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The protective effects of anthocyanins on neurons

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The neuroprotective effects of anthocyanins against oxidative stress in SH-SY5Y cells

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Introduction

Parkinson's and Alzheimer's are debilitating neurodegenerative diseases that are largely thought to be exacerbated, and perhaps even caused, by oxidative stress in and around neurons. At the same time, there has been increased research in the field of nutrition and how the foods we eat impact our short- and long-term health. These combined interests have resulted in fascinating studies that have found certain foods, namely plants, can have a variety of medicinal benefits. Many phytonutrients (nutrients found in plants) seem to reduce oxidative stress in various cell types, including neural cells. However, because the combination of these research fields has only recently increased in popularity, the antioxidative effects of relatively few phytonutrients have been studied, and almost none have been studied in conjunction with nutrients that belong to different subgroups of phytonutrients. The purpose of this study is to determine if plant extracts that have high levels of certain phytonutrients can increase the activity of cellular enzymes that reduce oxidative stress in SH-SY5Y neurons, and how their effects differ when combined with green tea extract (GTE), an extract that has been extensively studied for its antioxidative properties.

Polyphenols

There has been a recent uptick in research surrounding the antioxidative properties of phytonutrients. Polyphenols are one of the largest groups of phytonutrients, allowing them to be cheaply and abundantly obtained by readily incorporating plants into diets (Almeida et al., 2016). Flavonoids, of which anthocyanins are a subgroup, are one of the most prominent and most studied groups of polyphenols (Gamba et al., 2021). Anthocyanins have been observed as having antioxidative properties in neurological diseases by exerting a change in various biochemical pathways (Matrella et al., 2022). Some common anthocyanins (namely delphinidin, cyanidin,

and pelargonidin) have been found to be scavengers of free radicals, which cause significant oxidative stress (Noda et al., 2002). However, the impact of cyanins on antioxidative enzyme activity has not been extensively studied Additionally, they have not been studied in conjunction with green tea extract (GTE), which is rich in flavonoids and other polyphenols and has been demonstrated to consistently induce catalase and glutathione peroxidase (Bernatoniene & Kopustinskiene, 2018).

Oxidative stress

High concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that cause oxidative stress are toxic to neurons, and are believed to play a role in aging, especially in neurodegenerative diseases (Biswas et al., 2022). Indeed, deficiencies in antioxidative enzymes such as catalase and glutathione peroxidase, are associated with a number of neurodegenerative diseases such as Alzheimer's disease, bipolar disorder, Parkinson's disease, and schizophrenia (Nandi et al., 2019). For example, Glorieux et al. (2015) studied the effects of catalase deficiency in mice and found that the mice developed normally with low catalase expression but presented with defective oxidative phosphorylation in mitochondria after exposure to ROS. In contrast, the upregulation of catalase was associated with a nearly twenty percent increase in lifespan when exposed to ROS (Glorieux et al., 2015).

Oxidative stress can cause significant damage to DNA, RNA, and other crucial parts of a functioning cell (Kim & Jung, 2020). In particular, mRNA translation seems to be important in nearly every aspect of developing axons, including axon guidance, elongation, target selection, survival, and regeneration, as the proteins synthesized from mRNA translation are crucial for the function of axons (Kim & Jung, 2020). Axons carry electrochemical signals from neuron to neuron, so all of these developmental aspects are necessary for neuroplasticity, which is the

ability of the nerves to reorganize themselves following a neural injury, including those resulting from oxidative stress (Bavelier et al., 2002). Because catalase and glutathione peroxidase would mitigate oxidative effects, axon development and morphology can also be used to determine the ability of these enzymes to aid in neuroplasticity following oxidative stress.

Two common ways of introducing oxidative stress to *in vitro* mammalian cells are by exposing them to lipopolysaccharide (LPS) and D-galactose (DG). LPS is an extracellular component on the membrane of gram-negative bacteria, to which the innate immune response is to increase the production of ROS and RNS at the cellular level to breakdown the invading pathogens; however, this response acts as a sort of cellular grenade, harming nearby cells as well as the bacteria (Farhana & Khan, 2023). Similarly, DG is a reducing sugar that, while necessary in low concentrations, begins a cascade of biochemical reactions that result in the formation of ROS at high concentrations (Guo et al., 2020).

