Neuroprotective Properties of Phytochemicals against Paraquat-Induced Oxidative Stress and Neurotoxicity in *Drosophila melanogaster*

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ABSTRACT

Environmental toxicants like paraquat (PQ) induce the increase of oxidative stress, which is likely to lead to various neuropathological symptoms. Although the Drosophila system is applicable for anti-oxidative and neuroprotective drug discovery, this system remains to be more exploited in various aspects. In this study, using Drosophila as a model animal, we have examined the anti-oxidative and neuroprotective properties of plant-derived compounds against oxidative damage and neurotoxicity induced by paraquat,

which is a widely used herbicide. In this study, paraquat showed LC_{50} at 24.7 mM to adult males of *Drosophila melanogaster* within 24 hours. Dietary feeding of curcumin, quercetin, *Sanguisorba officinalis*, and *Zedoariae rhizoma* extracts prior to paraquat exposure extended lifespan and enhanced motor activities of flies. These compounds modulated the expression level of several genes associated with anti-oxidative and anti-aging effects such as *sod1*, *sod2*, *cat*, *gstD1*, and *mth* genes. Also, same treatments of phytochemicals to flies ameliorated other oxidative stress and neurotoxicity index factors such as ROS levels, superoxide dismutase. In contrast, no significant effects on catalase activities were observed. Additionally, the dietary feeding of phytochemical substances also reduced acetylcholine esterase activities, which were dramatically increased by paraquat treatment, implying that these phytochemicals also affected neuronal systems. Present study demonstrates that the dietary feeding of phytochemicals prior to paraquat exposure has anti-oxidative and neural protective effects, which leads to the recovery of behaviors and lifespan in fruit flies.

Keywords: *Drosophila melanogaster*, Oxidative stress, Paraquat, Neurotoxicity, Phytochemicals

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LIST OF ABBRIVIATION

AChE	Acetylcholinesterase
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood brain barrier
CAT	Catalase
cDNA	Complementary DNA
DCF	2,7-Dichlorofluorescein
DCF-DA	2,7-Dichlorofluorescein diacetate
DNA	Deoxyribonucleic acid
DNTB	5,5-Dithiobis 2-nitrobenzoic acid
GSH	Glutathione
GSHR	Glutathione reductase
gstD1	Glutathione S transferase D1
hUCP2	Human uncoupling protein 2
LC_{50}	Concentration of 50 % lethality
MAO-B	Monoamine oxidase - B
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger RNA
MTH	Methuselah
NDD	Neurodegenerative disease
Nrf2	Nuclear factor like 2

PBS	Phosphate-buffered saline
PD	Parkinson's disease
PQ	Paraquat (1,1-Dimethyl-4,4-bypyridylium chloride)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SOD	Superoxide dismutase
6-OHDA	6-Hydroxydopamine

CHAPER 1

GENERAL INTRODUCTION

The oxidative stress, caused by imbalance between generation and neutralization of reactive oxygen species (ROS), plays a key role in aging and the progression of neurodegenerative diseases (NDD) such as Alzheimer′s disease (AD) and parkinson′s disease (PD) (Sayre, Smith, and Perry 2001). Under a normal physiological condition, the level of ROS production is in equilibrium with the antioxidant capacity; however, when the generation of ROS overwhelms the capacity of cellular antioxidant defense system, oxidative damage occurs. One of the most important findings is the coincidental relationships between increased cases of PD and exposure to environmental factors such as agricultural agents, pesticides, and herbicides like paraquat and rotenone (Uversky 2004; Gorell et al. 1998; Vanacore et al. 2002). Paraquat (1,1-dimethyl-4,4-bipyridylium chloride, PQ) is an important ingredient of the bipyridyl herbicides, which generates free radicals during detoxification processes and thus increases ROS levels *in vivo* (Arking et al. 1991). Thus, this chemical causes extensive oxidative stress in mitochondria of the cell, resulting in the perturbation of biochemical processes, cell death, multi-organ failure, and NDD (Mohammadi-Bardbori and Ghazi-Khansari 2008).

PQ has also been considered a prime risk factor for PD because of both epidemiological evidence in the increased incidence of PD and its close structural similarity with MPP+, the active form of PD-inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Berry, Vecchia and Nicotera 2010). Various studies have shown that PQ penetrates the blood–brain barrier (BBB) and reduces

the number of dopaminergic neurons (Shimizu et al. 2003). It also accumulates subcellular organelles such as mitochondria and inhibits mitochondrial complex I by producing superoxide anions and other redox products (Yumino et al. 2002). The accumulation of PQ induced free radicals is believed to be due to decreased activities of the anti-oxidant defense enzymes like superoxide dismutase (SOD), catalase, glutathione (GSH), and glutathione reductase (GSHR). Subsequently, PD pathology has demonstrated the alteration of GSH levels in dopaminergic neurons of substantia nigra in the human brain (Pearce et al. 1997). Up to date, many researchers have focused on the existing PQ-induced PD models in vertebrates to screen several potential therapeutic compounds for neuroprotective effects (Orth and Tabrizi 2003).

Drosophila as a model system to contribute to drug screening for various NDD has been receiving gradual attraction because of its own advantages. Together with feasible genetic and molecular tools with a *Drosophila* model system, the fruit flies have served as a unique and powerful model to study human genetics and diseases and to screen potential therapeutic drugs (Marsh and Thompson 2006; Beckingham et al. 2005; Kim et al. 2011). Recently, *Drosophila melanogaster* has been extensively employed as a *in vivo* model system to characterize the effects of environmental toxicants such as PQ and rotenone, which would induce PD-like symptoms (Hosamani and Muralidhara 2009; Ortega-Arellano, Jimenez-Del-Rio and Velez-Pardo 2011; Dinis-Oliveira et al. 2006; Spivey 2011). There are several attempts to characterize the beneficial effects of phytochemicals that showed cellular defense mechanisms to PQ (Bonilla et al. 2006).

CHAPER 2

LITERATURE REVIEW

1. Paraquat

: The herbicide that induces the oxidative stress and kills dopaminergic neurons

Paraquat is an herbicide that was used worldwide until relatively recently, and which was banned in the European countries in 2007 (J. Bové and Perier 2012). It is known that it exerts its deleterious effects through oxidative stress (Jordi Bové et al. 2005). Paraquat toxicity is mediated by the induction of redox cycling with a cellular diaphorase such as nitric oxide synthase, which gives rise to the subsequent production of ROS (Suntres 2002). As its chemical name indicates, paraquat is a pyridinium that shares structural similarities with another herbicide used in the past called cyperquat, which happens to be MPP^+. This fact suggested that both neurotoxicants may also share some mechanisms of neurotoxicity, though the exact details of this are still a matter of debate (Gary Wright Miller 2007; Desplats et al. 2012; LoPachin and Gavin 2008)

Several cases of lethal poisoning resulting from ingestion of or dermal exposure to paraquat have been reported (Smith 1988). Over the course of many years, experimental studies using paraquat focused on its effects on the lung, liver, and kidney, probably because the toxicity induced by this herbicide in these organs is responsible for death after acute exposure. However, significant damage to the brain is seen in individuals who died from paraquat intoxication (Grant, Lantos,

and Parkinson 1980). Indeed, paraquat reaches the brain via neutral amino acid transporters (Richardson et al. 2005), but unlike rotenone is not able to freely cross the blood-brain barrier due to its hydrophilicity (Prasad et al. 2009).

Although the association of exposure to paraquat and higher risk of developing Parkinson's disease has been debated for several years (Liou et al. 1997; Berry, Vecchia, and Nicotera 2010), two recent epidemiological studies demonstrated that participants with PD were more likely than controls to have been exposed to paraquat in the past (Tanner et al. 2011; Anthony Wang et al. 2011). Those exposed to paraquat, along with exposure to the fungicides ziram and maneb, experienced the greatest increase in PD risk (Wang et al. 2011)

2. Mechanism of action of neurotoxins

6-hydroxydopamine (6-OHDA) enters neurons through the dopamine transporter. It is oxidized, producing hydrogen peroxide (H_2O_2) and para-quinone, following several consecutive reactions. On this account it induces neuronal cell death by production of reactive oxygen species (ROS). MPTP and rotenone which are lopophilic compounds can cross the blood-brain barrier. MPTP is metabolized into the toxic cation 1-methyl-4-phenylpyridinium (MPP^+) by the enzyme monoamine oxidase B (MAOB) (Jossan, Sakurai and Oreland 1989) in glial cells inside the brain. MPP^+ goes into dopaminergic cells via the dopamine transporter (Bezard et al. 2000). MPP^+ and rotenone accumulate in the mitochondria where

inhibiting mitochondrial respiratory chain complex I (Swerdlow et al. 1998). Complex I inhibition is caused to a reducing levels of ATP, increasing ROS level, and the activation of a mitochondria-dependent cell death pathway (Arthur et al. 2009) .

