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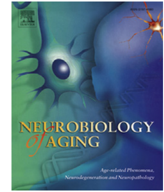

Pomegranate juice exacerbates oxidative stress and nigrostriatal degeneration in Parkinson's disease

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ABSTRACT

Numerous factors contribute to the death of substantia nigra (SN) dopamine (DA) neurons in Parkinson's disease (PD). Compelling evidence implicates mitochondrial deficiency, oxidative stress, and inflammation as important pathogenic factors in PD. Chronic exposure of rats to rotenone causes a PD-like syndrome, in part by causing oxidative damage and inflammation in substantia nigra. Pomegranate juice (PJ) has the greatest composite antioxidant potency index among beverages, and it has been demonstrated to have protective effects in a transgenic model of Alzheimer's disease. The present study was designed to examine the potential neuroprotective effects of PJ in the rotenone model of PD. Oral administration of PJ did not mitigate or prevent experimental PD but instead increased nigrostriatal terminal depletion, DA neuron loss, the inflammatory response, and caspase activation, thereby heightening neurodegeneration. The mechanisms underlying this effect are uncertain, but the finding that PJ per se enhanced nitrotyrosine, inducible nitric oxide synthase, and activated caspase-3 expression in nigral DA neurons is consistent with its potential pro-oxidant activity.

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1. Introduction

Selective degeneration of dopamine (DA) neurons in the substantia nigra (SN) underlies the cardinal motor impairments of Parkinson's disease (PD). The pathogenesis of PD is associated with mitochondrial dysfunction and increased oxidative stress, which may be caused in part by selective inhibition of complex I activity (Beal, 2005; Hu et al., 2010). Decreased activity and immunoreactivity of complex I have been observed in the SN of PD patients (Schapira et al., 1989) along with increased oxidative modification of lipids, proteins, DNA, and RNA (Jenner, 2003). Free radicals and other reactive oxygen species (ROS) and reactive nitrogen species (RNS) derived from DA metabolism and auto-oxidation (Cadet and Brannock, 1998), nitric oxide reactions (Beckman et al., 1990), lipid peroxidation (Liang et al., 2007), impaired mitochondrial function (Tapias et al., 2009), and alterations in defensive endogenous antioxidant systems (Chinta et al., 2007) may all lead to oxidative and nitrosative stress, contributing to a progressive loss of DA neurons. In this context, antioxidant strategies confer neuroprotection in several animal models of PD (Liang et al., 2007; Tapias et al., 2009). Evidence also suggests that microglial activation may perpetuate DA neuron degeneration, and anti-inflammatory therapies that inhibit microglial activation are protective in experimental PD (Wu et al., 2002, 2009).

Polyphenols are the major class of phytochemicals in pomegranate fruit and reportedly have antioxidant activity in PD models in vivo (Guo et al., 2007) and in vitro (Levites et al., 2002). The antioxidant activity of phenolic compounds is because of their ability to scavenge free radicals, donate hydrogen atoms, and chelate metal ions. Specifically, the antioxidant capacity of pomegranate juice (PJ) is mainly attributed to punicalagin and has been shown to be 3 times higher than that of red wine or green tea infusion (Gil et al., 2000). In addition, PJ has favorable pharmacological properties and may protect against inflammation, cancer, and neurodegeneration (Hartman et al., 2006; Koyama et al., 2010).

Systemic administration of rotenone reproduces behavioral, anatomic, and neuropathological changes occurring in idiopathic PD (Betarbet et al., 2000; Cannon et al., 2009). Uniform inhibition of

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mitochondrial respiratory chain complex I across brain regions by rotenone stimulates widespread oxygen free radical formation and nitric oxide ($NO^{\bullet}$) production (He et al., 2003) but degeneration is selective for the DA nigrostriatal system. Evidence suggests that microglia play a pivotal role in rotenone-induced degeneration of DA neurons (Sherer et al., 2003a).

This study was undertaken to determine whether dietary supplementation with PJ provides neuroprotective effects after rotenone exposure. We have focused our attention on the effect of PJ because its key components cross the blood-brain barrier, it is easy to administer, and it is safe. Because oxidative stress and inflammation may be prominent in PD pathogenesis (McGeer et al., 1988; Wu et al., 2002) we hypothesized that PJ would be protective.

2. Methods

2.1. Animals

Six-seven-month-old adult male Lewis rats were used for all experiments (Hilltop Lab Animals, Inc, Scottdale, PA, USA). The animals were maintained under standard conditions of 12 hours light and/or dark cycle, 22 ± 1 °C temperature-controlled room and 50%–70% humidity, and were provided water and food *ad libitum*. They were adapted for 2 weeks to the conditions described previously before experimentation. All studies were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and were performed in accordance with published National Institutes of Health guidelines.

2.2. Experimental design

Animals were randomly divided into the following 4 groups: vehicle + vehicle (VEH + VEH), pomegranate juice + vehicle (PJ + VEH), vehicle + rotenone (VEH + ROT), and pomegranate juice + rotenone (PJ + ROT). The experimental groups were comprised of 6 animals for vehicles (VEH + VEH and PJ + VEH) and 11 animals for rotenone groups (VEH + ROT and PJ + ROT). Before injections, body weights were recorded for each animal.

