories Vectastain Elite ABC-Peroxidase kit, mouse IgG (MJS BioLynx Inc., Cat. No. VECTPK6102). Following blocking, sections were incubated overnight at 4˚C in the following primary antibodies: beclin-1 (mouse IgG, 1 : 500, Cat. No. sc-48342) and C-Jun terminal kinase 1 (JNK1) (mouse IgG, 1:500, Cat. No. sc-137018) (Santa Cruz Biotechnologies). The slides were washed twice with TBS for 5min followed by incubation of secondary biotinylated antibody according to instructions from the Vectastain Elite ABC-Peroxidase kit. Slides were washed again with TBS, and then, tissue sections were incubated with avidin-conjugated horseradish peroxidase from the Vectastain Elite ABC-Peroxidase kit for 45 min. Slides were washed with TBS and incubated with 3,3′-diaminobenzidine (DAB) stain solution according to the Vector Laboratories DAB peroxidase substrate kit (MJS BioLynx Inc., Cat. No. SK-4100). Sections were dehydrated with two 5min anhydrous ethanol washes and a 7min xylene wash followed by cover slipping using Permount® mounting medium (Fisher Scientific Canada, Cat. No. SP15-500). Cells were imaged using bright-field microscopy via a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada).

Results

Autophagy-Related Gene/Protein Expression Was Enhanced in PS-1 Mutated Alzheimer's Disease Fibroblasts (PSAF) Treated with Ubisol-Q10

Mutations in PS-1 have been shown to reduce progression of autophagy leading to buildup of dysfunctional mitochondria and generation of increased ROS [11, 12, 25]. We compared expression levels of genes associated with autophagy and oxidative stress between NHF, untreated PSAF, and PSAF treated with either PTS (placebo/vehicle) or Ubisol-Q10 (Figure 1(a)). The gene expression profile of PSAF treated with Ubisol-Q10 was similar to that of NHF. Untreated PSAF and PSAF treated with PTS had lower overall expression levels of genes associated with autophagy/oxidative stress compared with PSAF treated with Ubisol-Q10 or NHF. In particular, beclin-1 (a major autophagy regulator) expression was enhanced in Ubisol-Q10-treated PSAF compared to untreated PSAF. Ubisol-Q10 caused enhancement of MAPK8/JNK1 (a major activator of beclin-1) expression at a similar level to that of healthy NHF. Confirming gene expression profiling results, Western blotting indicated increased expression of proteins beclin-1 and JNK1 in Ubisol-Q10-treated PSAF similar to NHF whereas untreated PSAF or the ones given PTS had significantly reduced expression of beclin-1 and JNK1 protein (Figures 1(b)–1(d)).

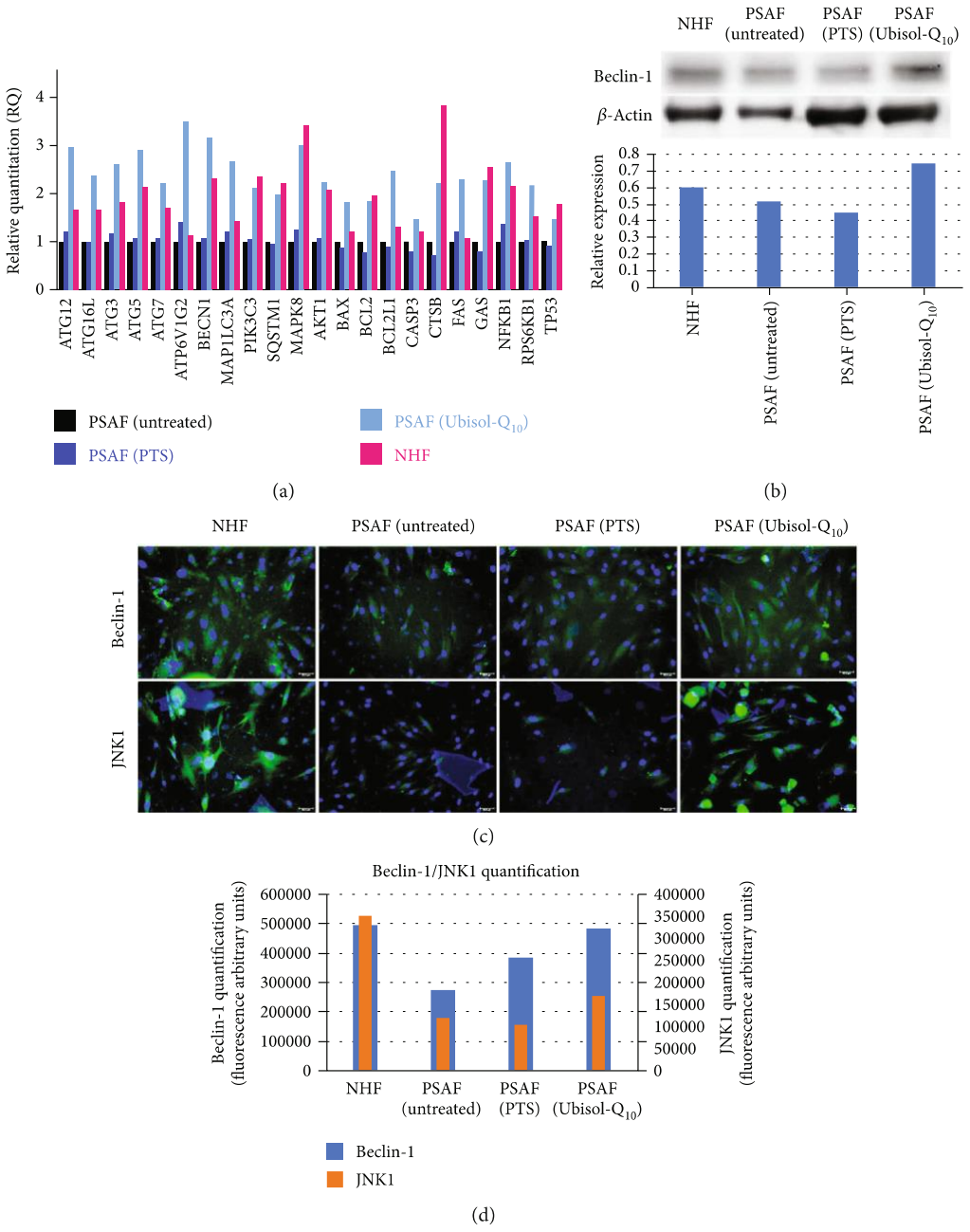

Figure 1. **Ubisol-Q10 enhances expression of autophagy related genes and proteins (a)** Autophagy-related gene expression profile for Normal Human Fibroblasts (NHF), untreated PSAF, PTS-treated PSAF, and Ubisol-Q10-treated PSAF. NHF cells served as a positive control whereas untreated and PTS-treated AD cells served as negative controls. Notably, autophagy-related genes beclin-1, MAPK8/JNK1, and CTSB were upregulated in AD cells treated with Ubisol-Q10 bringing expression levels similar to or higher than NHF. **(b)** Beclin-1 probing of whole cell lysates via Western blot from NHF and PSAF. Supporting gene analysis, Beclin-1 was upregulated in Ubisol-Q10-treated PSAF and not untreated or PTS-treated AD cells. **(c, d)** Immunofluorescence staining of NHF and PSAF

probing for beclin-1 (green) and JNK1 (green) and quantification of fluorescence, respectively. Treatment of PSAF cells with Ubisol-Q10 led to increased staining for beclin-1 and JNK1 compared to the untreated and PTS-treated groups indicating upregulation of autophagic proteins. These Ubisol-Q10-treated PSAF were stained in a comparable manner to NHF cells. Nuclei were counterstained with Hoechst for visualization. Micrographs were taken at 200x magnification. Scale bar = 50 μm.

Inhibition of Autophagy by SP600125 in Ubisol-Q10-Treated PSAF Leads to Return of SIPS Phenotype

It was previously reported that oxidative stress-induced premature senescence was prevented in PSAF in the presence of Ubisol-Q10 [8]. We investigated the role of autophagy in preventing SIPS in PSAF treated with Ubisol-Q10 by treating them with SP600126, a well-known inhibitor of autophagy by blocking JNK1, a major activator of beclin-1 [29, 30]. Confirming previous results, the relative amount of cells staining positive for blue SA-β-gal was reduced in Ubisol-Q10-treated cells compared to untreated PSAF. The proportion of cells staining positive for blue SA-β-gal in PSAF treated with Ubisol-Q10 and SP600125 was increased compared to that of Ubisol-Q10 PSAF not incubated with SP600125 (Figure 2). A similar observation was made when previously treated PSAF had Ubisol-Q10 treatment withdrawal for 48hrs in which staining for blue SA-β-gal increased. There was little to no observable difference in SA-β-gal staining between untreated PSAF and untreated PSAF incubated in SP600125.

In the presence of cell stressors such as ROS generation, p21 is known to promote cell cycle arrest. Previously, it was shown that PSAF had elevated expression of p21 compared to Ubisol-Q10-treated PSAF [8]. Indeed, the same observation was made when cells were probed for p21 via immunofluorescence (Figures 3(a) and 3(b)). PSAF treated with Ubisol-Q10 showed minimal staining for p21 compared to untreated PSAF and PSAF incubated in SP600125. In the presence of SP600125, Ubisol-Q10-treated cells showed increased expression similar to untreated PSAF. Similarly, when Ubisol-Q10-treated PSAF were starved of Ubisol-Q10 after 48 hours, p21 expression increased in a comparable manner similar to untreated PSAF.

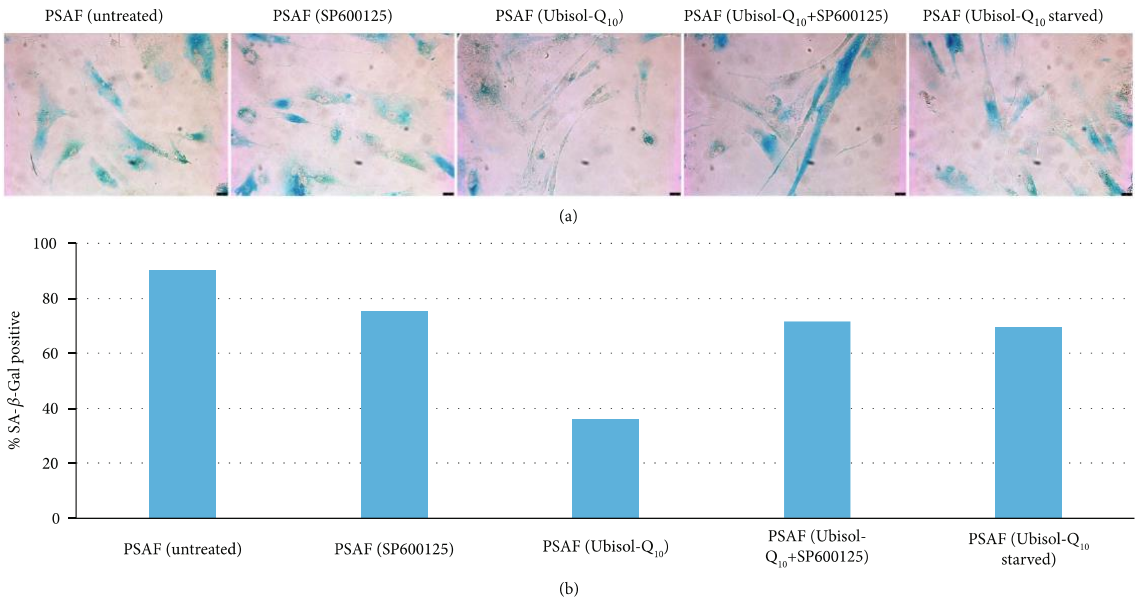

Figure 2. Ubisol-Q10-treated PSAF incubated with SP600125 JNK1 inhibitor showed a resumption of premature senescence bringing fibroblasts back to the original AD morphology. Cells were incubated in senescence-associated beta-galactosidase to identify senescent fibroblasts. Micrographs were taken at 200x. Scale bar = 25 μm.