Paraquat enters the brain with the assistance of neutral amino acid transporters and catalyzed the formation of ROS through two mechanisms: redox cycling and activation of ROS-producing enzymes such as NADPH oxidases (Liu et al. 2009) .

3. Neurotoxin-based models of neurodegenerative disease

The use of toxicity-induced animal models has been decisive elucidation of the pathophysiology underlying neurodegenerative diseases like a Parkinosn's disease (PD) and the development of therapeutic strategies aimed to treat motor disorders (J. Bové and Perier 2012). These models are being employed on the pathogenic mechanisms involved in PD associated neuronal cell death.

PD is a chronic and progressive neurodegenerative movement disorders that patients are in world widely. The average point of onset-age is sixty, with age being an dominant risk factors for this disease idiopathically (De Rijk et al. 1997). References to PD-like motor disorders can be found throughout the aging, Jean-Martin Charcot added more symptoms that PD gained recognition distinctively. PD

is characterized by resting tremor, slowness of voluntary movements, rigidity, and postural instability. These symptoms can be accompanied by non-motor disorders that were initially eclipsed by the obvious movement impairment. Non-motor symptoms happen early in PD based on motor problems (Gaig & Tolosa, 2009). These non-motor disorders also can show neuropsychiatic, sleep disorders, olfactory deficits and constipations (Chaudhuri et al. 2009)

4. Reactive Oxygen species (ROS)

Reactive oxygen species (ROS) are produced at the highest concentrations within the mitochondria and consist of superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), and hydroxyl radials ($OH\cdot$) (Celotto et al. 2012). ROS are a normal byproduct of mitochondrial oxidative phosphorylation and are kept in check by cytosolic and mitochondrial antioxidant enzymes. It is known that ROS play important roles in regulation of cell death and differentiation, suggesting their levels need to be tightly regulated for normal development, particularly within the brain (Finkel 2003; Ikonomidou and Kaindl 2011; Celotto et al. 2012).

Mitochondrial ROS has caused numerous diseases and aging, and many degenerative diseases, such as Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (ALS) diseases, normal and premature aging as well (Wallace 2005). ROS also have critical signaling roles, therefore, the levels should be regulated to avoid cellular damage and dysfunctions within mitochondria (Celotto et al. 2012).

ROS are normally scavenged by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase as the defense system against ROS systems (Balaban, Nemoto and Finkel 2005) and damaged proteins are considered to be repaired by responding of stress –related molecules (Hirano et al. 2012).

The generation of ROS and related oxidative stress and damages are researched to be in the pathogenesis of neurodegenerative disease (Fujita et al. 2012). There have been increasing the reports on ROS indicating that eliminating the ROS levels directly or inducing the resistant proteins and antioxidant enzymes to antagonize oxidative stress (Noda et al. 2011).

5. Phytochemicals

Plants (fruits, vegetables and medical herbs) may contain a variety of free radical scavenging molecules widely such as phenolic compounds (Phenolic acids, flavonoids, quinons, coumarins, lignans, tannins and so on), nitrogen compounds (alkaloids and amines and so on), terpenoids (carotenoids), vitamins and some other endogenous metabolites being rich in antioxidant activity (Cotelle et al. 1996; Uttara 2009; Velioglu et al. 1998).

Plants are biosynthesized by chemical metabolites in terms of their

chemical structures and biological functions to adapt to environmental stressors in nature, such as ultraviolet (UV) light, microorganisms and insects, and drought (Murakami and Ohnishi 2012). It is called as the secondary metabolites, which are continuously produced in plants. Flavonoids are well-known phytochemicals that have versatile biological function, containing self-protection substances from UV light and anti-fungal effects and so on. These secondary metabolites have been considered to have diverse bioactivities in a variety of evaluation systems. For example, a phytochemical seems to prevent chemical carcinogenesis through the suppression of oncogene induction, which is the role of upstream signaling molecules involved in oncogene induction.

6. Anti-oxidation

Anti-oxidation is defined as a fundamental self-defense mechanism which is distributed among organisms. ROS play numerous physiological and pathological roles by oxidizing at the molecular, cellular, and tissue specific levels (Frei and Higdon 2003). Polyphenols and carotenoids have naturally potential chemical substances to remove harmful ROS. Moreover, those functions of chemicals are able to have connections with their health promotion and to prevent diseases (Shih et al. 2008; Agarwal et al. 2001).

The Keap1/Nrf2 system functions to protect cells from oxidative damages (Kobayashi and Yamamoto 2005). Several anti-oxidation enzymes being Nrf2 affected by dependently and independently have been reported. For example, SOD

expressed in cells ubiquitously are Nrf2-dependent and catalyze the alternation of superoxide anion into molecular oxygen and hydrogen peroxide (Sun et al. 2004). They are classified, such as SOD1 (cytosolic, Cu/Zn-SOD) and SOD2 (mitochondrial, Mn-SOD).

7. *Drosophila melanogaster* model system

In recent years, *Drosophila melanogaster* has been used as a model of the neurodegenerative diseases (Pandey and Nichols 2011). This invertebrate shows a large panel of genetic approaches and the screening of potential therapeutic drugs can be allowed as advantages (Coulom and Birman 2004). Together with feasible genetic and molecular tools with a Drosophila model system, the fruit flies has served as unique and powerful model to study the wide range of human genetics and disease.

It has been employed with phenotype assessment such as structural changes because of gene modification (Lu and Vogel 2009). Moreover, the characteristics in behaviors and sensory processes are able to be assessed for the further investigation with complex behavioral properties, such as learning and memory and chemo sensations in *Drosophila*. Genetic modifications could be either enhancers or suppressors of a specific phenotype of flies (Kim et al. 2011) .

CHAPER 3

NEUROPROTECTIVE PROPERTIES OF PHYTOCHEMICALS AGAINST PARAQUAT-INDUCED OXIDATIVE STRESS AND NEUROTOXICITY

1. Introduction

Phytochemicals are plant-based chemical compounds that possess substantial health promoting properties. Numerous phytochemicals have been considered as the most abundant antioxidants, which may be able to counteract the main causes of age dependent NDD (Murakami and Ohnishi 2012; Salvioli et al. 2007). For instance, resveratrol is a polyphenol compound derived from red grapes, which shows antioxidant properties and augments neuronal survival in a rat model of PD (Jin et al. 2008). Traditionally, *Sanguisorba officinalis* and *Zedoariae rhizoma* were used as medicinal plants in Asia for the treatment of inflammation, diarrhea, chronic intestinal infection, duodenal ulcers, and bleeding. Recently, they have been reported to demonstrate anti-oxidative, anti cancer, anti-lipid peroxidation, and neuroprotective and anxiolytic activities (Yu et al. 2011; Ram and Kumari 2001).

Together with these anecdotally selected plant extracts and two promising antioxidant phytochemicals, curcumin and quercetin, we have here tested antioxidant and neuroprotective capacities using behavioral and enzyme assays using *D. melanogaster* system, which may provide further information on neuroprotective mechanisms as well as platform for drug discovery in the future using an invertebrate model system.

2. Materials and methods

2.1. Chemicals

PQ, H_2O_2 (30%), protease inhibitors, ethanol, DCF-DA, DTNB, acetylthiocholine iodide, quercetin (Que) and curcumin (Cur) were purchased form Sigma (St. Louis, USA).

2.2. Plant extracts

The dried roots of *Z. rhizome* and *S. officinalis* were purchased at Kyung-Dong traditional herb market in Seoul, Korea. The roots were dried at 50℃ in dry oven and homogenized into crude powders. The extraction was made by 70% methanol solution in water for 2 days. Subsequently, methanol extracts (c.a. 32% yield) were evaporated at 40℃ under pressure, after which these were dissolved in 100% methanol to make solutions.

2.3. Drosophila rearing

A Oregon R line of *D. melanogaster* was obtained from the Bloomington Drosophila stock center of Indiana University (Bloomington, USA). The flies were

raised at 25 ± 1℃ and 60–70% relative humidity with a light/dark cycle of 12:12 hours and fed on a standard fly food made of corn flour, white sugar, dried yeast, agar, and 1.8 ml of 100% ethanol containing 0.9 g of methyl 4-hydroxybenzoate (JUNSEI, Japan). Prior to each bioassay, 2–3 days old adult male flies were exposed to CO_2 for 3 sec to anesthetize for collection.