SH-SY5Y

SH-SY5Y cells are a subclone line of neural cells whose parent line was derived from a metastatic bone tumor (Kovalevich & Langford, 2013). Isolated cancerous cell lines are often used for cell culturing because of their proliferative properties, which allow for reproducible experimentation and results. The tumor that SH-SY5Y were derived from was comprised of neural stem cells, which are undifferentiated cells that are intended to undergo regulated proliferation (Dravid et al., 2021). This means that while some metabolic research can be done on the undifferentiated SH-SY5Y cells, they do not behave like neurons and should be differentiated for research that includes morphological and certain cell physiological factors (Dravid et al., 2021). This requires particular stimuli in order to differentiate the stem cells into neuronal cells with specific form and function (Hodgson et al., 1999). There are several

categories of differentiated neurons, each with unique properties. The neurons that are most involved in oxidative-stress-induced neurodegenerative diseases are dopaminergic (Kovalevich & Langford, 2013; Niaz, 2021). Dopaminergic differentiation of SH-SY5Y has been commonly achieved by introducing retinoic acid (RA) to the cells, along with other alterations to the cell media that supports the growth and stability of differentiated neurons (Dravid et al., 2021; Kovalevich & Langford, 2013; Messmer & Reynolds, 2005; Pandur et al., 2019; Wiatrak et al., 2022).

Methods

Treatment

SH-SY5Y cells were differentiated as detailed below and then pretreated with anthocyanin-rich extracts (elderberry, coffee berry, whole tart cherry), green tea extract (GTE), or a combination of an anthocyanin-rich extract and GTE for 24 hours (Fig. 1). Concentrations of phytonutrients were selected based on prescreened cell viability studies conducted in the Sharda lab. After pre-treatment, the cells were treated with 10µg/mL LPS for 8 hours or 300 mM D-galactose (DG) for 72 hours. LPS and DG serve as agents to cause oxidative stress in the cells. After treatment, the cells were lysed using a hypotonic lysis buffer (Shangari, 2006).

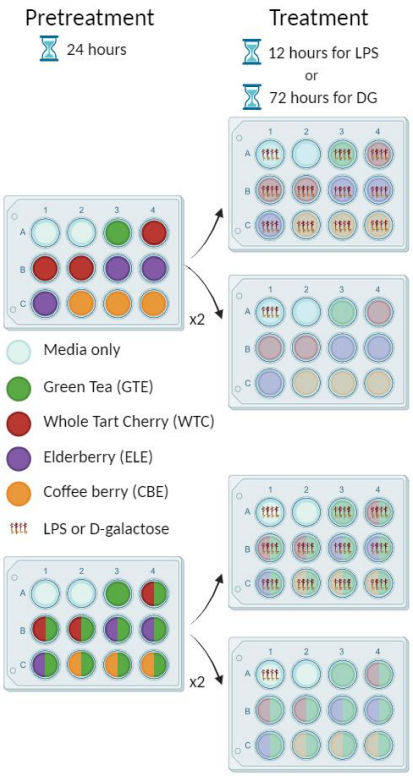

Fig. 1. Differentiated SH-SY5Y cells were pretreated with GTE, WTC, ELE, or CBE or with GTE and WTC, ELE, or CBE. Each plate contained positive, negative, and GTE controls. After 24 hours of pretreatment, half of the cells were treated with either LPS or D-galactose (DG). LPS treatment lasted for 12 hours, while DG treatment lasted for 72 hours.

Catalase Activity Assay

The assay for catalase is uses FOX 1 reagent, which is comprised of 250 µM ammonium ferrous sulfate, 100 µM xylenol orange, 0.1 M sorbitol, and 25 mM H_2SO_4 (Shangari, 2006). 10 uL 2.2 mM hydrogen peroxide was added to 100 µL cell lysate. 50 µL cell lysate was removed after 3 minutes and 10 minutes of incubation and were rapidly added to 950 µL FOX 1 reagent to stop the catalase reaction. Samples were incubated at room temperature for 30 minutes, micro centrifuged for 3 minutes at 12,000 × g (Hettich® Mikro 220R). A spectrophotometer (Agilent 8453) was used to read the absorbance at 560 nm (Shangari, 2006).