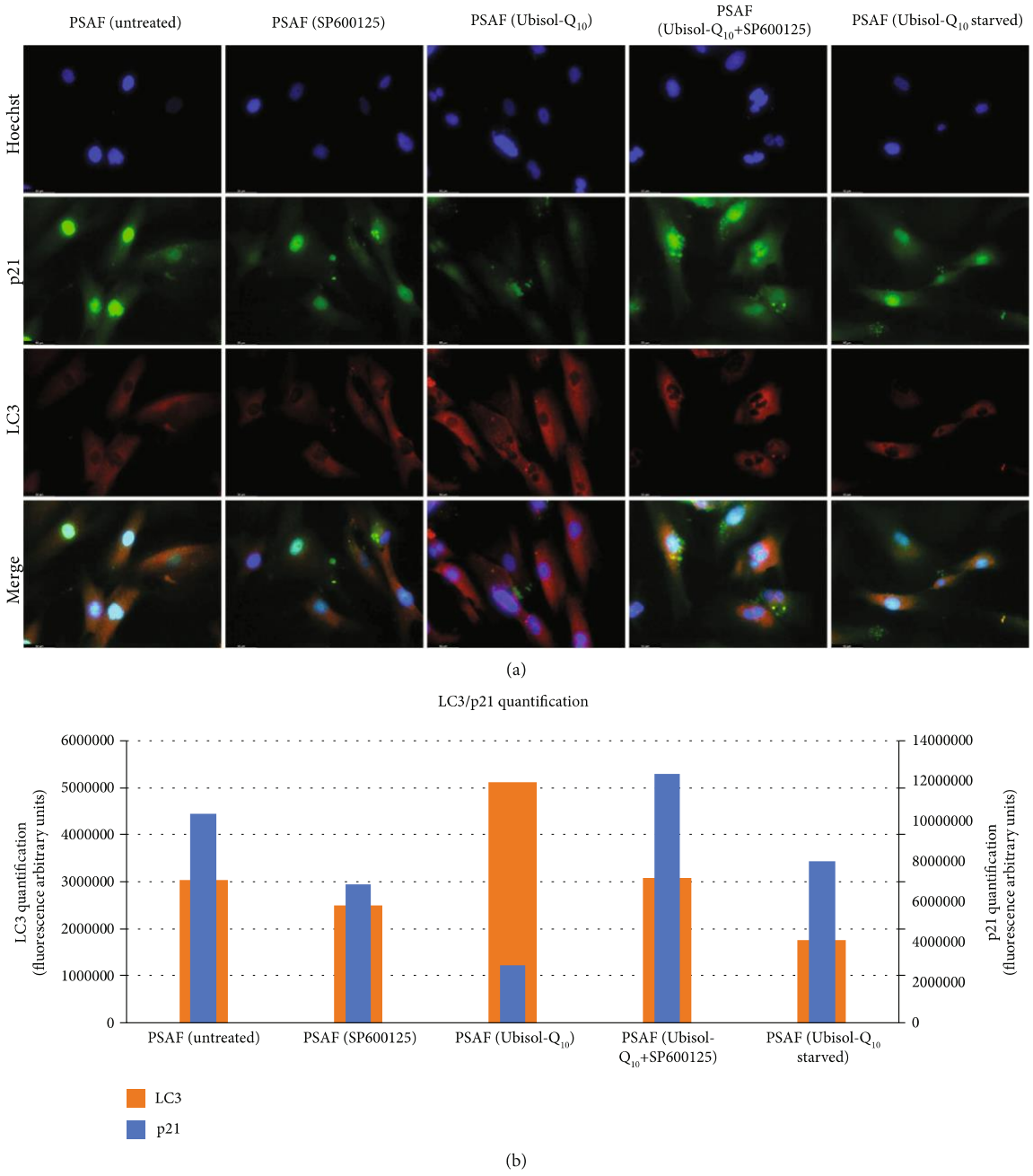

Figure 3. Treatment with SP600125 leads to resumption of senescence phenotype and reduced autophagosome formation in Ubisol-Q10-treated cells. (a, b) Immunofluorescence staining of PSAF probing for p21 (green) and LC3 (red) and quantification of fluorescence, respectively. Treatment of PSAF with Ubisol-Q10 leads to increased staining for LC3 puncta an autophagosome indicator. Ubisol-Q10 also lead to reduced staining for p21, an indicator of senescence. Ubisol-Q10-treated PSAF incubated in the presence of SP600125 resulted in increased staining for p21 and reduced staining for LC3 puncta similar to that of untreated PSAF, PSAF treated with SP600125, and PSAF

starved of Ubisol-Q10 for 48 hours. Nuclei were counterstained with Hoechst for visualization. Micrographs were taken at 400x magnification. Scale bar = 50 μm.

Ubisol-Q10 Treatment Withdrawal or Treatment with Autophagy Inhibitor SP600125 Results in Reduced Autophagosome Formation without Affecting Endogenous Levels of ROS

In a past study, generation of endogenous ROS was shown to be elevated in untreated PSAF compared to NHF [8]. Interestingly, the opposite observation was made for autophagosome formation (as demonstrated by MDC staining for autophagosomes). We investigated the effect autophagy inhibition has on autophagosome formation and endogenous ROS levels (Figure 4). Untreated PSAF or the ones given Ubisol-Q10 were incubated with autophagy inhibitor SP600125, and the presence of autophagosome formation and endogenous ROS was observed using MDC and DCF fluorescence staining, respectively.

Confirming results from the previous study, PSAF treated with Ubisol-Q10 had significantly higher levels of staining for MDC compared to untreated PSAF, indicating increased autophagosome formation (Figure 4). Ubisol-Q10-treated cells incubated with SP600125 had reduced autophagosome formation compared to those incubated without SP600125 as indicated by reduced proportion of cells staining positive for MDC. Similarly, when Ubisol-Q10 treatment was withdrawn, autophagosome formation decreased. Untreated PSAF incubated in SP600125 showed little to no observable difference in MDC staining compared to untreated PSAF not incubated in SP600125. Another method to measure autophagosome formation and overall autophagic flux is the detection of LC3 puncta [31]. Similar to MDC staining, LC3 puncta was increased in Ubisol-Q10-treated PSAF compared to untreated PSAF, PSAF incubated in SP600125, and Ubisol-Q10-starved cells (Figures 3(a) and 3(b)). In the presence of SP600125, LC3 puncta was reduced in PSAF treated with Ubisol-Q10.

Similar to results in the previous study mentioned above, endogenous levels of ROS were reduced in PSAF given Ubisol-Q10 compared to untreated PSAF (Figure 4). Ubisol-Q10-treated PSAF incubated with SP600125 or PSAF with withdrawn Ubisol-Q10 treatment showed minor increases in levels of endogenous ROS compared to the ones constantly given Ubisol-Q10 and not incubated in SP600125. Similar to DCF staining, 4-hydroxynonenal a lipid peroxidation by-product and indicator of oxidative stress was

reduced in cells given Ubisol-Q10. Ubisol-Q10-treated PSAF incubated with SP600125 or PSAF with withdrawn Ubisol-Q10 treatment showed slightly elevated levels of endogenous ROS compared to the ones constantly given Ubisol-Q10 and not incubated in SP600125 (Figures 4(b) and 4(d)).

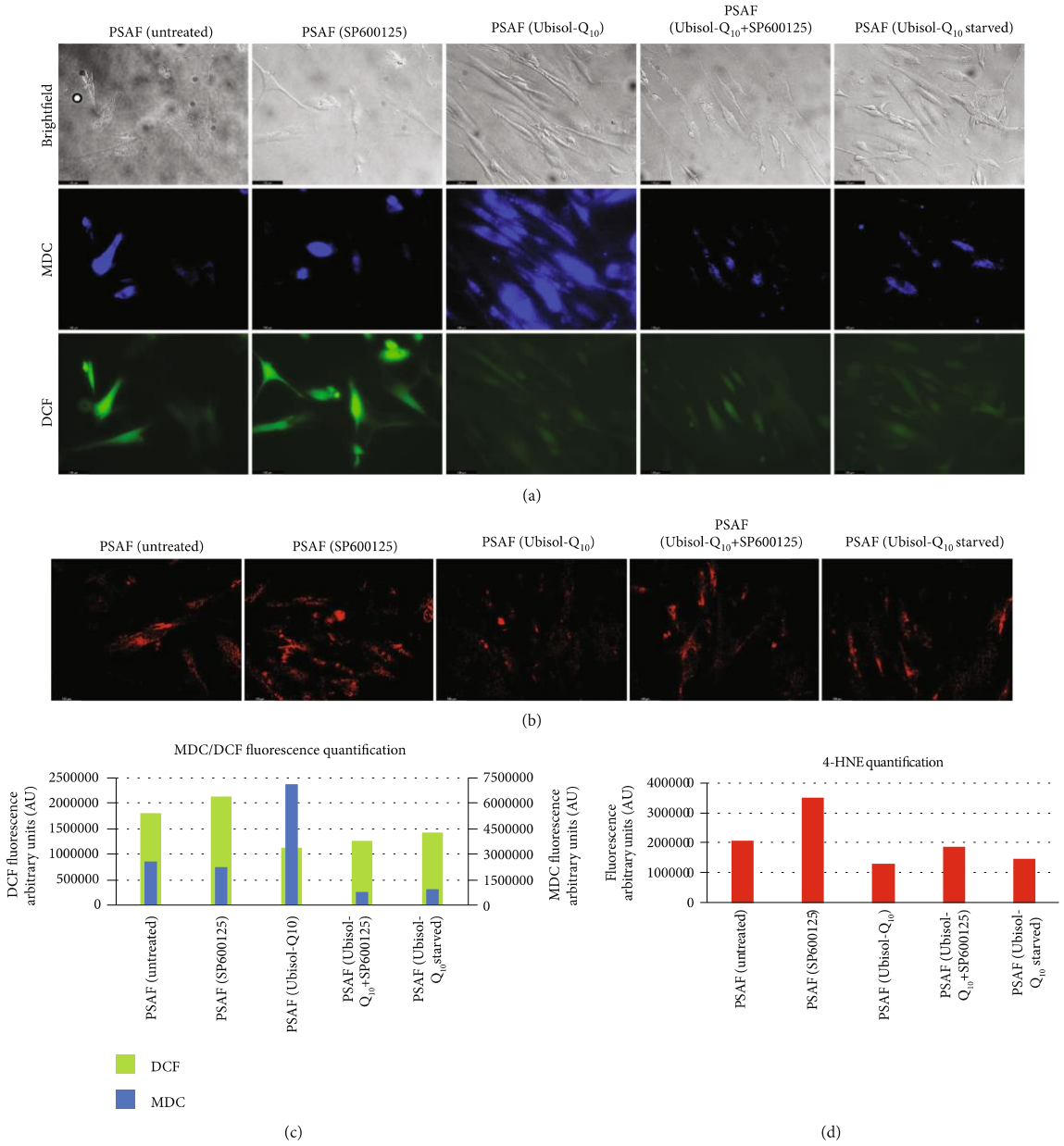

Figure 4. (a, c) Inhibition of autophagy via SP600125 leads to reduced autophagosome formation (blue) and an increase in oxidative stress (green) in PSAF following treatment with Ubisol-Q10. PSAF starved of Ubisol-Q10 returned to AD morphology. Cells were incubated with monodansylcadaverine (MDC) to visualize autophagic vacuoles. Cells were also incubated with 2′,7′-dichlorofluorescein diacetate which is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) for visualization of reactive oxidative species (ROS) production. **(b, d)** Similarly to DCF, immunofluorescence staining for 4-hydroxynonenal (4-HNE) a peroxidized lipid and an oxidative stress indicator was reduced in Ubisol-Q10-treated PSAF. Ubisol-Q10-treated PSAF incubated in SP600125 and PSAF starved of Ubisol-Q10 for 48 hours showed increased staining for 4-HNE similar to untreated PSAF. Micrographs were taken at 200x. Scale bar = 100 μm.

Ubisol-Q10 Treatment Leads to Increased Expression of Autophagy-Related Proteins In-Vivo

As mentioned above, we observed an increase in expression of autophagic proteins beclin-1 and JNK1 in PSAF treated with Ubisol-Q10 similar to NHF. We investigated if these same proteins are upregulated in double transgenic AD mice treated with Ubisol-Q10. Similar to PSAF, these double transgenic mice contain a PS-1 mutation [25]. Previously, it has been shown that oral feeding of Ubisol-Q10 to these mice ameliorated AD pathology [7]. The brain tissues of these same mice were analyzed using immunostaining (Figure 5). Indeed, beclin-1 and JNK1 were both upregulated in transgenic mice given drinking water supplemented with Ubisol-Q10 similar to the wild-type mice. Transgenic mice given unsupplemented or PTS-supplemented drinking water had significantly reduced expression of beclin-1 and JNK1 compared to wild-type mice or Ubisol-Q10-treated mice.