2.4. Determination of fly survivorships on PQ treatment

One hundred adult male flies were provided with supplemented food for 48 hours and then transferred to clean vials containing PQ soaked on the filter paper (2 cm I.D., HYUNDAI Micro, Seoul, Korea) with sucrose solution where PQ was applied at five concentrations of 5, 10, 20, 25, and 50 mM for 48 hours. Due to desiccation, filter papers were replaced every 24 hours. The survivorship of flies was subsequently observed at 12, 24, 36, and 48 hours after PQ treatment. The concentration of 50% lethality (LC_{50}) was determined 24 hours after PQ treatment. The LC_{50} from concentration-dependent mortality data was analyzed using Sigma-Plot program (Version 10, USA). All experiments were replicated four times. For further subsequent bioassays, 20 mM of PQ was employed as mentioned below.

2.5. Behavioral assays: climbing activity assays

To determine the locomotion patterns with a negative geotaxis assay, twenty male adult flies were placed in a glass jar (10 cm length, 2 cm diameter).

Flies were gently tapped to the bottom of the jar and left them climb up to the top of the jar (Hosamani, Ramesh and Muralidhara 2010). Distance zones from the bottom of the glass jar were divided into six zones, 0, 0–2, 2–3, 3–5, 5–7, and over 7 cm from the bottom to the top. The number of the flies migrating from the bottom to each zone was counted at 1 min observation time point after tapping. Each experiment was continued and repeated ten times at 10 min intervals.

2.6. Paraquat resistance assays

To investigate the effect of oxidative stress in the *D. melanogaster*, adult flies were fed with fly food for 2 days. After starving for 4 hours, flies were transferred to a vial displaced with a filter paper, which was soaked with 20 mM PQ (Methyl viologen, Sigma) in 5% sucrose solution. The survivorship of flies was observed and recorded at every 12 hours for 48 hours.

2.7. Feeding assay with phytochemical substances

Groups of 2–3 day-old male flies starved for 4 hours were transferred into new clean vials where flies were left to feed on sucrose 5% solution (w/v) as a control group. For tested groups, male flies were pre-fed for 48 hours with 5% sucrose solution (w/v) or 5% sucrose solution (w/v) mixed with following substances: 0.05% quercetin (Que), 0.02% curcumin (Cur), 0.05% *S. officinalis* extracts, and 0.025% *Z. rhizome* extracts. Afterwards, flies were allowed to feed on

water for 2 hours, which was soaked in a filter paper. Each group of treated flies was then transferred into a vial where 300 μl of 20mM PQ in a 5% sucrose solution (w/v) was soaked on a filter paper (Jimenez-Del-Rio, Guzman-Martinez and Velez-Pardo 2010). Due to desiccation of the soaked filter paper, the filter paper in each vial was replaced every 24 hours without disturbance to tested flies. The number of dead flies was counted at 12, 24, 36, and 48 hours after transferred to the vial.

2.8. Analysis of gene expression

Total RNAs were isolated from the head of fifty adult male flies using a RNA extraction kit (RNeasy Mini Kit, Qiagen, USA). Using 1 μg of total RNA, cDNA was synthesized with oligo-dT with a Superscript III enzyme (Invitrogen, USA). Then, using a template of 1 μl of synthesized cDNA, PCR amplification was performed with gene specific primer sets for target genes, *sod1* (NM_057387.4) (Yu et al. 2011), *sod2* (NM_057577.3) (Hirano et al. 2012), *cat* (NM_080483.2), *gstD1* (NM_079602.4), and *mth* (NM_079147.2). PCR conditions were performed by procedures at 95℃ for 5 min, followed by 35 cycles of 95℃ for 30 sec, 58℃ for 30 sec, 72℃ for 1 min, and a final extension at 72℃ for 5 min using a Thermal Cycler (Applied Bioscience, California, USA). *rp49* gene (Lee et al. 2009), a housekeeping gene, was used as a control. All experiments were performed in triplicate. In order to analyze the relative gene expression levels, gene bands on agarose gels were visualized using Gel Doc™ XR + Imaging system (Bio-Rad, USA). Subsequently, the band intensity was automatically computed by

densitometry standard by Multi-Gauge version 3.0 software (Fuji film, Tokyo, Japan). The relative gene expression levels (%) were obtained by calculation of the band intensity of tested groups over sucrose group, which received 5% sucrose with PQ without any phytochemial pre-treatment. Control groups did not receive PQ treatment.

2.9. Measurement of reactive oxygen species (ROS) levels

Fly heads were immediately prepared after feeding assays were finished. The heads of individual flies were homogenized in 500 μl of ice-cold PBST buffer containing 0.1% Tween 20 (v/v) with protease inhibitors to prevent protein degradation (Duttaroy et al. 2003). Homogenates were then centrifuged at 13,000g for 10 min at 4℃, after which 300 μl of each supernatant was transferred into 1.5 ml tube. The procedure of ROS measurement was modified from Strayer et al (Strayer et al. 2003). Briefly, non-fluorescent 2,7-dichlorofluorescein diacetate (DCF-DA) is a cell permeable dye and can be converted into 2,7 dichlorofluorescein (DCF) by interacting with hydrogen peroxide as a ROS, during which the intensity of emitted fluorescent light was able to be detected. 100 μl of each supernatant from fly heads was transferred into a 96-well plate. After adding 50 μM 2,7-dichlorofluorescein diacetate to the samples, the fluorescence intensity was measured every 5 min for 15 min using a fluorescence microplate reader with excitation 485 nm and emission 640 nm (Victor 3, Perkin Elmer, USA). The relative ROS levels were calculated by the ratio between control and treatment.

Flies in control did not receive PQ treatment nor any phytochemical pre-feeding. All experiments were performed in triplicate and fifty adult male flies were used in each test.

2.10. Enzyme bioassays

2.10.1. Preparation of homogenates from head

Flies (n = 40) in each sample were homogenized 1 ml of ice-cold 0.1 M Tris–HCl buffer (pH = 7.8 with 0.5% Triton X-100 and 1 µl protein inhibitor cocktail). The homogenized mixture was centrifuge at the speed of 12,000g for 10 min at 4℃. The supernatant was filtered through glasswool (Sigma, St. Louis, USA) to remove excess lipids and used for biochemical assays.

2.10.2. Superoxide dismutase (SOD) activity assays

An assay kit (SOD determination kit, Sigma) was used to measure the SOD activity. It was determined by using water-soluble tetrazolium salt WST-1 (2-(4-lodophenyl)-e-(4-nitrophenyl)-5-(2,4-disulfophenyl)-SH-tetrazolium, monosodium salt that produces a water-soluble formazan dye upon reduction with a superoxide anion. The principle is that the rate of the reduction with O_2 related to a xanthine oxidase activity which is inhibited by SOD. WST working solution and

enzyme working solution to each sample (20 μl) mixed thoroughly and incubated the plate at 37℃ for 20 min. Each sample from fly heads was analyzed at 440 nm absorbance in the microplate reader.

2.10.3. Catalase (CAT) activity assays

The measurement of catalase activities was followed by a procedure described previously (Aebi 1984). Briefly, 1 ml reaction mixture containing H_2O_2 (3%), 0.1 M sodium phosphate buffer, pH 7.0. in the disposable plastic cuvette (Mecasys, Seoul, Korea). The reaction was initiated by adding an equivalent to protein of 20 μg. The decrease in H_2O_2 was monitored for 3 min at 240 nm using UV spectrophotometer (OPTIZEN POP, Mecasys, Seoul, Korea) and expressed as μmol of H_2O_2 decomposed/min/mg protein.

2.10.4. Acetylcholinesterase (AChE) activity assays

AChE activities were assayed according to methods by Whittaker. Briefly, individual fly heads were homogenized in 500 μl of phosphate buffer (0.1 M, pH 8.0). Head homogenates were centrifuged at 10,000g for 5 min at 4℃, after which 300 μl of each supernatant was transferred into 1.5 ml tube. The assay reaction mixture consisted of 160 μl phosphate buffer (0.1 M, pH 8.0), 10 μl of 5,5-dithiobis 2-nitrobenzoic acid (1 mM DTNB, dissolve DNTB in phosphate buffer of

pH 7.0 and 0.1 M), 10 µl of acetylthiocholine iodide, and 20 µl sample were added. The AChE activity was measured by monitoring the increase in absorbance at 412 nm for 5 min. The results were expressed as nmol of substrate hydrolyzed/min/mg protein. Then, the relative AChE activity levels were calculated by the ratio between control and treatment. All experiments were performed in quadruplicate and fifty adult male fly heads were used in each test.