Rotenone (Sigma-Aldrich St. Louis, MO, USA) was administered intraperitoneally once a day at a dose of 3.0 mg/kg until the end of the treatment. The solution was prepared as a 50× stock dissolved in pure dimethyl sulfoxide at final concentration of 2%, then diluted in a medium chain fatty acid called Miglyol 812N at final concentration of 98% (Sasol North America, Inc, Houston, TX; distributed by Warner Graham, Baltimore, MD, USA), and administered at 1 mL/kg. This regimen produces relatively uniform bilateral nigrostriatal lesions, leading to loss of about 50% of DA neurons (Cannon et al., 2009). Control animals received an equivalent amount of vehicle (2% dimethyl sulfoxide + 98% Miglyol).

Rats were pretreated with PJ (POM Wonderful, Los Angeles, CA, USA) administered in their drinking water for 2 weeks. PJ from a single lot of PJ concentrate was diluted 1:40 in filtered water; importantly, this dose was selected after careful review of a manuscript that showed no statistically significant differences for any behavioral or neuropathological assessments between different concentrations of PJ (Hartman et al., 2006). The PJ used in our study is 4 times more concentrated than other conventional PJ sold commercially; thus, the final concentration of PJ at 1:40 dilution does not significantly differ from non-concentrated PJ.

The rats drank between 6.5–7.5 mL of fluid per day. The estimated polyphenol content in PJ is 3600 μg/mL (Aviram et al., 2008); thus, the amount of polyphenols consumed by the rats was estimated to be ~0.6–0.7 mg/d. The juice mixture was made fresh 2–3 times per week, filtered, and administered until the end of the study. Control rats (VEH + VEH) received sugar water that matched the sugar content of the 1:40 PJ dilution (85% sucrose, 7.5% D-(+)-glucose, and 7.5% D-fructose).

2.3. Behavioral study

2.3.1. Rearing behavior analysis

As a functional measure of loss of nigral DA neurons, spontaneous rearing activity was evaluated. Animals were placed in a clear plexiglas cylinder (height = 30 cm, diameter = 20 cm) for 5 minutes. While in the cylinder, animals typically rear and engage in exploratory behavior by placing their forelimbs along the wall of the cylinder (Schallert and Tillerson, 2000). All animals were observed and video recorded under red-light (10 lx), to encourage movement. To be classified as a rear, the animal had to raise the forelimbs above shoulder level and make contact with the cylinder wall with either one or both forelimbs. Removal of a forelimb from the cylinder wall and contact with the bottom surface of the cylinder was required before another rear was scored. Rearing behavior was assessed at baseline and again after 5 days of rotenone or vehicle injection for each rat. Importantly, this test has been used to assess bilateral DA-dependent behavioral deficits in the rotenone model (Cannon et al., 2009).

2.3.2. Postural instability test

To evaluate postural stability—a hallmark feature of PD (Woodlee et al., 2008)—rats were held almost vertically facing downward while 1 forelimb was allowed to contact the table surface which was lined with medium-grit sand paper. The tip of the rat's nose was aligned with the zero line of a ruler. One forelimb was gently restrained against the animal's torso by the experimenter allowing the rat to plant its unrestrained forelimb. The rat was then moved forward over the single planted forelimb until it made a "catch-up" step to regain its center of gravity. The new position of the tip of the nose indicated the displacement of the body needed to trigger a catch-up step in the unrestrained supporting forelimb. The postural instability test was carried out at baseline and after 5 days of rotenone treatment. In divergence with unilateral 6-hydroxydopamine (6-OHDA) models of PD, here, both forelimbs were averaged together because rotenone produces bilateral symptoms. This test has been previously used in rotenone-treated rats to assess bilateral deficits (Cannon et al., 2009).

2.4. Brain tissue processing

Tissue collection was carried out when the animals had reached endpoint, which was empirically determined for each individual animal when the behavioral phenotype became debilitating, that is, when akinesia, rigidity, and postural instability were manifested. Animals were euthanized by CO_2 inhalation followed by decapitation. The brains were quickly removed and sectioned sagittally along the midline. One hemisphere was fixed in 4% paraformaldehyde for 7 days and then placed in 30% sucrose in phosphate buffered saline (PBS) for cryoprotection until infiltration was complete (at least 3 days). The striatum and SN from the other half of the brain were dissected on ice, flash frozen in liquid nitrogen, and then stored at −80 °C until they were processed for neurochemical or immunoblotting analysis, respectively.

2.5. Neurochemistry

Striatal levels of DA and its metabolites, 3,4-dihydroxyphenylacetic acid, and homovanillic acid were analyzed using high-performance liquid chromatography with electrochemical detection (Cannon et al., 2009; Tapias et al., 2010). Frozen striatal tissue samples were suspended in cold 0.1 N perchloric acid ($HClO_4$) and 0.01% ascorbic

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