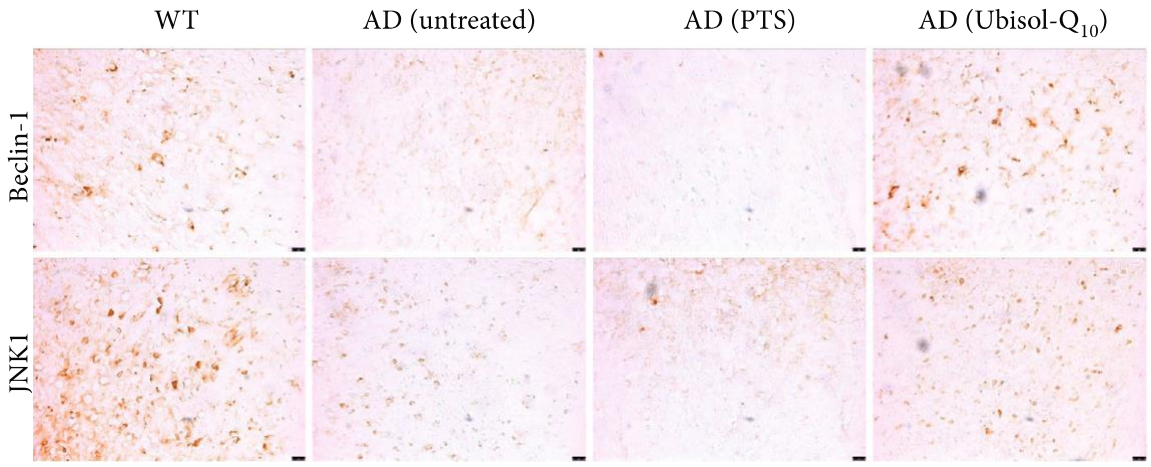

Figure 5. Immunohistochemical staining of the cerebral cortex from double transgenic mice probing for beclin-1 and JNK1. Oral supplementation of Ubisol-Q10 results in the upregulation of beclin-1 and JNK1 in transgenic mice similar to wild-type mice as indicated by the increased immunoactivity in the Ubisol-Q10-treated AD group compared to the untreated and PTS-treated groups. Micrographs were taken at 100x. Scale bar = 25 μm.

Discussion

In this report, we have shown that activation of autophagy is critical for cellular health. We have demonstrated that presenilin-1 mutations lead to inhibition of autophagy in PSAF as well as the brains of transgenic AD mice. Importantly, Ubisol-Q10 lead to resumption of autophagy and inhibition of senescence in PSAF. Furthermore, resumption of autophagy was also observed in Ubisol-Q10-treated transgenic AD mice. Thus, treatment with Ubisol-Q10 leads to amelioration of adverse effects of PS-1 mutation in vitro and in vivo by activation autophagy.

Presenilin-1 mutations are one of the major causes of early-onset AD. Fibroblasts from AD patients containing a PS-1 mutation as well as those from age-matched healthy individuals are easily available. The detrimental effect of PS-1 mutations has been studied in PSAF [8]. These cells undergo premature senescence earlier (around 12 population doublings) compared to healthy fibroblasts which undergo senescence around 40 population doublings. PSAF are under constant elevated levels of oxidative stress. Generally, when cells are faced with increased oxidative stress, autophagy is triggered as a pro-survival response. It is hypothesized that if autophagy is unable to progress, cells would undergo senescence. Ubisol-Q10 a water-soluble formulation of coenzyme-Q10 has been shown to protect neuronal cells from oxidative stress and excitotoxicity [28, 32]. Interestingly, Ubisol-Q10 treatment prevented oxidative stress and SIPS in PSAF and enhanced autophagy [8]. Indeed, when we compared the gene expression of oxidative stress/autophagy-related genes in NHF, PSAF, and Ubisol-Q10-treated PSAF, we found that several autophagy-related genes were upregulated in Ubisol-Q10 PSAF. The gene expression pattern of Ubisol-Q10-treated PSAF was found similar to that of healthy NHF (Figure 1(a)). These results indicate that Ubisol-Q10 treatment of PSAF enables these cells to overcome the deleterious effects of the PS-1 mutation. Expression of some of these autophagy-related genes was confirmed at the protein level by Western blotting and immunofluorescence (Figures 1(b) and 1(c)). Indeed, this was indicated by the increased expression of beclin-1 via Western blotting and immunofluorescence in Ubisol-Q10-treated PSAF. Interestingly, beclin-1 was expressed in higher amounts in Ubisol-Q10-treated PSAF compared to NHF. Furthermore, JNK1 a major activator of beclin-1 was also

upregulated in Ubisol-Q10-treated PSAF comparable to NHF. These observations indicate that Ubisol-Q10 treatment could be triggering autophagy and inhibiting senescence at the same time. The question proposed here is whether autophagy is preventing PSAF from undergoing SIPS, and inhibiting autophagy could result in the return of the SIPS phenotype in PSAF. Indeed, when Ubisol-Q10-treated PSAF were treated with autophagy inhibitor SP600125 (a known inhibitor of beclin-1 via JNK1 inhibition [29, 30]), we saw a drastic decrease in autophagosome formation (Figures 4(a), 4(c), and 3) as well as the return of the SIPS phenotype (Figures 2, 3(a), and 3(b)). It should also be noted that when Ubisol-Q10 treatment was withdrawn, autophagosome formation decreased and the SIPS phenotype returned in PSAF indicating that constant treatment with Ubisol-Q10 is required to maintain a healthy cell morphology (Figures 2 and 4(a)). Another important observation was that despite reduced ROS production (Figure 4), withdrawal of Ubisol-Q10 resulted in reduction of autophagy and resumption of senescence (Figures 2 and 3). This could indicate that Ubisol-Q10-induced autophagy is independent of antioxidative effects. Thus, it seems that stress (such as oxidative stress, DNA damage, starvation, and mitochondrial dysfunction)-induced autophagy and SIPS have an inverse relationship and could play a very important role in homeostasis and maintenance of neuronal cells.

Mitochondrial dysfunction has been shown to be involved in autophagy, senescence, and apoptosis by several researchers [33]. Previously, Ubisol-Q10 has been shown to inhibit bax-induced destabilization of mitochondria in mammalian cells [34]. Thus, it seems that Ubisol-Q10 has the potential to prevent the deleterious effects of PS-1 mutations and reverse the SIPS phenotype by resumption of autophagy in PSAF. Most importantly, Ubisol-Q10 treatment has demonstrated very clear amelioration of AD pathology in double transgenic mice containing mutated PS-1 and amyloid precursor protein (APP) [7]. Could this effect be the result of activation of autophagy via Ubisol-Q10 in these mice? When we used brain sections from the mice of the same experiment and stained for autophagy-related proteins, we observed upregulation of beclin-1 and JNK1 in the cortex of Ubisol-Q10-treated mice appearing similar to brains of wild-type mice (Figure 5). This is extremely important as Ubisol-Q10, a simple/GRAS approved nutritional supplement, has shown unprecedented activation of autophagy leading to reversal of SIPS in vitro and halting of the progression of AD pathology in vivo.

Furthermore, this is a nutritional supplement that can be taken perpetually without any side effects.

Patents

Ubisol-Q10 is a patented formula.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 5: Investigation into efficacy and mechanisms of neuroprotection of ashwagandha root extract and Ubisol-Q10 in an aged transgenic mouse model of Alzheimer's Disease

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List of Abbreviations

AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
PS-1	Presenilin-1
ROS	Reactive Oxygen Species
SIPS	Stress Induce Premature Senescence
NHF	Normal Human Fibroblast
PSAF	PS-1 Mutated AD Familial Type 3 Fibroblast
PTS	Polyoxyethanyl-α-Tocopheryl Sebacate
ASH	Ethanolic Ashwagandha Extract
4-HNE	4-Hydroxynonenal
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
GFAP	Glial Fibrillary Acidic Protein
Iba-1	Ionized Calcium-Binding Adapter Molecule 1
BDNF	Brain Derived Neurotrophic Factor
GDNF	Glial Derived Neurotrophic Factor
NeuN	Neuronal Nuclei

Introduction

Alzheimer's Disease (AD) is the most common neurodegenerative disease and the leading form of dementia across the globe. AD is characterized by progressive neuron loss in the hippocampus and cortex leading to memory loss/cognitive decline, morbidity, and eventually death [1]. A key pathological feature of AD is the accumulation and deposition of β-amyloid fibrils forming extracellular neuritic plaques [2]. Other pathological/biochemical mechanisms/features of AD include mitochondrial destabilization [3-6], generation of reactive oxygen species (ROS) [3, 7-11], inhibition of autophagy [8,12,13], inflammatory modulation of microglia and astroglia [7], and reduction in the thickness of CA1/CA3 neurons in the hippocampus (as indicated by increased staining of neuron nuclei (NeuN) indicating more neurons [7,14]. As a result of these multiple mechanisms, AD should be considered a multi-factorial disease and targeting only one mechanism may prove futile in preventing the neurodegeneration that occurs in AD [15]. Furthermore, many therapies used to treat AD only provide symptomatic relief and do not halt the neurodegeneration. Also, these symptomatic relief therapies are chemo modulators and extended use has shown to have toxic side effects and adverse psychological effects [16]. Therefore, to successfully treat AD, a multifaceted approach using natural health products that are well tolerated would be preferable.

Ubisol-Q10 is a water-soluble formulation of coenzyme-Q10 and has shown to reduce oxidative stress, improve long-term and working memory, and drastically inhibit β-amyloid plaque formation in 16-month-old transgenic mice that display AD type pathology [7]. While Ubisol-Q10 was effective in treating some AD pathologies in transgenic mice such as oxidative stress, it does not target all mechanisms of AD and there were still some β-amyloid plaques in the brains of mice (though reduced compared to untreated transgenic mice). Ashwagandha root extract has been used as a promoter of brain health in Ayurveda (the traditional school of Indian medicine). Several groups have shown ashwagandha root extract to be neuroprotective in in-vitro models of AD [17,18]. In particular, ashwagandha root extract was shown to reduce amount of key inflammatory cytokines and modulated stress response [19,20]. Since both ashwagandha and Ubisol-Q10 target multiple and different mechanisms such as oxidative stress and inflammation, these two treatments

could be combined to be an even more effective therapy for Alzheimer's Disease compared to if they were used alone.

As mentioned earlier, inflammation plays a key role in Alzheimer's Disease. While Ubisol-Q10 was shown to reduce oxidative stress, ashwagandha has been shown to be immunomodulator and suppress neuroinflammation. Certain glial cells have been implicated with neuroinflammation. In particular astrocytes and microglia play a major role in inflammation with AD. Microglia become activated in the presence of β-amyloid plaques in a process known as microgliosis. During microgliosis, microglia change from their ramified (inactive) state to their ameboid (active) state [21]. In this active/ameboid state, microglia release various inflammatory cytokines which can cause the surrounding neurons around the β-amyloid plaques to become stressed and potentially undergo apoptosis [22].

Unlike microglia, astrocytes typically provide a supporting role to neurons. Astrocytes are involved in providing metabolic support, supporting synaptic connections, neurotrophic factor secretion, and even preventing microglia activation among other support roles [23-25]. In their inactive state, astrocytes have few processes extending from the cell body while active (reactive) astrocytes have a significant amount of branching of cytoplasmic processes extending from the cell body [26]. In transgenic AD mice, the amount of reactive (activated) astrocytes were reduced compared to wild-type mice. Furthermore, microglia and astrocytes are known communicate with each other and that they can either activate or inactivate each other [27]. As mentioned earlier, ashwagandha was shown to reduce the amount of pro-inflammatory cytokines. This could be possible due to either direct inactivation of microglia via the ashwagandha extract or as a result of astrocyte activation which then could be inactivating microglia.

In this study, we will investigate if Ubisol-Q10 combined with ashwagandha is a more potent therapeutic for reducing the number of β-amyloid plaques in the brains of double transgenic mice AD mice compared to if either therapeutic was used alone. Based off the anti-inflammatory properties of ashwagandha, it is expected that transgenic mice given the extract will have reduced activation of microglia and enhanced astroglia activation. To investigate status of neurodegeneration, the thickness of the CA3 region will

be assessed via probing for NeuN (neuronal nuclei) which is a marker of neurons in the CA3. Thickness of the CA3 region of the hippocampus is also expected to be thicker with treatment with Ubisol-Q10 and ashwagandha (as indicated by increased neuronal nuclei staining in the CA3) as it's been shown previously that the treatments prevented neurodegeneration when used separately [7,18].

Materials and Methods

Extraction of ashwagandha root and phytochemical assessment

The same protocol for extraction was used as described by Vegh et al [28]. The same batch of extract was used in both this experiment and the experiment by Vegh et al. Ashwagandha root powder (Premier Herbal Inc., North York, ON, Canada) was agitated in anhydrous ethanol at a ratio of 1:10 (w/v) at 70°C for 24hrs. The crude extract was then filtered through a P8 paper filter, and ethanol was removed using a rotary evaporator. The solid extract was resuspended at 200mg/mL with anhydrous ethanol. The resuspended extract was analyzed using ultra-performance liquid chromatography coupled with ultra-violet spectroscopy to assess withanolide content and colorimetric analysis based on Dowd's reagent for flavonoid content. Phytochemical analyses were performed by Laboratoire PhytoChemia (Chicoutimi, QC, Canada). Report of the extract contents is shown in Table 1 of CHAPTER 2.