3. Results

3.1. Paraquat-induced lethality response: LC_{50} determination

Flies were exposed to five concentrations of PQ, 5, 10, 20, 25, and 50 mM. Five and 10 mM of PQ treatment did not show significant mortality after 24 hours (round and filled circles in Fig. 1A). The survivorships at the exposed concentrations of 5, 10, 20, 25, and 50 mM after 48 hours were 42%, 19%, 4%, 2%, and 0%, respectively, indicating PQ concentration over 20 mM in the filter paper was highly toxic to the flies ($p < 0.05$).

Notably, fly survivorships 24 hours after PQ treatment showed remarkable distribution patterns compared to other observation time points (Fig. 1A), which allowed us to determine LC_{50} value for PQ toxicity to flies. The values at the tested concentrations were considered to be significantly different from one another ($r = 0.98$), indicating that 24.7 mM of PQ represented 50% of survivorships and lethality at 24 hours time point (Fig. 1B).

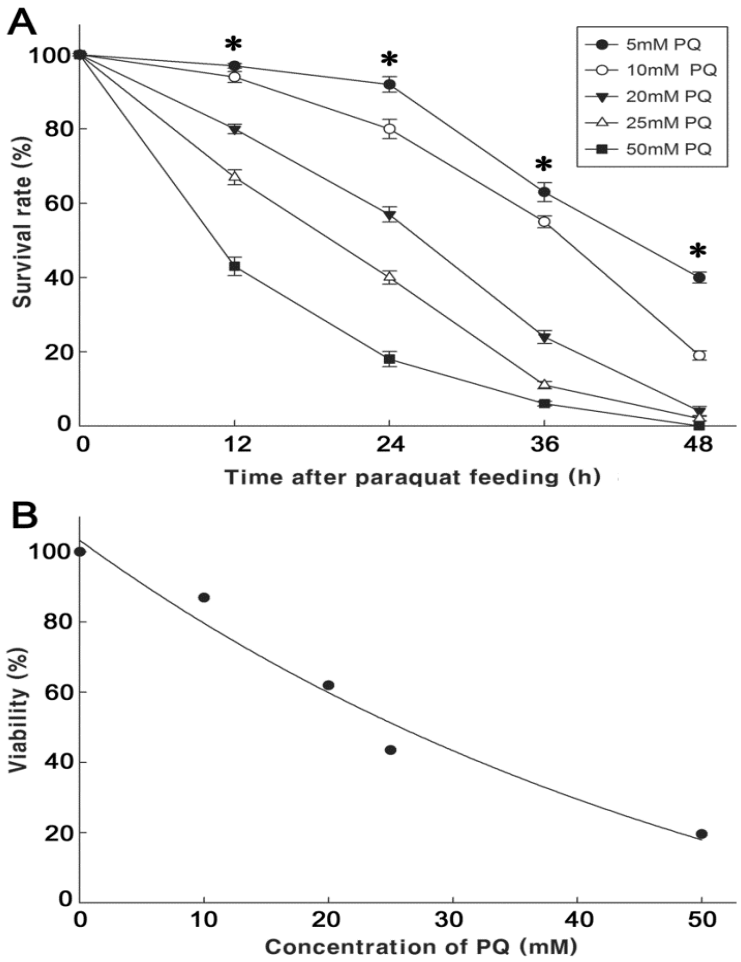

Figure 1. Survivalships of *Drosophila melanogaster* induced by paraquat (PQ) treatment

(A) Survival rate among adult male flies exposed to five different concentrations of PQ (5, 10, 20, 25, and 50 mM), showing different lethality for 48 hours. Indicates statistical significance of survival rates on each time point observed by one-way ANOVA, Duncan's test (N = 4, *p < 0.01). (B) Regression curve to analyze a LC_{50} value from concentration-dependent mortality at 24-hours after paraquat treatment (r = 0.98). This indicated 24.7 mM of PQ as the LC_{50} value. Error bars depict mean ± SE.

3.2. Effects of phytochemicals on PQ-induced mortality of flies

We investigated whether or not flies pre-treated with phytochemicals tended to survive more from PQ treatment than nontreated control groups. Our result showed that tested flies pre-fed with 0.05% *Z. rhizoma*, 0.025% *S. officinalis*, 0.05% quercetin, and 0.02% curcumin were survived with 99 ± 0.9%, 97 ± 1.7%, 100%, and 99 ± 0.8%, respectively, 12 hours after PQ treatment, compared with 93 ± 1.1% survival rate in fly group fed only with sucrose. Four fly groups pre-fed with phytochemical substances tended to represent the increase of survival rates after 12 hours despite no statistical significance (Fig. 2). Subsequently, the survival rates of flies pre-fed with *S. officinalis* extracts, quercetin, and curcumin after 24 hours demonstrated 79 ± 1.2%, 82 ± 4.1%, and 78 ± 1.6%, respectively, which showed better survival rates than a fly group fed with *Z. rhizoma* extracts (Fig. 2A) and non-pre-treated group (66 ± 2.1%) (Fig. 2, $p < 0.01$). No difference of the survival rate was found in the fly group fed with *Z. rhizoma* extract after 24 hours, indicating that *Z. rhizoma* extract was not able to restore the fly survival rate, compared to other tested phytochemicals. Flies fed with *Z. rhizoma* and *S. officinalis* extracts after 36 hours showed low survival rates compared to controls (Fig. 2A and B).

Notably, flies fed with quercetin and curcumin showed the significant increase of survival rates, which were 17 ± 2.1% and 10 ± 2.2% after 36 hours,

respectively. Compared to flies fed with *Z. rhizoma* and *S. officinalis* extracts showing no survivorships after 48 hours, querecetin- and curcumin-fed fly groups showed 5 ± 0.2% and 4 ± 2.4% (Fig. 2).

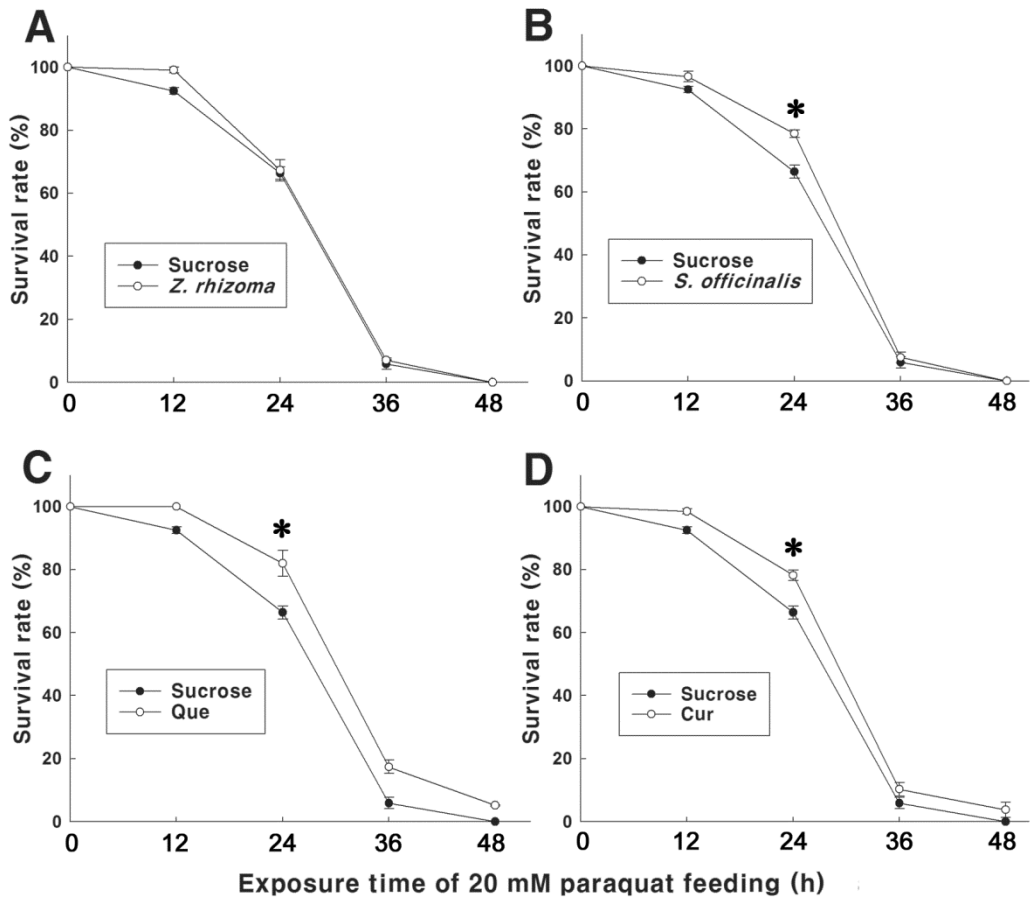

Figure 2. Effects of phytochemicals on PQ-induced mortality of flies

Survival rate was observed from wild type flies pre-fed with phytochemical substances containing (A) 0.025% *Z. rhizoma* (B) 0.05% *S. officinalis* extracts, (C) 0.05% quercetin (Que), and (D) 0.02% curcumin (Cur). The effect of 20 mM paraquat to fly lifespan demonstrated that most flies with no phytochemical provision did not survive after 48 h (open circles). Flies pre-fed with S. officinalis extract, quercetin, and curcumin showed significant increase of survivorship 24 h after paraquat treatment. Statistical significance was determined by one-way ANOVA with a group analysis by Duncan′s test (N = 3, *p < 0.01). Error bars depict mean ± SE.