BCA Protein Assay

Protein concentration was determined spectrophotometrically using the Thermo Scientific™ Pierce™ BCA Assay Kit (ThermoFisher Scientific, 23225) according to the kit instructions. Results were used to standardize catalase activity to protein concentration.

DCFDA Assay and Imaging

A dichlorofluorescin diacetate (DCFDA) assay protocol was adapted from Rahimi et al. (2018) and was run on some of the treated cells to assess the presence of ROS. This will provide additional evidence that the extracts are meaningfully affecting antioxidative enzyme activity. Stained cells were imaged on a fluorescent microscope (Nikon Eclipse Ti) with neurite-tracing analysis using NeurphologyJ, a free software of the NIH ImageJ suite. The morphology of the cells in the images can be used as indicators of overall cell health as well as the effects of ROS and the antioxidant treatments.

Statistical Analysis

The validity of the results was analyzed using a 2-sample t-test assuming unequal variances in Microsoft Excel. A p-value of ≤ 0.05 was considered statistically significant.

Differentiation

Undifferentiated SH-SY5Y cells were maintained in basic growth media at 37 ºC/5% CO_2. The differentiation protocol was adapted from Kovalevich & Langford (2013) and Dravid et al. (2021). 12-well plates (MidSci, TP92012) were coated with ECM GFR Gel (from Engelbreth-Holm-Swarm Mouse Sarcoma) diluted 1:100 in basic media and incubated at 37 ºC/5% CO_2 for 24 hours before plating cells. Any additional liquified ECM GFR left in wells were aspirated 1 hour before cells were plated. Cells were plated at a concentration of 12,987 cells/cm^2. The following day, the media was changed to differentiation media. Differentiation media was

changed every 48 hours. After 8 days of differentiation, cells were ready for experimentation. Cultivated cells were maintained at 37 °C/5% CO_2.

Media

Basic Growth Media

Dulbecco's Modified Eagle Medium (DMEM) (Sigma, D6546), 10% heat-inactivated fetal bovine serum (hiFBS) (ThermoFisher Scientific, 10082147), 1× glutaMAX™ Supplement (glutamax) (ThermoFisher Scientific, 35050061), 1×penicillin streptomycin solution (pen/strep) (Sigma, P4333).

SH-SY5Y cells were attained from ATTC (CRL-2266) and grown from frozen samples. This was done by warming 9 mL and 20 mL of basic growth media in separate 50 mL centrifuge tubes. The frozen cells thawed in a 37ºC bead bath for about 2 minutes. The entire contents of the cryovial were then removed and resuspended in 9 mL warmed media, then centrifuged at 1500 rpm for 5 minutes (Eppendorf Centrifuge 5810 R). The supernatant was aspirated, and the cell pellet was resuspended in 20 mL of basic growth media and placed in a sterile T75 tissue culture flask (MidSci, TP90076). The cells were then allowed to incubate at 37 ºC/5% CO_2 for about a week until the cells reach about 80% confluency before being passaged to new flasks. At least 2 passages occurred before cells were plated and differentiated.

Differentiation Media

Neurobasal medium (ThermoFisher Scientific, 10888022), 1×serum-free B-27™ Supplement (B27) (ThermoFisher Scientific, 17504001), 1×glutamax, 20 mM KCl, and10 μM all-*trans*-retinoic acid (RA) (ThermoFisher Scientific, 207340010). B27, glutamax, and KCl were added

to neurobasal medium in advance, and RA was added immediately before media was applied to cells.

Results

In order to gain a better understanding of the effects of anthocyanin rich polyphenols on neuronal catalase activity, we pretreated differentiated dopaminergic SH-SY5Y cells with whole tart cherry (WTC), elderberry extract (ELE), and coffee berry extract (CBE). Cells were then stressed with either DG as a model of senescence or with LPS as a general inflammatory model prior to assessing catalase activity. As shown in figure 2, consistent with published findings, treatment with DG alone reduced catalase activity. DG-induced senescent cells that were pretreated with only GTE or WTC had significantly higher catalase activity than those that were stressed with DG without any extract pretreatment (Fig. 2). We did not observe statistically significant differences in catalase activity in DG-stressed cells that were pretreated with ELE or CBE, although trends show that they may have a positive effect.