Animal Care

All animal care, treatments, and procedures were approved by the University of Windsor's Animal Care Committee in accordance with the Canadian Council for Animal Care guidelines. The experiments were conducted on 32 male double-transgenic APP/PS1 mice (Jackson Laboratory; Strain: B6C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax) and 8 male C57BL/6 wild-type mice (Charles River Laboratories). The transgenic mice co-expressed a chimeric mouse/human APP (Mo/HuAPP695swe) and mutant human presinilin-1 (PSEN1-dE9) transgenes. The gene mutations in the double-transgenic mice are linked with early-onset familial AD; thus, these mice begin to show amyloid plaque deposition by 6 months of age, which progressively increases until 12 months. The double transgenic and wild-type (WT) mice arrived at one month of age. They were acclimated over a two-week span, after which the treatment period began. Transgenic mice were randomly assigned to one of four groups: untreated group receiving regular drinking water (n=8), treated groups receiving Ubisol-Q10 (n=8), ashwagandha extract (ASH) (n=8), or ashwagandha and Ubisol-Q10 (tonic) (n=8). All 8 wild-type mice received regular drinking water. The animals were housed at a constant temperature of 20°C and were under a

controlled 12-hour dark-light cycle to ensure they were awake during the day for the behavioural assessments. The animals also had access to food and their respective water treatments and their body weight was measured weekly.

Tissue Preparation for Immunohistochemistry

When the mice reached 18 months of age, they were euthanized while under anaesthetization with 3% isoflurane at a flow rate of 1.5L of oxygen per minute. Once animals showed lack of withdrawal reflex (stage 3 anesthesia/lack of pain), the animals were perfused using ice cold 1x PBS containing 28μg/mL heparin (Sigma-Aldrich, Oakville, ON, Canada, Cat. No. H3393) which was followed by tissue fixation with ice cold PBS with 10% formaldehyde. The brains were then dissected and stored in 10% formaldehyde made in PBS at 4°C. before sectioning, the brains were incubated in 30% sucrose (w/v in PBS) until brain sank in solution. Following cryoprotection in sucrose, the brains were cryosectioned at 30μm thickness with Shandon™ M-1 embedding matrix (Thermo Scientific, Mississauga, ON, Canada, Cat. No. 1310 TS) onto glass microscope slides.

Antibodies

The following antibodies were used at the described dilutions during immunohistochemistry (IHC) to assess the morphological changes and levels of various proteins in the brain: β-amyloid antibody (mouse IgG, 1:500; Novus Biologicals Cat No. NBP2-13075)*, glial fibrillary acidic protein (GFAP) (rabbit IgG, 1:500; Novus Biologicals, Centennial, CO, USA, Cat. No. NB300-141)*, ionized calcium-binding adapter molecule 1 (IBA1) (rabbit IgG, 1:300; Novus Biologicals, Cat. No. NB100-1028)*, beclin-1 (mouse IgG, 1:500; Santa Cruz Biotechnology, Dallas, TX, USA, Cat. No. sc-48342)*, brain-derived neurotrophic factor (BDNF) (rabbit IgG, 1:200; Santa Cruz Biotechnology, Cat. No. sc-20981)*, 4-hydroxynonenal (4-HNE) (rabbit IgG, 1:500; Abcam Inc., Cambridge, UK, Cat. No. ab46545)*, LC3B (rabbit IgG, 1:500, Abcam Inc., Cambridge, UK, Cat No. ab192890)*, neuronal nuclei (NeuN) antibody (mouse IgG,

1:600; EMD Millipore Cat No. MAB 377X)*, Vector Laboratories fluorescein horse anti-mouse IgG (1:500; MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. FI-2000)**, and Alexa Fluor™ 568 goat anti-rabbit IgG (Thermo Scientific Canada, Brockville, ON, Canada, Cat. No. A11011)**. Antibodies denoted with * and ** were used as primary and secondary antibodies respectively for IHC.

Immunohistochemistry

Tissue sections were washed twice in 1X tris buffer saline (TBS) for 5min each. The tissue sections were then blocked with DAKO serum-free protein block (Agilent Technologies Canada Inc., Cat. No. X0909) for 1hr to prevent binding of non-specific IgG. After blocking, tissue sections were incubated in the above-mentioned primary antibodies overnight at 4°C (antibodies were diluted in DAKO antibody diluent (Agilent Technologies Canada Inc., Cat. No. S0809) containing 1µL 10% TWEEN/100 µL total antibody solution). The following day, sections were washed twice with TBS for 5min each to remove unbound primary antibody. Tissue sections were then incubated for 2hr at room temperature in above mentioned secondary antibodies (antibodies were diluted in DAKO antibody diluent (Agilent Technologies Canada Inc., Cat. No. S0809) containing 1µL 10% TWEEN/100 µL total antibody solution). Sections were then washed twice for 5 min in TBS followed by incubation with 10µM Hoechst 33342 (Molecular Probes, Eugene, OR, USA Cat. No. H3570). Sections were washed twice for 5 min in TBS followed by coverslipping with VECTASHIELD® Vibrance® antifade mounting medium (MJS BioLynx Inc., Brockville, ON, Canada, Cat. No. VECTH18002). Slides were cured for 1hr before imaging under epifluorescence with a Leica DMI6000 B inverted microscope (Leica Microsystems, Concord, ON, Canada). Corrected total fluorescence (CTF) was quantified using ImageJ software.

Results

Effect of treatment with Ubisol-Q10 and ashwagandha extract on β-amyloid plaque load and staining for microglia in the brains of double transgenic AD mice

It has been previously reported that presence of amyloid plaques leads to activation microglia [7]. Seen in figure 1, Wild-type mice (positive control) did not have any staining for β-amyloid plaques (as indicated by zero green staining) and showed minimal staining of microglia (as indicated by reduced red staining). Transgenic mice given regular drinking water (untreated/negative control) showed the greatest number and largest sized β-amyloid plaques (indicated by intensity/size/number of green spots) and greatest/brightest amount of staining for microglia (brightest/greatest amount of red staining). Transgenic mice given Ubisol-Q10 supplemented drinking water had a reduced number of β-amyloid plaques, decreased β-amyloid plaque size, and reduced amount of microglia compared to the negative control. Transgenic mice given ashwagandha supplemented drinking water had a reduced amount of staining for microglia (reduced amount of red staining) and reduced β-amyloid plaque number (reduced number of green spots) compared to the negative control but β-amyloid plaque size appeared unchanged. Transgenic mice given drinking water supplemented with both Ubisol-Q10 and ashwagandha (tonic) had the greatest reduction in β-amyloid plaque size/number (minimal green stain) compared to either Ubisol-Q10 or ashwagandha used alone, as well as reduced staining for microglia similar to mice given only ashwagandha.

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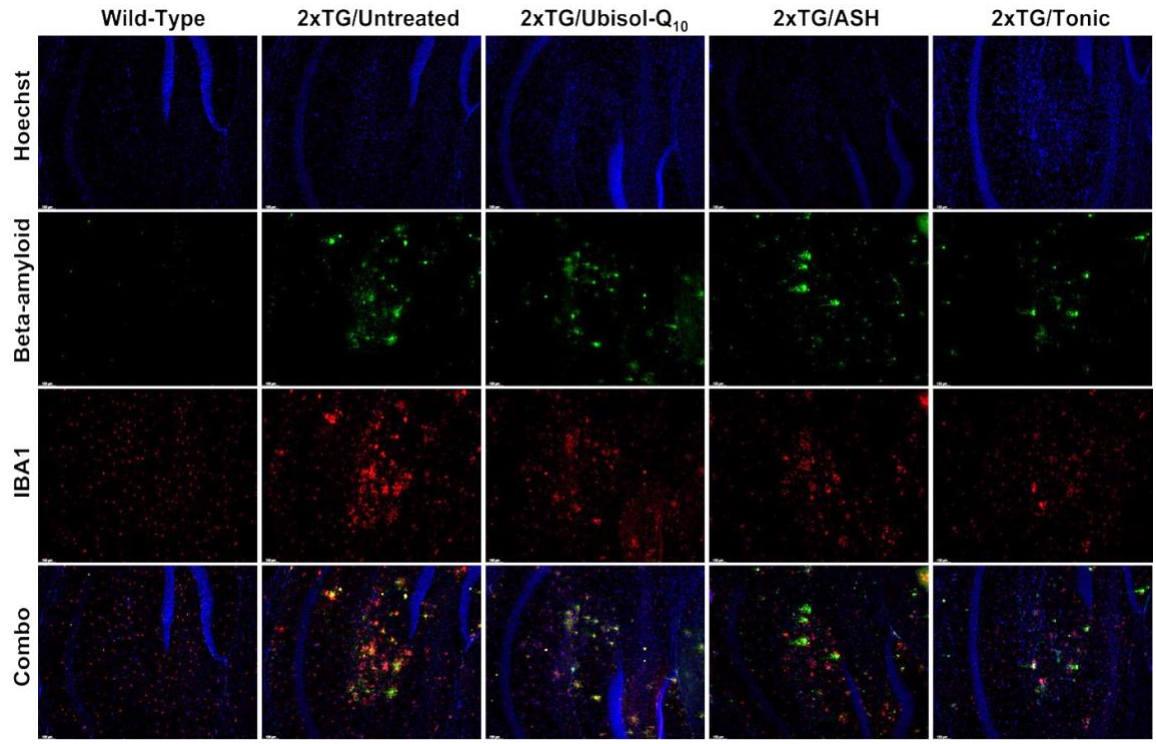

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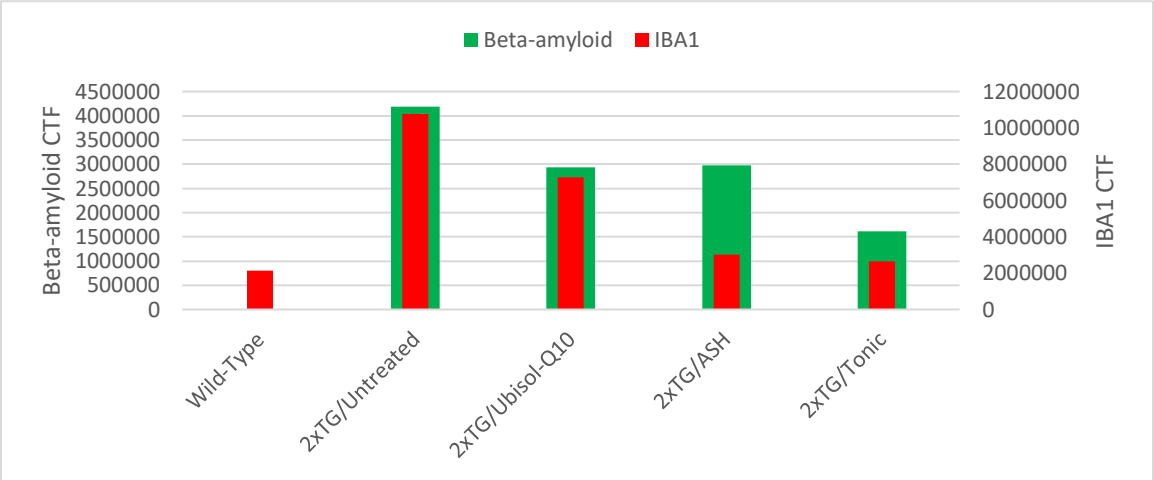

Figure 1. Effect of Ubisol-Q_{10} and ashwagandha on beta-amyloid plaque deposition and IBA1 expression. (**A**) Immunofluorescent staining in hippocampus in midbrain sections probing for beta-amyloid plaques and ionized calcium-binding adapter molecule 1 (IBA1) and (**B**) quantification of fluorescence (corrected total fluorescence/CTF) of beta-amyloid and IBA1. Nuclei were counterstained with Hoechst. Micrographs were taken at 200X magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Effect of Ubisol-Q10 and ashwagandha extract treatment on levels of beclin-1 and LC3B

We have previously shown that autophagy regulator beclin-1 was upregulated in fibroblasts from AD patients and the brains of double transgenic mice treated with Ubisol-Q10 [8]. Furthermore, we have also shown that beclin-1 is upregulated in the brains of Parkinsonian rats injected with paraquat [28]. Here we probed for beclin-1 as well as autophagosome marker LC3B which is also shown to be modulated in response to Ubisol-Q10 (figure 2) [8]. Untreated transgenic mice showed reduced levels of both beclin-1 and LC3B compared to wild-type mice. Transgenic mice given Ubisol-Q10 showed elevated expression of both beclin-1 and LC3B similar to wild-type mice. Ashwagandha fed transgenic mice appeared similar to untreated mice in expression of both autophagy related proteins. Transgenic mice fed water supplemented with both Ubisol-Q10 and ashwagandha showed elevated expression of both beclin-1 and LC3B similar to wild-type mice and transgenic mice fed Ubisol-Q10.