3.3. Effects of phytochemicals on fly locomotion

All flies that were not treated with PQ showed movement to over 7 cm height in a tested vial. In contrast, flies exposed to 20 mM PQ for 12 and 24 hours showed completely different patterns of movement. That is, flies tended to stay at the middle or bottom areas of the vials, implying that PQ may increase toxic effects to fly locomotion and illness (Fig. 3A).

Together with this idea, we found that *Z. rhizoma* and *S. officinalis* extracts, quercetin, and curcumin were able to alleviate the locomotion function of flies caused by PQ treatment. Our study also indicated that the climbing abilities to the top of the vial were dramatically decreased by PQ treatment in flies that was not fed with phytochemical substances (control in Fig. 3B). In contrast, flies pre-fed with phytochemicals before PQ treatment showed increased locomotion activities, compared to the control group (Fig. 3B, $p < 0.01$). Compared to survivorship presented in Fig. 2, the extracts of *Z. rhizoma* and *S. officinalis* showed the significant prevention of fly locomotion deficits caused by PQ.

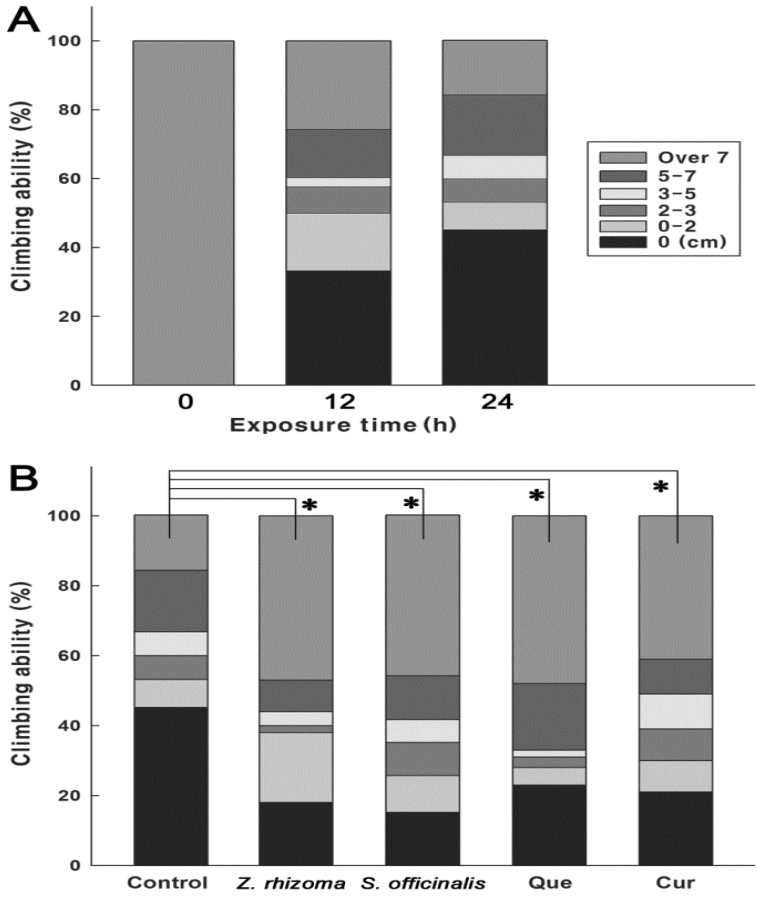

Figure 3. Improvement of fly locomotion pre-fed with phytochemical substances after PQ exposure

Five different zones were allocated by the distance, which were 0, 0–2, 2–3, 3–5, 5–7, and over 7 cm from the bottom of the jar. (A) 20 mM PQ treatment induced the deficits in locomotion of flies after 12 and 24 hours. (B) Effect of PQ-induced climbing ability after pre-fed with phytochemical substances supplementation. Flies pre-fed with the extracts of *Z. rhizoma* and *S. officinalis*, quercetin (Que), and curcumin (Cur) showed significant restoration improvement of climbing activities, compared to non-pre-fed control group. Statistical significance was determined by one-way ANOVA, Duncan′s test ($N = 5$, $*p < 0.01$). Error bars depict mean ± SE.

3.4. Modulation of gene expression related to oxidative stress by phytochemicals

To examine the effect of phytochemical substances on antioxidant- and aging related gene expression in flies caused by PQ treatment, RT-PCR amplification from each fly sample was performed. It has been reported that *sod1*, *sod2* and *cat* activities generally decreased with aging, which are indicative of one of the factors inducing oxidative stress (Peng et al. 2011). These genes are thought to play an important role in molecular and cellular modulation in lifespan and further expression of antioxidant genes (Honda, Tanaka and Honda 2010). Also, overexpression of *sod1* and *sod2* genes in yeast *Sacchromyces cereviseae* and *D. melanogaster* had been reported to decrease oxidative damage and to increase lifespan.

In our present study, *sod1* and *sod2* genes were substantially up-regulated in the flies fed with *S. officinalis* extracts and quercetin, compared to non-treated flies 24 hours after PQ exposure (Fig. 4B, $p < 0.01$). Flies fed with *Z. rhizoma* showed similar patterns but *sod2* level was not significantly increased. In contrast, cat gene showed up-regulation patterns in flies pre-fed with *Z. rhizoma* extracts and quercetin, compared to non-treated flies 24 hours after PQ exposure. Two phytochemical materials, *S. officinalis* and *Z. rhizoma* extract showed the increase of expression patterns, compared to non-pre-fed group (Fig. 4B, $p < 0.01$).

Compared to up-regulation of *sod1*, *sod2*, *cat*, and *gstD1* genes, *methuselah* (*mth*) gene was significantly down-regulated in flies pre-fed with the

extracts of *Z. rhizoma* and *S. officinalis* as well as quercetin after 24 hours (Fig. 4B, $p < 0.01$). Interestingly, it has been reported that *mth* mutant flies showed 35% increase of lifespan and having resistance of a variety of stress such as starvation, high temperature, and PQ-induced free radical generation (Lin, Seroude and Benzer 1998). The decrease patterns of *mth* expression could prolong the survivorships of flies pre-fed with querecetin where the average levels of *mth* expression in this fly group were approximately fivefold less than non-fed fly groups (Fig. 4B). Flies pre-fed with the extracts of *Z. rhizoma* and *S. officinalis* also showed the decreased patterns of *mth* gene expression (Fig. 4), implying that more flies were able to survive in comparison of other non-treated controls (Fig. 2).

It has been reported that chronical exposure to PQ may contribute to the gradual development of neurodegenerative disorders such as Parkinson′s disease (Dinis-Oliveira et al. 2006). Thus, the alternation of expression patterns in tested genes here would improve survival rate and locomotion activities, resulting in reducing the risk of neurodegenerative diseases.