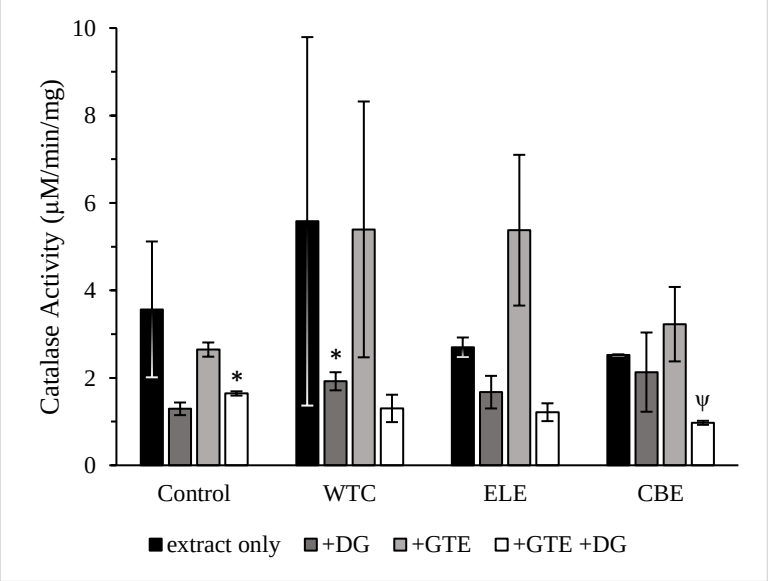

Fig. 2. Individual GTE and WTC extracts increase catalase activity when challenged with DG-induced senescence, but not when combined. Differentiated SH-SY5Y cells were (1)

treated with the given anthocyanin extract alone for 96 hours, (2) pretreated with the anthocyanin extract for 24 hours and induced into senescent stress with DG treatment for 72 hours, (3) pretreated with the anthocyanin extract and GTE to determine any synergistic interactions for 96 hours, and (4) pretreated with the anthocyanin extract and GTE for 24 hours and then induced to senescence with DG for 72 hours to determine if synergistic protection would be observed. Experimental values were expressed as mean ± standard deviation. $^{\Psi}P < 0.05$ vs. GTE + DG.

Our lab has previously observed synergistic effects of these phytonutrients when combined with green tea extract (GTE) in a macrophage model of inflammation. Interestingly, WTC or ELE combined with GTE exhibited strong catalase induction suggesting the possibility of synergy in SH-SY5Y differentiated neurons (Fig 2). However, to our surprise, when induced to senescence with DG, all protective effects appear to be lost. In fact, DG-stressed cells that were pretreated with ELE or CBE when compared to DG-stressed cells that did not receive pretreatment, although trends show that they may have positive effect. As seen in Fig. 2, DG-stressed cells that were pretreated with both CBE and GTE had significantly lower catalase activity than those that were pretreated with GTE alone. Although not statistically significant, DG-stressed cells pretreated with WTC and GTE or ELE and GTE also demonstrated lower catalase activity than non-pretreated senescent cells.