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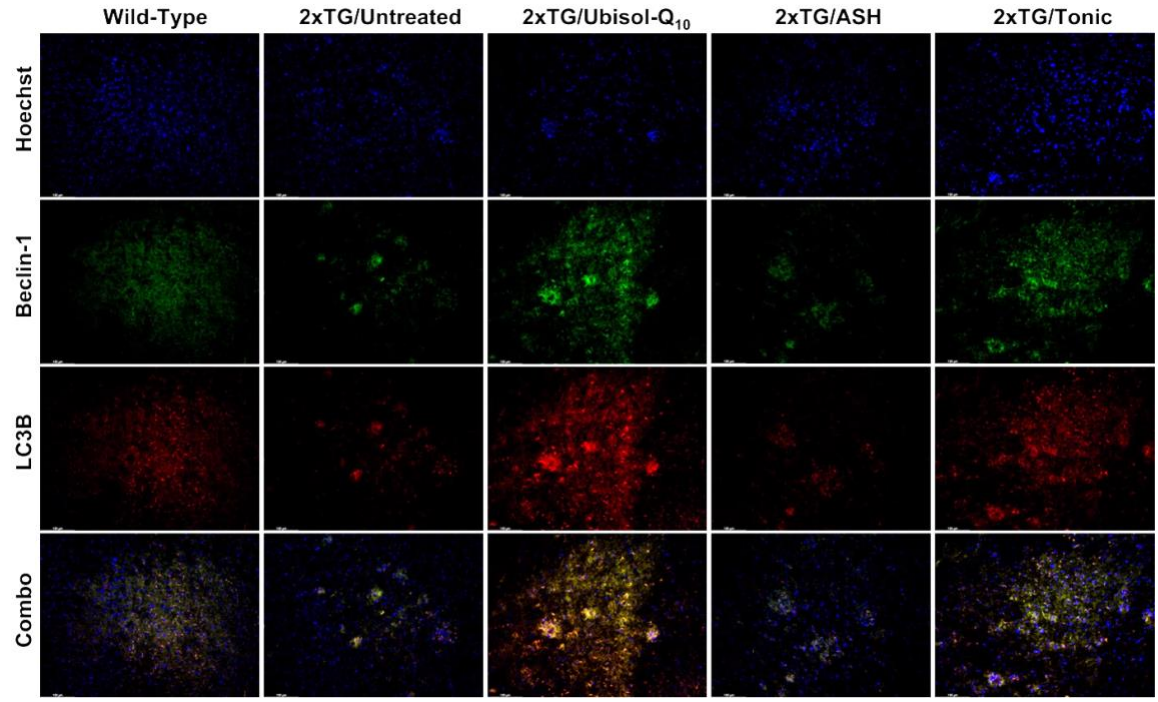

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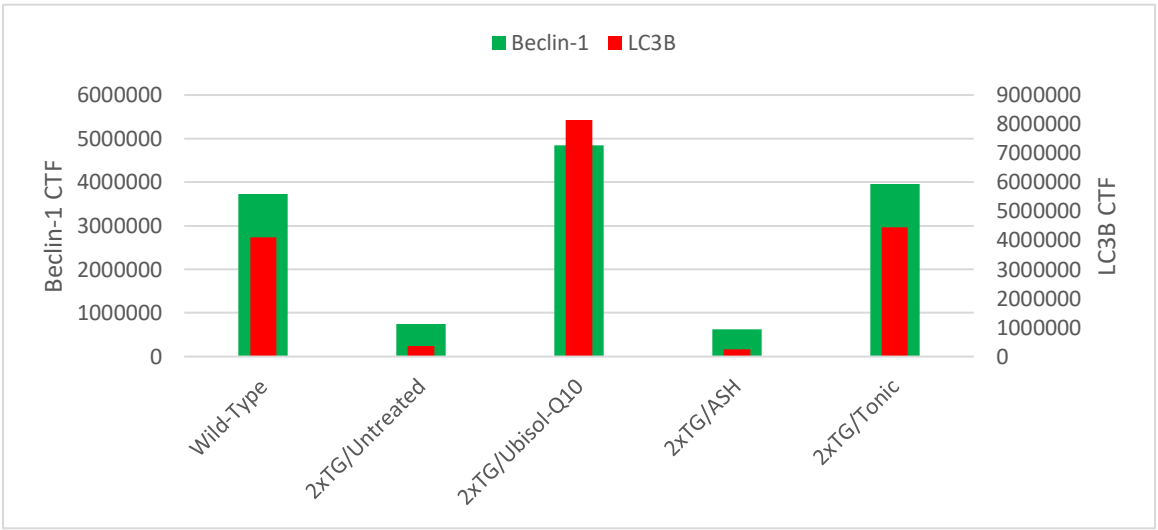

Figure 2. Effect of Ubisol-Q_{10} and ashwagandha on autophagy activity. (**A**) Immunofluorescent staining in cortex in midbrain sections probing for autophagy regulator beclin-1 and essential autophagy related protein LC3B and (**B**) quantification of fluorescence (corrected total fluorescence/CTF) of beclin-1 and LC3B. Nuclei were counterstained with Hoechst. Micrographs were taken at 200X magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Effect of Ubisol-Q10 and ashwagandha extract treatment on levels of 4-hydroxynonenal

It is well known that levels of reactive oxygen species and oxidative stress is elevated in Alzheimer's Disease [3,7,8]. Previously we have seen that 4-hydroxynonenal (4-HNE) a lipid peroxidation product and marker of oxidative stress is elevated in Alzheimer's Diseased fibroblasts and double transgenic mice [7,8]. In this experiment we investigated the effect of treatment with Ubisol-Q10 and ashwagandha extract on 4-HNE presence in the brains of double transgenic mice (figure 3). Wild-type expressed minimal expression for 4-HNE. Transgenic mice given plain drinking water (untreated) showed elevated levels of 4-HNE compared to wild-type mice. Transgenic mice given Ubisol-Q10, ashwagandha, or the tonic showed reduced presence of 4-HNE appearing similar to wild-type mice.

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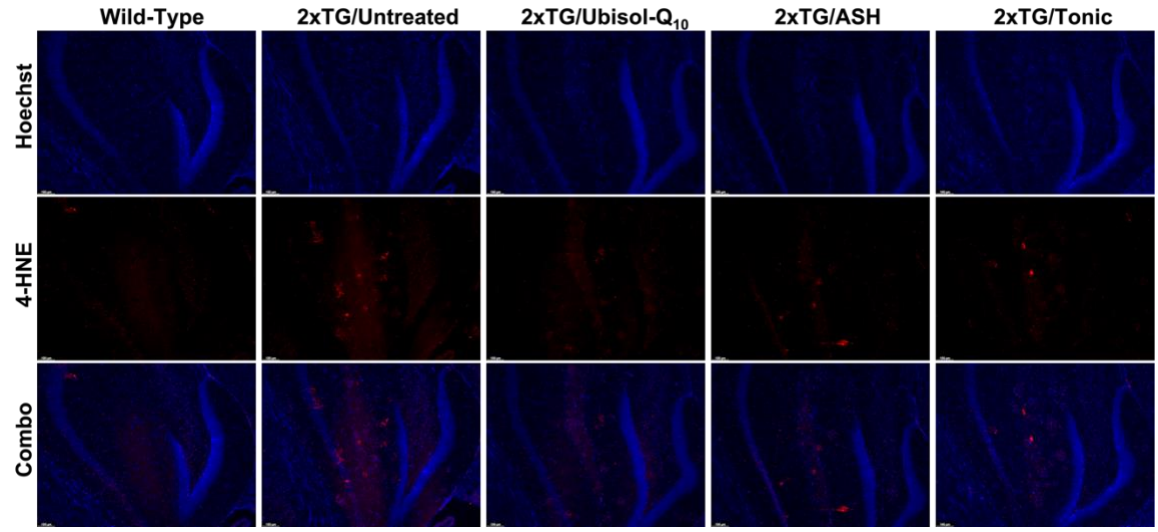

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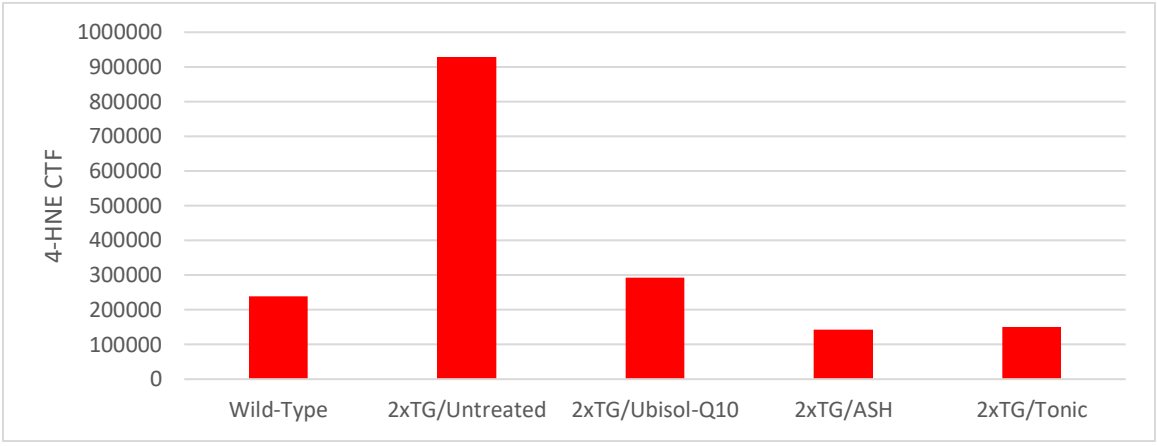

Figure 3. Effect of Ubisol-Q_{10} and ashwagandha on lipid peroxidation. (**A**) Immunofluorescent staining in hippocampus in midbrain sections probing for 4-hydroxynonenal (4-HNE), a lipid peroxidation by-product and marker of oxidative stress and (**B**) quantification of fluorescence (corrected total fluorescence/CTF) of 4-HNE. Nuclei were counterstained with Hoechst. Micrographs were taken at 200X magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Effect of treatment with Ubisol-Q10 and ashwagandha extract on staining for astrocytes and neuronal nuclei in the brains of double transgenic AD mice

Previously, it was shown that the CA1 layer and astrocyte activation was reduced in double transgenic mice [7]. Here we wanted to see if another region of the hippocampus, the CA3, was affected in double transgenic mice. We also wanted to investigate the status of astrocytes in response to Ubisol-Q10 and ashwagandha treatment. As seen in figure 4, there was no observable difference between staining/thickness for NeuN (green) in the CA3 region between any of the groups. Wild-type and transgenic mice given regular water (untreated) showed similar levels of staining for astrocytes (similar staining for red). Transgenic mice given Ubisol-Q10 supplemented drinking water showed a slightly greater number of cells staining for astrocytes compared to wild-type or untreated transgenic mice. Transgenic mice given ashwagandha or both ashwagandha and Ubisol-Q10 had greater number of cells staining for astrocytes (more red cells staining positive for GFAP) as well as staining for GFAP compared to mice given other treatments.

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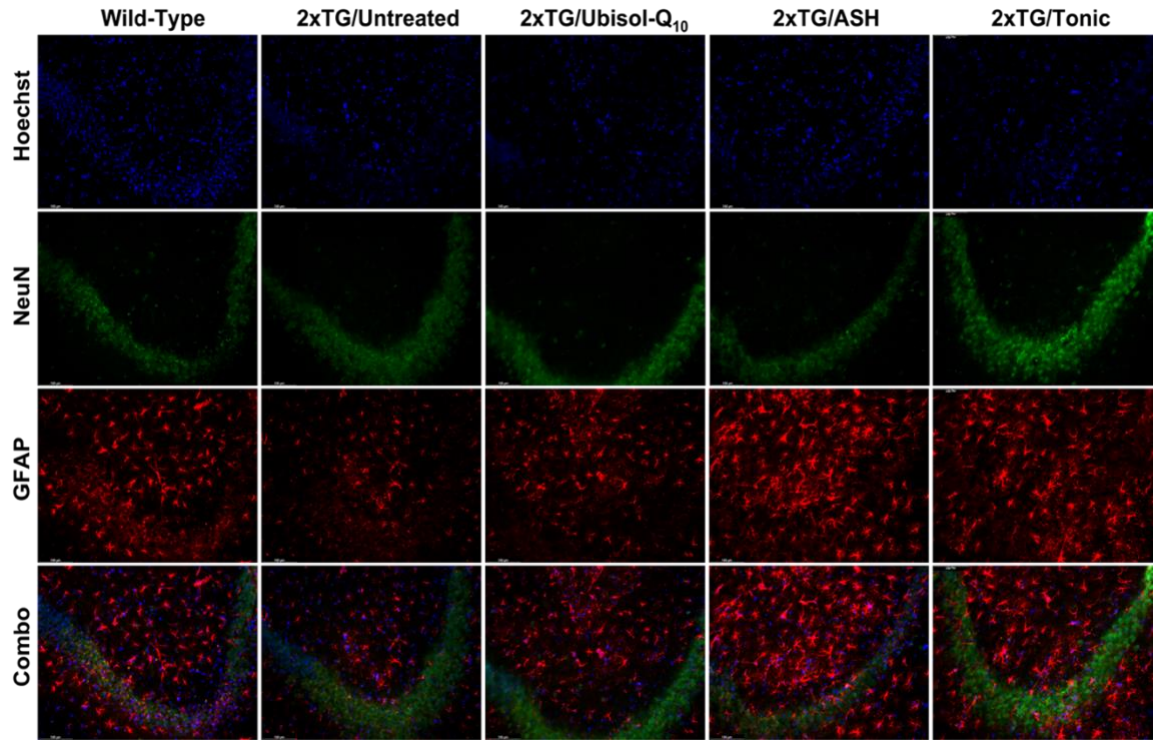