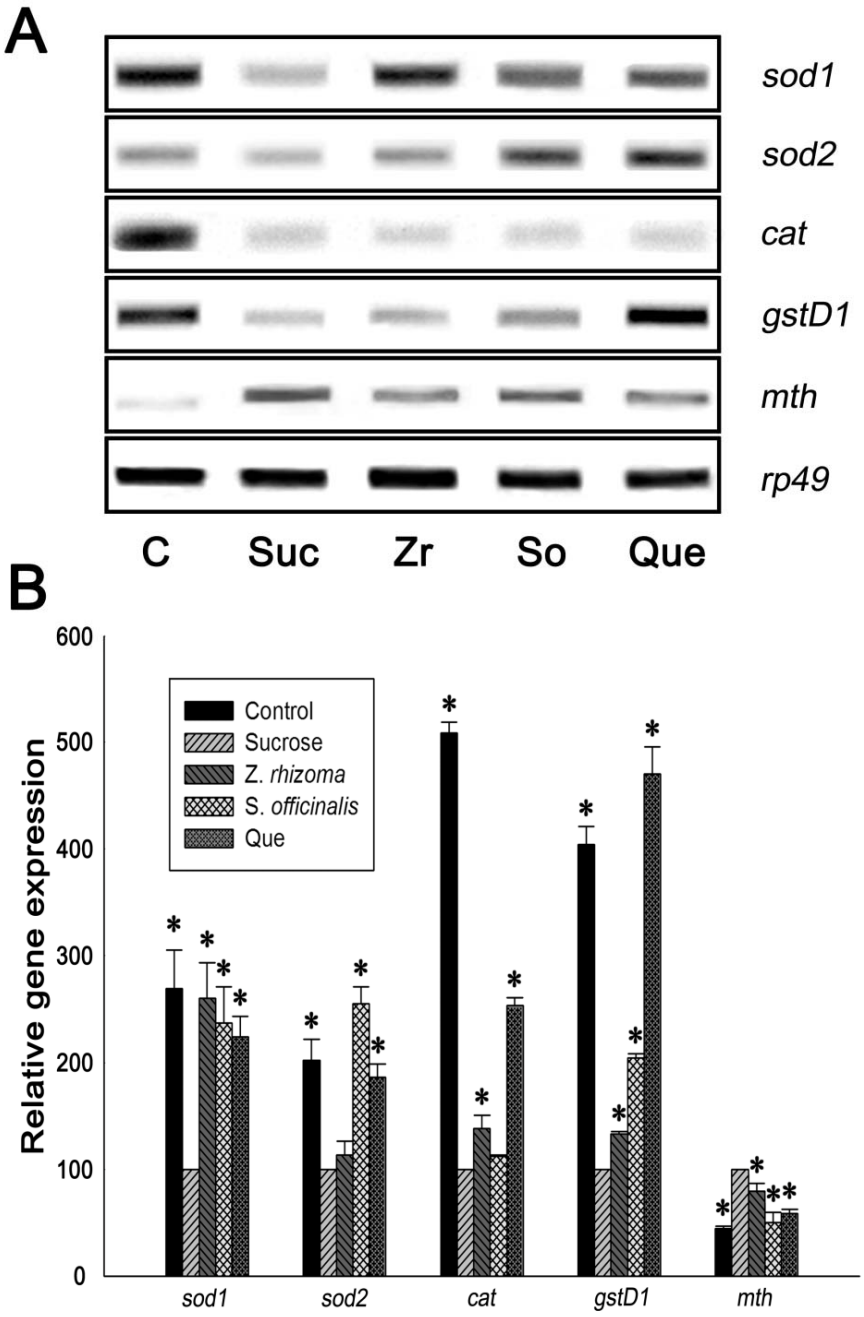

Figure 4. RT-PCR analysis for the gene expression of *sod1*, *sod2*, *cat*, *gstD1*, and *methuselah*(*mth*) in heads of male flies

Pre-fed with three phytochemical substances (Zr: *Z.rhizoma* extract, So: *S.*

officinalis extract, Que: quercetin) after 24-hours exposure of 20 mM paraquat (A) and (B) relative gene expression patterns upon pre-feeding of phytochemical substances compared to non-fed control group. Gene expression of each gene by three phytochemical substances after PQ treatment showed slightly different patterns. mth gene was down regulated by phytochemical substances, compared to negative control. *rp49* gene was used as a control gene, showing no change in gene expression upon any treatment. Statistical significance was determined by one-way ANOVA with Duncan's test (N = 3, *p < 0.01). Error bars depict mean ± SE.

Table 1. Information of the primer sequences used for RT-PCR analysis

Gene	Sequence (5' → 3')	GenBank
	Forward and Reverse	**Accession No.**
sod1	CACGGTTTTCTTCGAACAGG CATTGGTGTTGTCACCGAAC	NM_057387.4
sod2	GCCCGTAAAATTTCGCAAAC TCTCCCGGCAGATGATAG	NM_057577.3
cat	ACCAGGGCATCAAGAATCTG ACCTTCTTGGCCTGCTCGTA	NM_080483.2
gstD1	GACTCCCTGTACCCTAAGTGC TCGGCTACGGTAAGGGAGTCA	NM_079602.4
mth	GTGGAAAACCAGGATTGGAA TGCGCAAAGTTCTGAATGTC	NM_079147.2
rp49	AGGGTATCGACAACAGAGTG CACCAGGAACTTCTTGAATC	NM_079843..2

3.5. Alternations of ROS levels by phytochemicals

Previous research demonstrated that the increase of ROS levels in the cell triggered abnormal cellular functions, which thus increased cell mortality by affecting various biochemical pathways (Cho, Hur and Walker 2011). Therefore, neutralizing ROS levels produced by PQ is likely to be important cellular process to maintain cell homeostasis.

In our present study, we attempted to delve into the modulatory effects by the extracts of *S. officinalis* and *Z. rhizoma* and quercetin on ROS metabolisms by measuring ROS levels from a head homogenate of flies. Control flies exposed to PQ showed significant increase of a ROS level, compared to non-treated flies, demonstrating that ROS was induced by PQ treatment (Fig. 5). Interestingly, ROS levels in three groups of flies pre-fed with phytochemicals were significantly reduced, implying that the protective capacity of phytochemicals may be attributed to scavenging free radicals or alternatively to up regulation of anti-oxidative defense machineries.

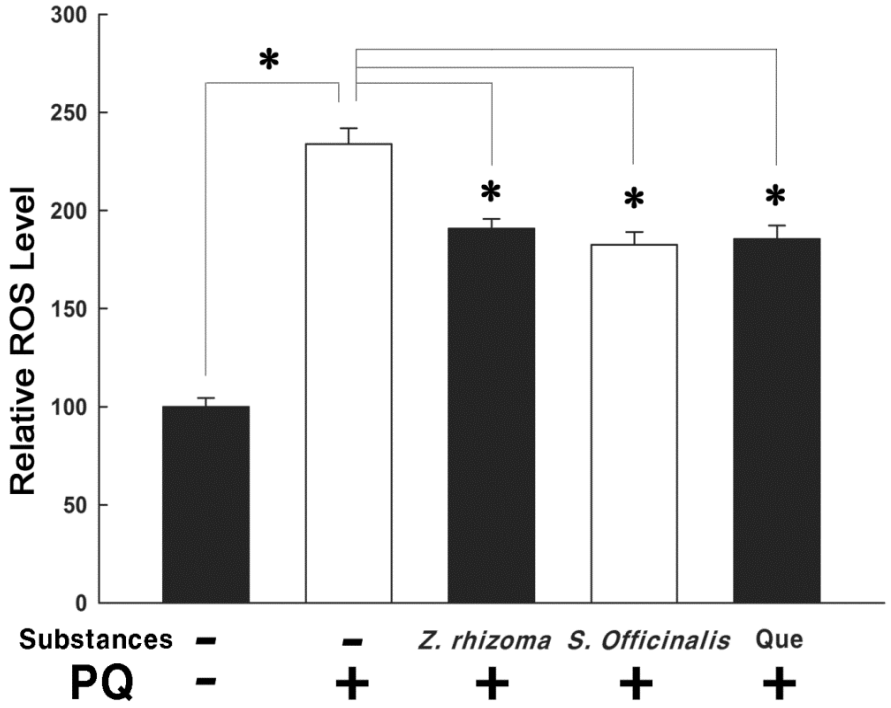

Figure 5. The effects of phytochemical substances on the production of reactive oxygen species (ROS) induced by PQ treatment in *Drosophila melanogaster*

Compared to control, the exposure to PQ increased ROS level. The production levels of ROS in fly groups pre-fed with three phytochemical substances were reduced compared with flies exposed to PQ with no pre-feeding with phytochemical substances. Statistical significance was determined by one-way ANOVA with Duncan′s test (N = 6, $*p < 0.01$). Error bars depict mean ± SE.

3.6. Effects of phytochemicals on enzyme activities associated with anti-oxidative responses

It has been demonstrated that the repeated enzyme activities of SOD and catalase (CAT) play a pivotal role in removing superoxide anion (O^{2-}) and changes in gene expression of SOD and CAT were believed to be closely related with the lifespan of *D. melanogaster* (Hirano et al. 2012; Griswold et al. 1993; Li et al. 2007). In our current study, the activity of SOD enzyme induced by PQ exposure was significant lower in fly groups pre-fed with the extracts of *Z. rhizoma* and *S. officinalis* and quercetin, compared to non-pre-fed group (group indicating substance -, PQ + in Fig. 6). Interestingly, the quercetin-fed group showed the lowest level of SOD activities amongst three groups pre-fed with the phytochemical substances, indicating that quercetin as a single compound has strong effects to modulate anti oxidative pathways in the cell by restoring SOD activities.