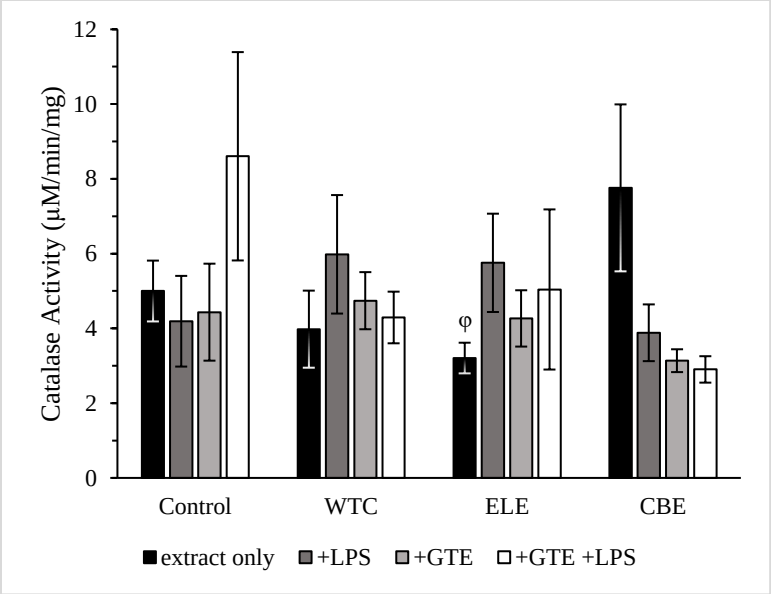

Fig. 3. Twelve hours of LPS stimulation was insufficient to observe discernable differences between anthocyanin extract treatments. Differentiated SH-SY5Y cells were (1) treated with the given anthocyanin extract alone for 36 hours, (2) pretreated with the anthocyanin extract for 24 hours and induced into senescent stress with LPS treatment for 12 hours, (3) pretreated with the anthocyanin extract and GTE to determine any synergistic interactions for 36 hours, and (4) pretreated with the anthocyanin extract and GTE for 24 hours and then induced to senescence with LPS for 12 hours to determine if synergistic protection would be observed. Experimental values were expressed as mean ± standard deviation. $^{\varphi}P < 0.05$ vs. negative control.

We next wanted to determine if these anthocyanin rich phytonutrients could protect neuronal cells in an LPS-induced inflammatory model (Fig. 3). While differentiated SH-SY5Y cells that were pretreated with ELE had significantly lower catalase activity levels than those that were not pretreated, results comparing either WTC or CBE to those that were not pretreated were inconsistent. In fact, our data suggests that twelve hours of LPS treatment was insufficient to measurably impact catalase expression in control or phytonutrient pretreated cells.

Discussion

As a whole, anthocyanin-rich extracts by themselves seem to have little effect on catalase activity in both stressed and unstressed cells, although ELE did have significantly lower catalase

activity in than the negative control in the results from the LPS-treatment experiment (Fig. 2). Contrary to expectations, stressed SH-SY5Y cells demonstrated diminished catalase activity when pretreated with both anthocyanin-rich extracts and GTE. This indicates that there is an antagonistic interaction between these extracts, whether directly or through the signaling pathways they initiate.

Previous studies regarding catalase activity levels in DG- or LPS-stressed cells yielded results that included statistically significant results between positive and negative controls, as well as between GTE-pretreated cells and controls, including those that have used SH-SY5Y cells as their *in vitro* model. However, the results in this study did not show significant differences between the positive and negative controls or GTE and the controls. The reason for this is unclear, although there are many possibilities. Many previous studies used varying concentrations of DG and LPS in their stimulated cells. Due to limited time and funds, this study was conducted using only one low concentration of DG or LPS. The use of higher concentrations of these cell-aging molecules might have yielded clearer differences in catalase activity among the various treatment combinations. Furthermore, although some previous studies did use whole extracts as pretreatments, many only pretreated using purified components of said extracts, which may have enabled them to have greater contact and effects on the cells. It may be beneficial to study how the individual components of these extracts impact SH-SY5Y catalase activity.

Further research may include what specific molecules in WTC, ELE, CBE, and GTE interact with neuronal cells, and which receptors they each interact with. This could aid in understanding the biochemical pathways that these extracts use to signal. Additionally, anthocyanin-rich extracts do not necessarily negatively affect the cells as a whole. If they were acting as free-

radical scavengers, they would eliminate some oxidative stress in the cells, in which case it may not be necessary for catalase to be upregulated. Additionally, there are other antioxidant enzymes that are prevalent in these cells that may have been positively impacted by these extracts. Studying the activity levels of other common antioxidant enzymes such as glutathione peroxidase and superoxide dismutase would help in better understanding the effects of anthocyanin-rich extracts, both alone and in conjunction with GTE, on the overall oxidative stress in SH-SY5Y cells.

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