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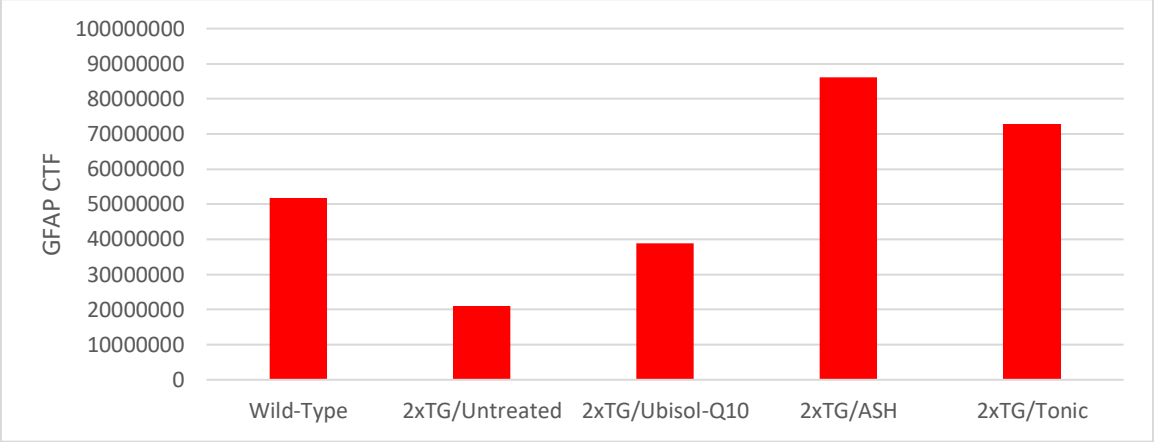

Figure 4. Effect of Ubisol-Q_{10} and ashwagandha on astroglia activation. (**A**) Immunofluorescent staining in hippocampus in midbrain sections probing for glial fibrillary acidic protein (GFAP) a marker of astroglia and neuronal nuclei (NeuN) and (**B**) quantification of fluorescence (corrected total fluorescence/CTF) of GFAP. Nuclei were counterstained with Hoechst. Micrographs were taken at 200X magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Effect of Ubisol-Q10 and ashwagandha extract on levels of brain derived neurotrophic factor

Following investigation of astroglia status, we probed for brain derived neurotrophic factor (BDNF) to determine its association with astroglia status. Seen in figure 5, untreated transgenic mice showed reduced expression of BDNF compared to wild-type mice similar to GFAP in figure 4. Transgenic mice fed Ubisol-Q10, ashwagandha extract, or the tonic all showed elevated expression of BDNF compared to untreated mice.

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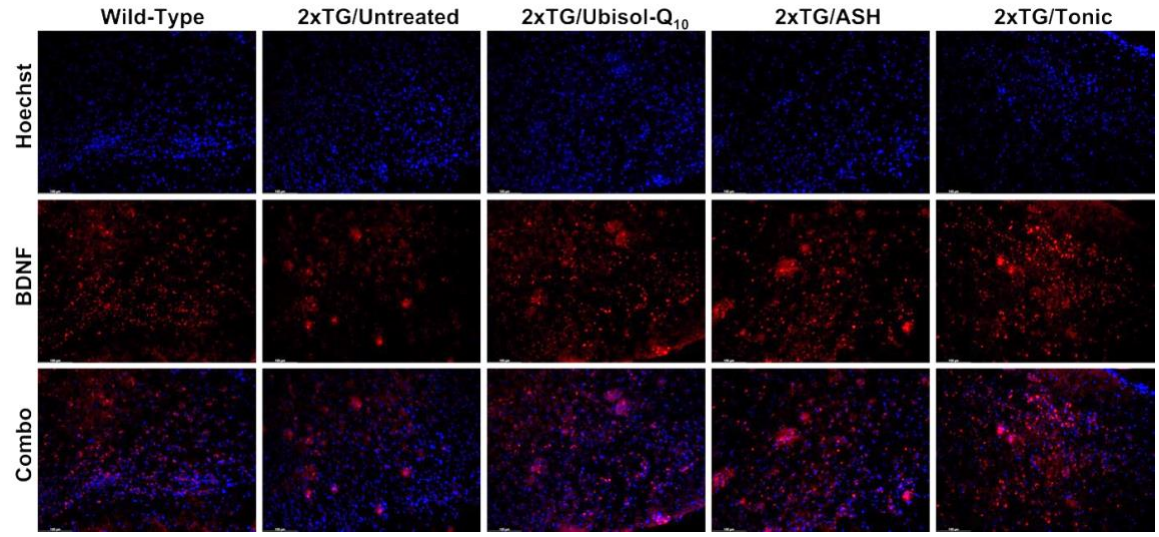

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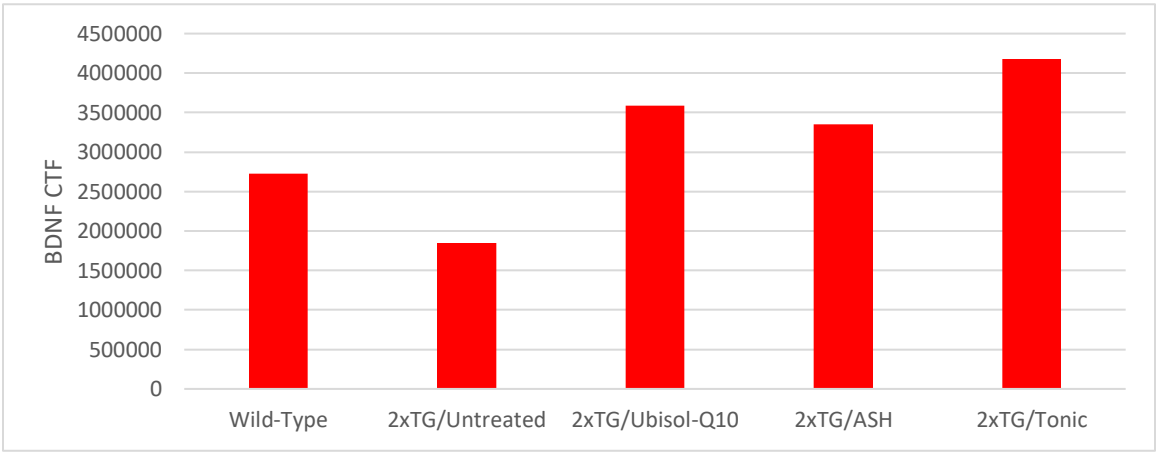

Figure 5. Effect of Ubisol-Q$_{10}$ and ashwagandha on neurotrophic factor expression. (**A**) Immunofluorescent staining in cortex in midbrain sections probing for brain derived neurotrophic factor (BDNF) and (**B**) quantification of fluorescence (corrected total fluorescence/CTF) of BDNF. Nuclei were counterstained with Hoechst. Micrographs were taken at 200X magnification. Scale bar = 100 microns. Images are representative of 3 independent experiments with similar trends.

Discussion

Alzheimer's Disease (AD) is complex neurodegenerative disease with one of the main pathological features being the development of β-amyloid plaques which can cause stress to neurons leading to cell death [1,2]. Furthermore, inflammatory activation of astrocytes and microglia has also been observed in in-vivo models of AD [7]. Previously, treatment with either Ubisol-Q10 or ashwagandha root extract (which target different pathologies of AD) when used alone have shown to reduce the number of β-amyloid plaques in the brains of transgenic mice [7,18]. We have demonstrated the neuroprotective efficacy of using combined treatment of Ubisol-Q10 with ashwagandha root extract in a transgenic mouse model of AD.

Administration of Ubisol-Q10 in drinking water was able to reduce the size and number of β-amyloid plaques in the brains of double transgenic mice compared to the untreated transgenic mice (mice given regular drinking water) as indicated by the reduced staining for β-amyloid plaques (figure 1). Previously, it has been reported that Ubisol-Q10 was able to increase levels of autophagy (the mechanism for which cells eliminate defective organelle and misfolded proteins) in fibroblasts containing AD mutations. Indeed, the same mechanism was observed in the transgenic mice given Ubisol-Q10 or the tonic. Levels of both autophagy proteins beclin-1 and LC3B were upregulated in the brains of animals fed solutions containing Ubisol-Q10 but not ashwagandha. This is the fourth time we have reported this conserved mechanism of autophagy activation with Ubisol-Q10 [3,7,8]. Similarly, ashwagandha was reported by Seghal et al. to reduce β-amyloid load in transgenic mice brains. But instead of resumption of autophagy, ashwagandha treatment resulted in removal of β-amyloid via enhancement of liver low-density lipoprotein receptor-related protein (LRP) (a protein involved in clearing debris from various areas of the body through cholesterol) [18]. While we did not probe for this protein in the brain or liver of this experiment, it is possible the same mechanism is occurring with mice given ashwagandha in this study, but further analyses (such as IHC staining of liver LRP) are required to confirm this role of LRP enhancement. When Ubisol-Q10 and ashwagandha root extract were combined, there was greater enhancement of β-amyloid plaque clearance. It is possible that Ubisol-Q10 is increasing levels of autophagy and ashwagandha is

enhancing LRP resulting in two different protein clearance pathways working together to clear out the β-amyloid plaques from the brains of these double transgenic mice.

Interactions between neurons and glia cells are also important to maintain brain homeostasis [7]. Previously, it was seen that pro-inflammatory microglia are active around β-amyloid plaques in transgenic mice brains [7]. Treatment with Ubisol-Q10 resulted in reduced microglia activity as indicated by reduced ameboid/active microglia compared to untreated transgenic mice. A possible reason for this is that Ubisol-Q10 is reducing the size and number of β-amyloid plaques, and as a result, there are less/smaller β-amyloid plaques resulting in fewer microglia detecting these toxic protein aggregates. Similarly, microglia activation was shown supressed in ashwagandha treated groups. In previous studies, ashwagandha was shown to directly inhibit microgliosis [20]. When Ubisol-Q10 and ashwagandha were combined, there were greater levels of microgliosis inhibition compared to if either treatment was used alone. This was indicated by a reduction of ameboid microglia and a greater number of ramified/resting microglia.

In contrast to pro-inflammatory microglia, astrocytes provide support to neurons such as providing nutrition to neurons from the blood brain barrier or even providing synapse support between neurons. While astrocyte activation was observed in the untreated transgenic group similar to wild-type mice, this is a common phenomenon, in response to stressors such as toxic β-amyloid plaques. We saw a slight enhancement of activated astroglia in Ubisol-Q10 treated transgenic mice compared to the untreated transgenic mice. This was shown via increased number of cells staining for GFAP as well as cells showing a greater number of cytoplasmic extensions indicating reactive/active microglia. A possible reason for this is that Ubisol-Q10 is also protecting astroglia as well from β-amyloid plaques via acting as an antioxidant. β-amyloid plaques have also been shown to be cytotoxic to astroglia by increasing levels of reactive oxygen species (ROS) [26]. By reducing the amount of ROS, there will be healthier/more activated astroglia. Indeed, levels of 4-hydroxynonenal, a lipid peroxidation product due to elevated ROS was reduced in transgenic mice fed Ubisol-Q10 compared to untreated transgenic mice. Ashwagandha and the tonic also showed reduced levels of 4-HNE. Similar to Ubisol-Q10, ashwagandha extracts to act as antioxidants due to presence of several phytochemicals which are known

antioxidants such as withaferin A which was present in our extract. Ashwagandha treatment resulted in enhanced activation of astroglia as indicated by increased fluorescence, greater number of cells staining for GFAP, and greater number of processes coming from astrocyte cell bodies. In previous studies ashwagandha was shown to enhance activation of astroglia [20]. Therefore, ashwagandha could be acting in the same manner in this study. When Ubisol-Q10 and ashwagandha were given together there was even greater activation of astroglia cells compared to the mice only given ashwagandha as indicated by increased fluorescence intensity, greater number of cells staining for GFAP, and greater number of processes coming from astrocyte cell bodies. Mentioned earlier, astrocytes are also involved in the secretion of pro-survival neurotrophic factors. Brain derived neurotrophic factor (BDNF) was observed to be increased in all treatment groups of transgenic mice compared to untreated mice which had reduced expression of BDNF.

Previously, it has been reported that neurons in the CA1 pyramidal region in the brains of transgenic AD mice were reduced [7]. CA1 neurons are found in the hippocampus and involved in the process of forming short-term memory. Similar to CA1 neurons, there are CA3 neurons which are also involve in short-term memory formation. In this study we assessed whether CA3 neurons are reduced in transgenic AD mice. With the use of immunohistochemistry and epi-fluorescent microscopy we did not find a qualitative difference between the thickness of the CA3 region in any of the mice groups. It is possible these neurons are not affected by the β-amyloid plaques in these double transgenic mice, but more research is required to assess effects of β-amyloid on the CA3 pyramidal layer as other varieties of transgenic mice show neurodegeneration in the CA3 region [14].