In contrast, we found no significant change in the CAT activities by feeding three phytochemical substances, even though CAT activities showed the significant increase after PQ exposure in comparison with non-treated group (Fig. 7, $p < 0.05$).

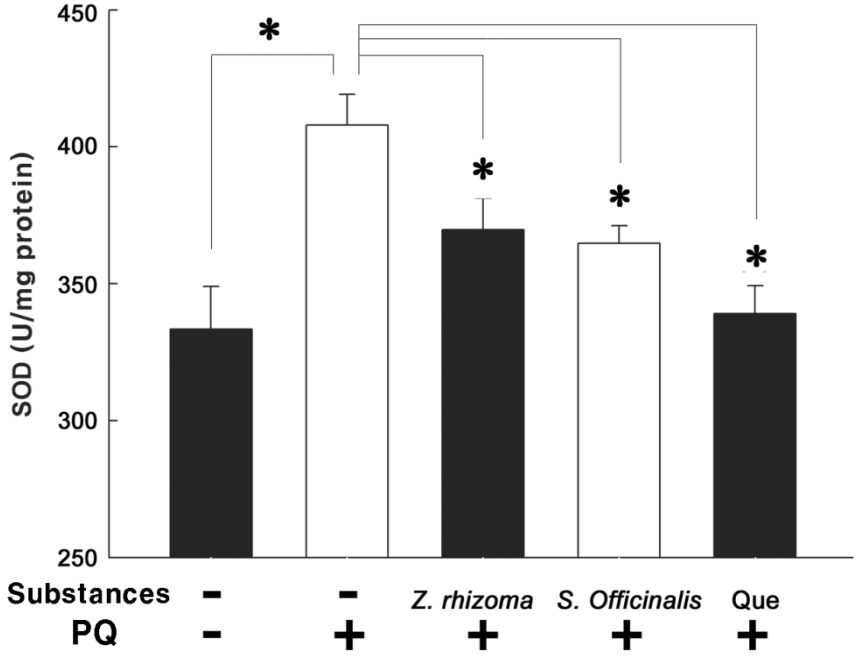

Figure 6. The effects of phytochemical substances on the activities of superoxide dismutase (SOD) induced by PQ treatment in *Drosophila melanogaster*

Restoration patterns of SOD activities after quercetin (Que) supplementation returned to a nearly normal state compared to control group where flies received no substances nor PQ. Also, SOD activity was significantly reduced when flies were fed with the extracts of *Z. rhizoma* and *S. officinalis*. Statistical significance was determined by one-way ANOVA with Duncan′s test (N = 5, ∗p < 0.01). Error bars depict mean ± SE.

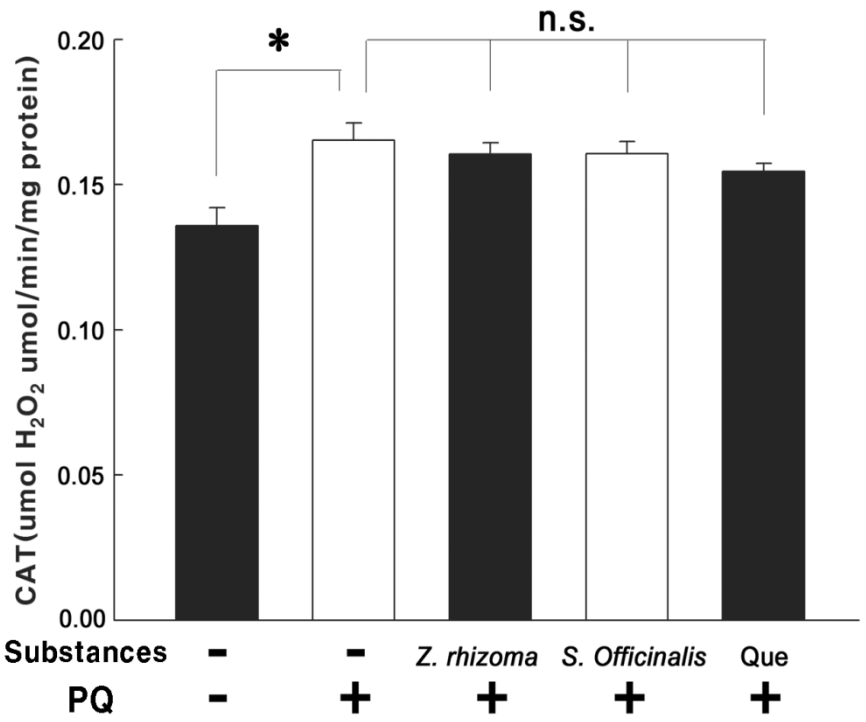

Figure 7. The effects of phytochemical substances on the activities of catalase (CAT) induced by PQ treatment in *Drosophila melanogaster*

CAT activities were not noticeably different (n.s.), compared to a fly group received PQ exposure only. Statistical significance one-way ANOVA wit Duncan's test (N = 5, *p < 0.01). Error bars depict mean ± SE.

3.7. Effect of phytochemicals on acethylcholinesterase (AChE) activity

Control flies exposed to PQ showed the significant increase of an AChE activity level, compared to non-treated flies (Fig. 8). AChE activity levels in three groups of flies pre-fed with phytochemicals were significantly reduced. Flies pre-fed with *S. officinalis* extracts and curcumin showed lower AChE activity levels, compared to non-pre-fed group (group indicating substance -, PQ + in Fig. 8), *Z. rhizoma* and quercetin induced significantly lower levels of AChE activities compared to non-treated PQ group (group indicating substance -, PQ - in Fig. 8). Interestingly, the quercetin-fed group showed the lowest level of AChE activities amongst four groups pre-fed with the phytochemical substances, demonstrating that quercetin as a single compound has much stronger effects on alternation in cholinergic function.

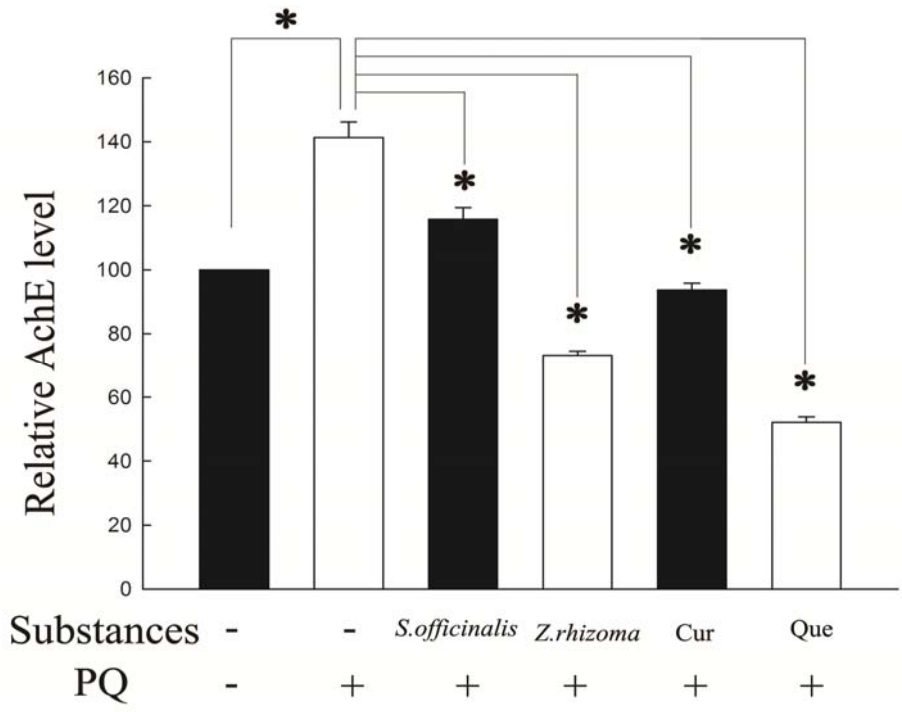

Figure 8. The effects of phytochemical substances on the activities of acetylcholine esterase (AChE) induced by PQ treatment in *Drosophila melanogaster*

Exposure to PQ increased AChE activities, while dietary feeding of phytochemical substances prior to PQ treatment reduced AChE activities. The dietary feeding of *Z. rhizoma* and quercetin (Que) showed the dramatic decrease of AChE activities. *S. officinalis* extracts and curcumin (Cur) also decreased AChE activities compared to flies treated with PQ alone. Statistical significance was determined by one-way ANOVA with Duncan's test (N = 4, *$p < 0.01$). Error bars depict mean ± SE.