Conclusions

Treatment with Ubisol-Q10 and ashwagandha combined showed enhanced β-amyloid plaque reduction compared to either treatment alone. The combined treatment of Ubisol-Q10 and ashwagandha also showed enhanced activation of astrocytes and inhibition of microgliosis compared to either treatment used alone. While there was no observable difference between the thickness of the CA3 region of the hippocampus in any of the mice more work is required to fully assess the status of neurons in the pyramidal tract/region of the hippocampus. Furthermore, Ubisol-Q10 was observed to enhance autophagy similar to what we have reported previously. It is possible Ubsiol-Q10 and ashwagandha target different pathologies of AD such as inflammation, oxidative stress, and autophagy which is resulting in enhanced removal of these β-amyloid plaques in the brains of these transgenic mice. With the reduction of amyloid plaques, inflammation, and oxidative stress due to the combination treatment, the neurodegeneration that occurs in AD might be being halted but further analyses are required to confirm neurons are protected. This combination treatment should prove to be an effective treatment for targeting pathologies of AD (potentially resulting in prevention of neurodegeneration leading to improvement of memory/cognition) but clinical trials are required to confirm neuroprotective effects in humans. Furthermore, these treatments are classified as natural health products and have been shown to be non-toxic in both animals and humans supporting Ubisol-Q10 and ashwagandha as potential effective therapeutics for AD, as these treatments target multiple pathologies of AD instead of only providing symptomatic relief like current AD therapies.

Patents

Ubisol-Q10 is a patented formulation.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 6: GENERAL DISCUSSION

List of Abbreviations

E-ASH	Ethanolic Ashwagandha Extract
WS-ASH	Water-Soluble Ashwagandha Extract
PQ	Paraquat
PD	Parkinson's Disease
AD	Alzheimer's Disease
DA	Dopaminergic
4-HNE	4-Hydroxynonenal
PS-1	Presenilin-1
PSAF	Mutated AD Familial Type 3 Fibroblast
APP	Amyloid Precursor Protein
CAPR1	Cell Division Cycle and Apoptosis Regulator 1
BDNF	Brain Derived Neurotrophic Factor
GDNF	Glial Derived Neurotrophic Factor
PTS	Polyoxyethanyl-alpha-tocopheryl Sebacate
NGF	Nerve Growth Factor
LC3B	Microtubule-associated Proteins 1A/1B light chain 3B
SASP	Senescence Associated Secretory Phenotype
SIPS	Stress Induced Premature Senescence
BECN1	Beclin-1
JNK1	c-Jun N-terminal Kinase 1

MAPK8	Mitogen-activated Protein Kinase 8
CTSB	Cathepsin B
LRP	Low Density Lipoprotein Receptor-Related Protein
NF-**κ**B	Nuclear Factor Kappa-B
iNOS	Inducible Nitric Oxide Sythase
HPLC	High Performance Liquid Chromatography
IBA1	Ionized Calcium Binding Adaptor Molecule 1
GFAP	Glial Fibrillary Acidic Protein

General Discussion

In this dissertation, a novel combined formulation of a water soluble-formulation of coenzyme-Q10, Ubisol-Q10, and several forms of extract of ashwagandha root (ethanolic ashwagandha extract (E-ASH) or water-soluble E-ASH (WS-ASH)) were evaluated for their efficacy and mechanism of neuroprotection in a paraquat (PQ) induced rat models of Parkinson's Disease (PD) as well as an in-vitro and in-vivo model of Alzheimer's Disease (AD).

In Chapter 2, the efficacy and mechanism of neuroprotection of Ubisol-Q10 combined with E-ASH was evaluated in a rat model of PD. Previous work has shown that Ubisol-Q10 or extracts of ashwagandha when used alone, reduced neurodegeneration of dopaminergic (DA) neurons in various in-vivo and in-vitro models of PD [1-4]. While either agent did reduce loss of DA neurons, they weren't effective of protecting all neurons compared to control groups. This is likely due to the agents not targeting all biochemical mechanisms of PD. As mentioned before, PD is a complex multifactorial disease where there are several different processes intertwined together including: mitochondrial dysfunction, oxidative stress, autophagy impairment, inflammation, and accumulation of senescent cells. Even if one or a few mechanisms were to be targeted, there are still other ones that could be contributing to progression of neurodegeneration. Here we showed interventive therapy of orally administered Ubisol-Q10 combined with E-ASH (the Tonic) was observed to be more effective at protecting DA neurons in rat brains from the neurotoxic effects of PQ in rat brains compared to either Ubisol-Q10 or E-ASH alone. A key observation made between Ubisol-Q10 and E-ASH in the way they protected DA neurons. Ubsiol-Q10 primarily protected DA neuron numbers yet the morphology of these neurons was not maintained compared to the saline control as indicated by reduced number of fibers extending from the cell body. E-ASH on the other hand, while not as effective at protecting overall neuron count, neuron morphology was better maintained as they had greater amount of fibers extending from their cell bodies similar to the saline control rats. This better protection was likely attributed from Ubisol-Q10 and E-ASH targeting different biochemical mechanisms implicated PD. Furthermore, this protection of DA neurons was complemented by reduced PQ-induced motor deficits in behavioural analyses of these rats.

Ubisol-Q10 and E-ASH both acted as antioxidants as indicated by reduced levels of lipid peroxidation product, 4-hydroxynoneal (4-HNE). Indeed, Ubisol-Q10 has been previously shown to be a potent antioxidant as well as ashwagandha [1-5]. Along with acting as an antioxidant, Ubisol-Q10 was shown to enhance expression of major autophagy regulator beclin-1 in PQ-injected rat brains. This observation was quite exciting as this was the third instance were Ubisol-Q10 was reported to enhance this autophagy regulating protein. Previously, Ubsiol-Q10 was observed to enhance autophagy activation in presenilin-1 (PS-1) mutated AD familial type 3 fibroblasts (PSAF) and the brains of double transgenic AD mice containing mutant amyloid precursor (APP) and PS-1 genes [5,6]. Treatment with the tonic or either Ubisol-Q10 or E-ASH alone resulted in enhanced expression of cell division cycle and apoptosis regulator 1 (CARP1). CARP1 was previously thought to be primarily involved in mediating apoptosis but in this case the contrary is happening. This was not totally unexpected as CARP1 is known to be a co-activator of cell/cycle regulator APC/C E3 ligase which is involved in survival signaling [7].

Along with mitochondrial dysfunction, oxidative stress, and impaired autophagy, neuroinflammation is also reported to have a major role in PD development. Pro-inflammatory microglia are known to surround DA neurons and their degree of activation has been correlated with DA neuron loss in early PD. Decreased activity of pro-survival astroglia are also reported to be impacted in PD [8]. Its been observed that inactivation of astroglia could be a direct result of elevated oxidative stress and they are unable to cope or they are inactivated by the previously mentioned pro-inflammatory microglia. Ubisol-Q10 or E-ASH, treatment resulted in a marked decreased in activation of microglia and increased activation of astroglia of PQ-injected rats. The greatest anti-inflammatory response was observed with the tonic. Interestingly, PQ-injected animals that had treatments containing E-ASH, had greater levels of pro-survival brain and glial derived neurotrophic factors (BDNF and GDNF) despite similar levels of activation of astroglia among treatment groups with Ubisol-Q10, E-ASH, and the tonic. It’s possible some component in E-ASH is stimulating enhanced expression of these neurotrophic factors. Indeed, components of ashwagandha including withaferin A (which was present in our extract) were shown to enhance expression of GDNF and BDNF [9].

Chapter 3 involved the synthesis of a water-soluble formulation of E-ASH to improve bioavailability. While E-ASH and Ubisol-Q10 were effective at protecting PQ-induced Parkinsonism in rats, there were was the major issue of being able to utilize E-ASH as a therapeutic for PD. While ashwagandha doses were able to be greatly reduced compared to previous literature (with some groups using doses of up to 1000mg/kg/day) by combining with Ubisol-Q10 (E-ASH dose was 120mg/kg/day), there were still issue with being able to deliver E-ASH economically/effectively. With most of the phytochemical constituents of E-ASH being hydrophobic and poorly water-soluble, the extract would crash out of solution when added to the rats' drinking water. With that in mind, this poses the same issue with regular coenzyme-Q10, where there is poor absorption of the drug during digestion, and therefore most of it is excreted and not bioavailable. Furthermore, yields of the E-ASH were low with around a 10-20% yield of solid extract from raw root powder. To remedy the poor bioavailability of E-ASH, a patented process using amphipathic PTS (polyoxyethanyl-alpha-tocopheryl sebacate), similar to Ubisol-Q10, was used to create a water-soluble formulation of E-ASH (WS-ASH). Following successful completion of WS-ASH synthesis, its neuroprotective efficacy at varying doses was compared to E-ASH in a PQ-induced rat model of PD. WS-ASH better protected DA neurons in PQ-injected rat brains compared to E-ASH at the same dose (2mg/mL). Interestingly, WS-ASH at 1mg/mL appeared as effective as WS-ASH at 2mg/mL indicating WS-ASH neuroprotection efficacy plateaus beyond 1mg/mL. A dose dependency was observed with WS-ASH as a concentration of 0.2mg/mL (one tenth the original E-ASH dose) was minimally effective at protecting DA neurons.

Following, E-ASH and WS-ASH comparison and WS-ASH dosing, the protective efficacy of WS-ASH combined with Ubisol-Q10 was re-evaluated to investigate potential changes in the properties of E-ASH due to nano-micellization with PTS (Chapter 3). Similar observations were made between the tonic containing WS-ASH (Chapter 3) or E-ASH (Chapter 2). WS-ASH, Ubisol-Q10, or the tonic, behaved as potent antioxidants in PQ-injected rats given these treatments as all had reduced 4-HNE levels compared to untreated PQ-injected rats. Changes in glia activation were similar between E-ASH and WS-ASH (i.e., reduced microglia activation and enhanced astroglia activation). Interestingly, WS-ASH was just as effective if not better at protecting DA neurons

compared to Ubisol-Q10 which is possibly due to the enhanced solubility/bioavailability of WS-ASH. Changes in BDNF and GDNF expression were also similar between E-ASH and WS-ASH. Furthermore, expression of neurotrophic factor essential for neuron survival, nerve growth factor (NGF), was maintained with Ubisol-Q10, WS-ASH, and tonic treatment compared to saline control animals.

Unexpectedly, WS-ASH similar to Ubisol-Q10, enhanced activation of autophagy proteins beclin-1 and microtubule-associated proteins 1A/1B light chain 3B (LC3B). The mechanism for which the expression of these autophagy proteins were being enhanced by WS-ASH was not investigate but withaferin A of the extract has been reported to enhance LC3 expression [10]. Unfortunately, there are conflicting reports with regards to the autophagy activating potential of withaferin A as it has also been shown inhibit autophagy [11,12]. It must be noted that these previous reports showing autophagy inhibition/activation were done in cancer cell lines which have widely different metabolisms and physiologies compared to neurons present in whole intact brains as depicted here. Furthermore, E-ASH was shown to enhance autophagy activation in PSAF (Appendix B). It's possible the reason autophagy activation wasn't observed initially with E-ASH was due to the poor bioavailability.

As mentioned previously, accumulation of senescence cells has also been implicated in PD development. In particular, senescent microglia and astroglia due to oxidative stress have been implicated in PD due to their display of the senescence associated secretory phenotype (SASP) resulting in release of pro-inflammatory element creating a vicious feedback loop of senescence cell accumulation, inflammation, and oxidative stress [13]. Previously, Ubisol-Q10 was shown to prevent oxidative stress induced premature senescence (SIPS) in PSAF [5]. While not a model of PD, PSAF exhibit similar pathophysiology to neurons in transgenic AD, which like the PQ-induced PD rat model, all share similar biochemical mechanisms leading to develop of the respective disease (i.e., mitochondria dysfunction, oxidative stress, autophagy inhibition, inflammation, and senescence cell accumulation). Here, Ubisol-Q10 was also observed to reduce cell senescence in PQ-injected rat brains as indicated by reduced expression of senescence associated protein p21 (Chapter 3). WS-ASH and the tonic were also shown to

reduce levels of p21. It's possible all three treatments may be acting in a similar way to prevent senescent cell accumulation (preventing oxidative stress that can lead to SIPS) though further experiments would be needed to confirm this mechanism. Along with acting as an antioxidant, the ashwagandha extract used in these experiments was shown to contain the senolytic quercetin which could also be involved in preventing senescence in the brain of PQ-inject rats [13].