4. Discussion

Several studies reported that sustained exposure of flies to subchronic doses of PQ reproduced the characteristic pathological features of PD (i.e., a progressive deficiency of dopaminergic neurons, alteration of locomotor behavior) (Zhou et al. 2011; McCormack et al. 2002). Likewise, in the present study, we evaluated the PQ-induced locomotion deficits of *D. melanogaster* by measuring negative geotaxis. It has been reported that PQ induced the alternation of behavioral patterns in flies such as motor behaviors, including resting tremors, rotation of the body, and postural instability, which was likely to be the symptoms of human PD (A. Chaudhuri et al. 2007). In addition, this behavioral phenotype has been explained that PQ might affect the loss of dopaminergic neurons and induce the mobility deficits characterized by decreased walking activities (Brooks et al. 1999). Chaudhuri et al. (A. Chaudhuri et al. 2007) demonstrated that flies exposed to PQ exhibited significant neuronal cell death of dopaminergic neurons. Therefore, further studies on the molecular and neural correlations of PQ induced effects on behaviors and oxidative stress remains to be addressed in order to understand the parkinsonian symptom-like processes in *D. melanogaster*.

Subsequently, we then asked the questions if co-exposure or pre-treatment with potential neuroprotective chemicals can prevent or relieve PQ-induced locomotion deficits. This idea stemmed from the fact that mammalians taking functional foods or phytochemicals regularly would have less oxidative stress by

environmental toxicants (Hosamani and Muralidhara 2010), indicating that potential neuroprotective properties are likely to be involved in specific subsets of cellular and molecular systems governing fly locomotion such as dopaminergic neuron-specific population or subcellular organs like mitochondria *in vivo*, which need further experiments in the future.

It was demonstrated that *Nrf2* transcription factor was the one of candidate genes that could be responded by oxidative stress factors, which can bind to regulatory elements encoding enzymes involved in glutathione biosynthesis (Yates and Kensler 2007; Dinkova-Kostova and Talalay 2008; Trinh et al. 2010). *Glutathione Stransferase D1*, *gstD1*, is an original response gene induced by oxidative stress which encodes widely recognized detoxification and antioxidant genes (Sawicki et al. 2003; Sykiotis and Bohmann 2008). Our result demonstrated that high expression level of *gstD1* gene was induced by the neuroprotective effects by quercetin. Further possibility of relationships with *Nrf2* and *gstD1* in this particular experiment needs to be investigated.

Mitochondrium is the major source of cellular ROS and its dysfunction appears to contribute to aging. Another line of research has demonstrated that the activation of the human uncoupling protein 2 (hUCP2) led to reduce ROS generation and to decrease oxidative damage, which in turn showed to extend fly lifespan (Fridell et al. 2005). Our present study also supported this idea that the reduction of ROS production in mitochondria extended lifespan of *Drosophila melanogaster*. Although it remains to be examined to identify cellular mechanisms

associated with this physiological process, it would be obvious that the reduction of ROS generation may result in the changes of gene expression levels, which promote lifespan.

Previous reports indicated that green tea extracts and *Bacopa monnieri* herb prolonged the life span of flies, showing the elevation of both SOD and CAT activities (Li et al. 2007; Hosamani and Muralidhara 2009). Compared to other research, our results demonstrated that three phytochemical substances may have different cellular mechanisms to modulate separately between CAT and SOD activities. Further studies to pinpoint the detailed mechanisms remain to be explored in the future.

Abnormal induction of AChE activities by environmental toxicants tended to induce oxidative stress in neurological dysfunction, leading to decrease in acetylcholine levels in the brain (Melo, Agostinho and Oliveira 2003). Also, it has been reported that acetylcholine plays an important role in scavenging superoxide anions, which in turn reduced lipid peroxidation (Pillay et al. 2003).

CHAPER 4

CONCLUSION

Phytochemicals and plant extracts have been widely used for preventive and therapeutic substances to inflammatory- and oxidative stress-based disease symptoms. Our current study has attempted to characterize the behavioral and molecular modulation by phytochemical treatments in a *Drosophila* model system prior to PQ exposure. The evidence on neuroprotective effects of two Korean medicinal plant extracts, *Z. rhizoma* and *S. officinalis* and quercetin against PQ toxicity was presented here, which caused the impairment of locomotion and survival in the fruit flies. Treatment with tested phytochemicals and plant extracts prior to PQ exposure to flies indeed improved survival and locomotor activities, suggesting that these substances could serve as potent anti oxidative and neuroprotective agents. In addition, these phytochemicals and plant extracts were able to modulate the activities of anti-oxidative genes and enzymes such as *sod1, sod2, cat, gstD1*, and *mth*. Also, in flies fed with phytochemicals and plant extracts prior to PQ exposure, the level of other oxidative stress index factors such as ROS and SOD enzyme was attenuated. However, we did not find substantial alternation patterns of CAT gene and enzyme that plays an important role in removing oxidative radicals, implying that mechanisms and targets of these phytochemicals remain to be determined.

Taken together, our findings demonstrated that dietary feeding of phytochemicals to *Drosophila* significantly attenuated PQ-mediated oxidative stress and neural damages. Finally, these in vivo screening methods for neuroprotective properties using *Drosophila* model per se further confirm the reliability of this model for further studies on cellular and molecular mechanisms and applications for potential chemical screening.

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노랑초파리 (*Drosophila melanogaster*)에서 파라쿼트로 유도된 산화스트레스와 신경독성에 대한 파이토케이컬의 신경보호 특성 연구

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국문초록

파라쿼트 같은 다양한 외부환경에 대한 독성 자극원은 생체내의 산화적 손상을 일으켜 산화스트레스의 증가시킨다. 이는 다양한 신경병증을 유도하는 것으로도 잘 알려져 있다. 초파리(*Drosophila melanogaster*) 모델 시스템은 항산화 효과와 신경 보호 차원의 약물 탐색을 위해서 적용할 수 있기 때문에 다양한 측면으로 유용하게 이용될 수 있도록 계속 발전시켜 나아가야 한다.

이 연구에서는 동물 모델로서 초파리를 사용하여 살충제로 널리 사용되고 있는 파라쿼트로 산화적 스르레스를 유도로 인하여 생체 내부에 산화적인 손상과 신경독성에 대하여 식물 유래 기능성 파이토케미컬 성분들의 항산화적 효과와 신경보호적인 특성을 확인하였다.

성충으로 태어나 2-3일 지난 수컷 초파리에 파라쿼트를 24시간 노출시켰을 때 파라쿼트의 LC_{50} 값은 24.7mM 이라는 결과를 얻었다. 커큐민과 퀘르세틴 단일 성분과, 지유와 봉출 추출물을 설탕물과 혼합하여 먼저 섭취시킨 후 파라쿼트에 노출시켰을 때, 식물 유래 성분 및 추출물들을 섭취하지 않은 초파리들과 비교하여 수명이 연장되었고 운동능력의 증가를 보였다. 이러한 식물 유래 성분 및 추출물은 *sod1*, *sod2*, *catalase*, *gstD1* and *mth* 등 항산화 및 항노화와 관련이 있는 유전자의 발현 패턴 변화를 조절하는 기능을 한다. 또한 위의 네 가지 물질은 산화적 스트레스의 마커인 ROS 레벨과 생체 내 존재하는 항산화 효소인 SOD 활성에 영향을 주어 개선시키는 것을 확인하였다. 이와는 대조적으로 SOD 처럼 생체 내 존재하는 항산화 효소인 Catalase 활성에서는 직접적인 영향을 미치지 못하는 것으로 관찰되었다. 식물 유래 성분과 추출물의 섭취는 파라쿼트의 노출로 인하여 급격하게 증가된 Acetylcholine esterase 활성을 눈에 띄게 감소시키는 것으로 식물 유래 파이토케미컬 성분들이 신경 시스템에 영향을 주는 것을 추측할 수 있다.

이러한 결과들을 통해서, 본 연구는 산화적 스트레스를 유도하는 파라

쿼트에 노출되기 전 식물 유래의 기능성 물질인 파이토케미컬 성분을 섭취하였을 때 산화적 스트레스에 대하여 예방 차원의 항산화 효과와 신경보호 기능을 수행하며, 종합적으로 초파리의 수명과 운동성 및 행동의 개선을 이끌어 낼 수 있음을 시사한다.

따라서 본 연구는 산화적 스트레스로 인한 질병 모델을 연구하는데 다양하게 이용될 수 있음을 제시하여 산화적 스트레스 유도로 인한 신경퇴행성질환 연구의 기반을 마련하여 질병 원인에 대한 기초 연구와 식물 유래 기능성 성분인 파이토케미컬을 토대로 하는 약물 탐색 분야의 발전에 기여를 할 것이라 사료된다.

검색어 : 노랑초파리, 산화스트레스, 파라쿼트, 신경독성, 파이토케미컬

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