During behavioural testing covered in Chapter 2 of this dissertation, the mechanism of how Ubisol-Q10 protects PSAF from the deleterious effects of familial PS-1 mutations was further investigated in Chapter 4. Initial pilot data of quantitative polymerase chain reaction of genes associated with apoptosis, autophagy, and oxidative stress revealed expression of several key genes of proteins associated with autophagy to be enhanced in PSAF treated with Ubisol-Q10. These genes included: beclin-1 (BECN1), a major autophagy regulator; c-Jun N-terminal kinase 1/mitogen-activated protein kinase 8 (JNK1/MAPK8), an activator of beclin-1; and cathepsin B (CTSB), a lysosomal cysteine protease. Previously, Ubisol-Q10 was shown to prevent oxidative SIPS as well as enhance production of autophagosome formation [5]. With this information, the role of autophagy enhancement due to Ubisol-Q10 in protecting PSAF was further investigated. This was done be treating cells with small molecule autophagy inhibitor SP600125 which blocked activation of JNK1 and therefore beclin-1. As expected Ubisol-Q10 pre-treated cells that were incubated with SP600125 showed reduced autophagy activity as indicated by reduced autophagosome formation and reduced LC3B puncta. Furthermore, these cells also resumed their SIPS phenotype as indicated by enhanced expression of senescence marker p21 and increased senescence β-galactosidase activity. This indicates that along with acting as a potent inhibitor of oxidative stress, Ubisol-Q10 also acts as an activator of autophagy that was critical in prevent SIPS in PSAF. This mechanism was also observed in the cortices of APP/PS-1 mutant double transgenic AD mice where Ubisol-Q10 resulted in enhanced expression of JNK1 and beclin-1. As mentioned previously, beclin-1 expression was also enhanced in the brains of PQ-injected rats treated with Ubisol-Q10 further supporting the observation of autophagy activation seen in PSAF.

Chapter 5 examined the neuroprotective efficacy of Ubisol-Q10 and E-ASH combined together compared to the agents alone in a transgenic AD mouse model. Similar to PD, oxidative stress, mitochondrial dysfunction, autophagy inhibition, inflammation, and senescence are also implicated in AD development [13-18]. Treatment with Ubisol-Q10 or E-ASH alone were both shown to reduce β-amyloid load in the brains of double transgenic mice. The tonic was most effective at reducing β-amyloid load likely as a result of the agents working via different mechanisms to remove these plaques. As mentioned previously, Ubisol-Q10 was shown to enhance beclin-1 activation and ashwagandha has been shown to activate low density lipoprotein receptor-related protein (LRP). It's possible these same mechanisms are occurring here together to remove the β-amyloid plaques. Indeed, autophagy was shown to be enhanced with Ubisol-Q10 due to increased expression of beclin-1 and LC3B. Ubisol-Q10 and E-ASH where both shown protect the brains of transgenic mice from the pro-oxidant effects of β-amyloid as indicated by reduced levels of 4-HNE around what look to be β-amyloid plaques (though this would need to be confirmed with β-amyloid counter staining, previous work has shown β-amyloid plaques elicit a pro-oxidant effect in their periphery) [19].

Pro-inflammatory microglia have been shown to be activated around β-amyloid plaques [20,21]. Similar to what was observed in PQ-inject rats discussed in Chapter 2, Ubisol-Q10, E-ASH, and the tonic were all shown to reduce microgliosis in the brains of transgenic mice. In contrast to microglia, presence of active astroglia have been shown to be reduced in AD [22]. Treatment with Ubisol-Q10 result in slight increased activation of astroglia compared to untreated transgenic mice. This might be due to Ubisol-Q10 reducing antioxidant burden as discussed earlier. E-ASH and tonic treatment resulted in an even greater increase in astroglia activation compared to Ubisol-Q10. The exact mechanism of how ashwagandha activates astroglia and suppresses microglia was not investigated but previous work has shown that withaferin A inhibits nuclear factor kappa-B (NF-**κ**B) and inducible nitric oxide synthase (iNOS) both of which are involved in regulating inflammation and suppressing and activating astroglia and microglia respectively [23]. Similar to PQ-injected rats discussed in Chapter 2, increased BDNF expression with all treatments also coincided with increased astroglia activation which would further aid in protecting neurons in the brains of these double transgenic mice.

Conclusion

This dissertation demonstrates the therapeutic potential of a novel combined formulation of Ubisol-Q10 and two forms of extract of ashwagandha root against two of the most common neurodegenerative diseases in the world, Alzheimer's, and Parkinson's Disease. Using in-vitro and in-vivo models of Alzheimer's and Parkinson's Disease, it was determined that both diseases share several biochemical mechanisms (oxidative stress, mitochondrial dysfunction, autophagy impairment, inflammatory modulation of glia, and accumulation of senescent cells) leading to their development. Ubisol-Q10 combined with either E-ASH or WS-ASH was more effective at reducing the deleterious effects of AD and PD compared to the agents alone as they targeted all biochemical mechanisms of the diseases rather than just one or a few. Furthermore, delivery of E-ASH was improved using PTS to create the water-soluble and more neuroprotective WS-ASH. In addition, these treatments were shown to be well tolerated over the use of the animals' entire lifespans and showed not visible negative side effects. The information presented here shows the important need for development of new therapies for AD and PD that have potential halt progression of neurodegeneration and improve quality of life for those living with these diseases.

Future Work

The work presented here highlighted the neuroprotective effectiveness of a combinatorial therapy composed of two well tolerated natural health products for targeting biochemical mechanisms of AD and PD. Most of the work shown involving animals presented biochemical data only. Along with biochemical data, behavioral data is also essential when using rodents to ensure biochemical changes result in an observable improvement of the animals' behaviour as well. Currently, raw behavioural data for animals discussed in Chapters 3 and 5 is being processed with the assistance of Dr. Cohen's animal behaviour laboratory. It would also be ideal to refine the effective dosage required for WS-ASH as only 3 doses were used and within a large range (2mg/mL, 1mg/mL, and 0.2mg/mL). At 1mg/mL a plateau in WS-ASH effectiveness was observed indicating doses could be further reduced. Furthermore, to validate/ensure that PTS is in fact enhancing absorption of E-ASH in WS-ASH, bioavailability studies should be conducted to ensure higher amounts of the extract are entering the brain. This could be done similar to what was done with Ubisol-Q10 where mass spectrometry/high performance liquid chromatography (HPLC) was used to analyze levels of coenzyme-Q10 of brain tissue lysates. Amounts of certain key phytochemicals of therapeutic interest such as withaferin A could be measured in the brains of animals either given E-ASH or WS-ASH at varying doses. Further phytochemical analysis of E-ASH should also be conducted as analysis of E-ASH in Chapter 2 only measured 5 different components. Following greater analysis, the individual components should be tested for their ability to affect the biochemical mechanisms implicated in AD and PD. Further work should also be conducted with different models of PD and AD. Here only a PQ-induced rat of PD and double transgenic mouse model of AD was utilized. Another model of PD includes DJ-1 mutated mice as a genetic susceptibility model PD. For AD, there exist other genetic mouse models including triple transgenic mice which carry a mutation that results in hyperphosphorylation of tau leading to development of neurofibrillary tangles along with APP and PS-1 mutations that lead to β-amyloid plaque accumulation. The work presented here also only probed for changes in oxidative stress, autophagy impairment, inflammation, and senescence and lacked analysis of mitochondria dysfunction markers (though numerous in-vitro studies prior have shown the mitochondria preserving effects of Ubisol-Q10 and ashwagandha).

The reason for this is that mitochondria are drastically affected during perfusion of animals. Once blood is flushed from the animal, mitochondria are almost immediately affected by the lack of oxygenated blood. And in the case of the brain where neurons almost exclusively use oxidative phosphorylation, mitochondria in neurons are almost immediately affected by lack of oxygenated blood and begin to self-destruct making it difficult to analyze mitochondria related proteins in the brain. This could be remedied though by sacrificing the animals without perfusing and immediately snap freezing the brains in liquid nitrogen to preserve mitochondria status. Furthermore, while well-known markers of oxidative stress (4-HNE), autophagy (beclin-1 and LC3B), inflammation (IBA1/GFAP), and senescence (p21) were consistently shown to be affected in several models of AD and PD, other markers for changes in these biochemical processes should be investigated. Finally, based on the exciting findings observed in Chapter 3 with WS-ASH in the PQ-induced rat model of PD, it would be interesting to see the neuroprotective effectiveness of WS-ASH in an AD transgenic rodent model.

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APPENDICES

Appendix A: *Copyright Permissions*

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Appendix B: ***Immunofluorescent staining of PSAF treated with Ubisol-Q10 and ashwagandha extract***

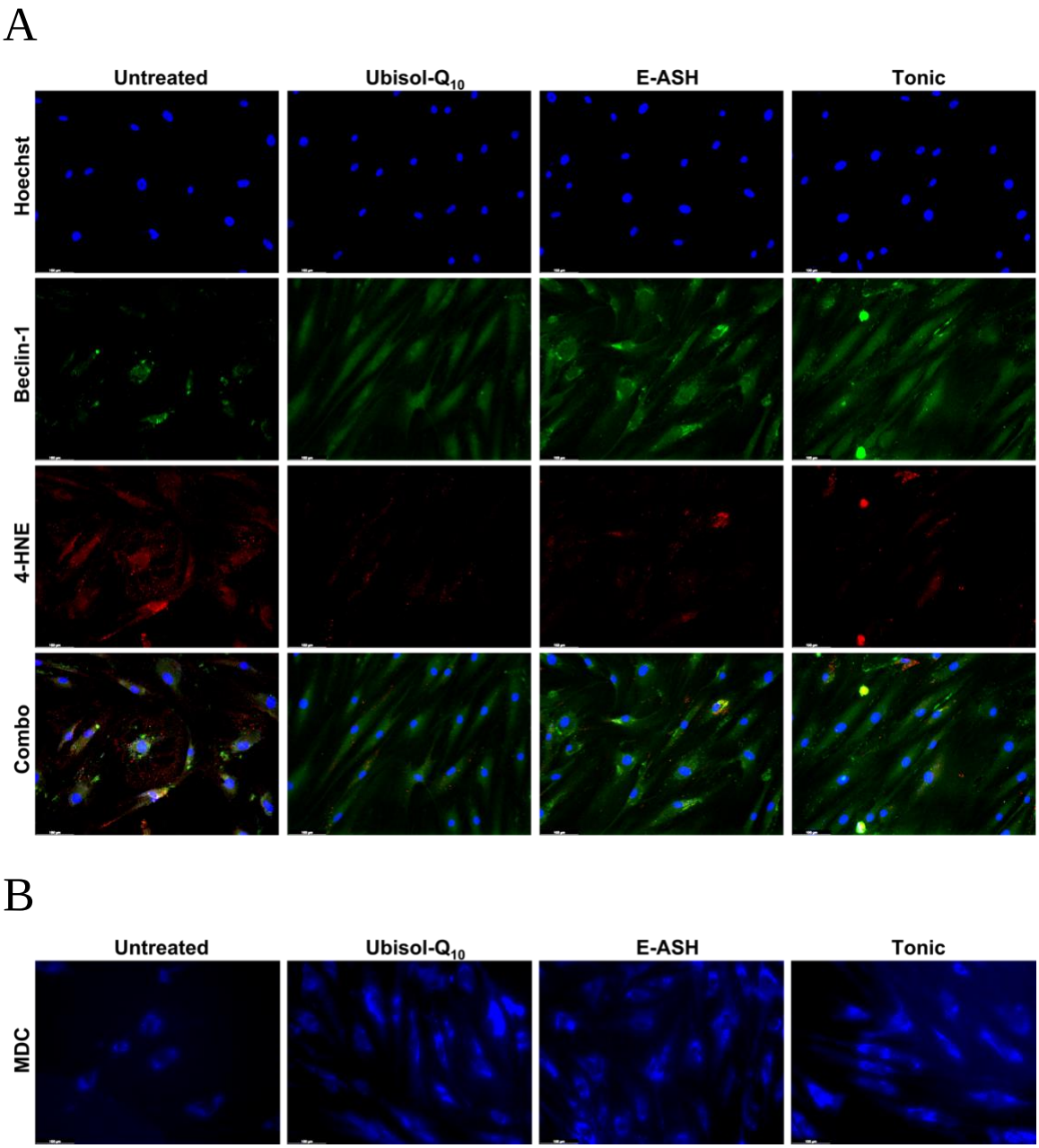

Figure 1. Ubisol-Q10 and ethanolic ashwagandha extract (E-ASH) treatment reduce oxidative stress and enhance autophagy in PSAF. **(A)** Immunoflourescent staining of PSAF probing for autophagy regulator beclin-1, and oxidative stress marker 4-HNE. **(B)** MDC staining of autophagosomes in PSAF. Micrographs were taken at 200x magnification (scale bar = 100 microns).

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2020-21	Ontario Graduate Scholarship
2020	G&C Morgan Graduate Scholarship
2020	Loaring Memorial Award – Breast Cancer Research
2018-2020	Mitacs Accelerate Fellowship
2019	NHPRS Best Poster Presentation (2nd Place)
2019-2020	Ontario Graduate Scholarship

2019	NHPRS Travel Award
2019	Windsor Prostate Cancer Award
2018-2019	Queen Elizabeth II Graduate Scholarship
2018	NHPRS 3 Minute Thesis Competition (2nd Place)
2018	NHPRS Travel Bursary
2017-2018	University of Windsor Graduate Entrance Scholarship

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