PROTECTIVE EFFECTS OF PHENOLIC ACID DERIVATIVES OF COFFEE AND INDIAN GOOSEBERRY EXTRACTS ON RETINAL DEGENERATION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2015

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Cornell University 2015

The human retina is a thin layer of tissue on the inner back wall of the eye, containing millions of photoreceptor cells that receive and combine visual information. The retina sends visual information to the superior colliculus of the brain through the optic nerve. Because the retina ranks as one of the highest energy-consuming systems in the body, it requires a constant and abundant oxygen supply from the blood. Therefore, the retina is relatively susceptible to damage induced by oxidative stressors. Excessive oxidative stress is a key contributing factor in the pathophysiology of retinal disorders such as age-related macular degeneration, glaucoma, and retinopathy, which can lead to visual loss or complete blindness. Clinical studies have shown that the progression of retinal diseases can be slowed down by dietary treatment with antioxidant compounds such as vitamins, carotenoids, zinc, and phenolic phytochemicals. Coffee is one of the most frequently consumed beverages worldwide and its popularity continues to increase. Although coffee is rich in phenolic compounds and is the main contributor of dietary antioxidant intake in the diet, its protective effect against retinal degeneration has never been studied. In these studies, we employed several approaches to examine the protective effect of phenolic compounds in coffee on the retina. Specifically, the studies presented here investigated (i) the effects of coffee extracts and chlorogenic acid, a major phenolic compound in coffee, on retinal degeneration and (ii) the effects of phenolic acid metabolites formed after coffee consumption on retinal degeneration. The results showed that chlorogenic acid and coffee extracts could significantly

reduce the apoptosis induced by hypoxia or optic nerve crush stress in the retina. In addition, coffee metabolites, especially chlorogenic acid, caffeic acid, and dihydrocaffeic acid, could reach the eye and protect the retina against hypoxia and optic nerve crush *in vivo*. Moreover, we show that extracts of Indian gooseberry, which is widely consumed in Asia as an ingredient in traditional medicine, juice, and cosmetics, has a retinal protective effect against amyloid beta-induced neuronal degeneration. Collectively, these findings suggest that coffee and Indian gooseberry consumption may help to prevent retinal degeneration.

BIOGRAPHICAL SKETCH

Holim Jang was born on September 1, 1983 in Seoul, South Korea as the eldest of two sons to Se Kun Jang and Kyung Sook Lee. Holim Jang attended Yonsei University, Seoul, South Korea from 2002 to 2009, where he graduated with a B.S. degree, with a major in Bioengineering and a minor in Business Administration. As an undergraduate student, Holim Jang was a part-time technician at a biomaterials processing laboratory under the direction of Dr. Jiyong Park. Holim Jang served as a medic in the Korean Augmentation Troops to the United States Army from 2004 to 2006, where he learned about medicine, nutrition, and food in a practical, rather than academic, context. Holim Jang decided to pursue an M.P.S. degree in the field of Food Science and Technology under the direction of Dr. Chang Yong Lee at Cornell University in the fall of 2009. Following completion of his degree in January 2011, Holim Jang began a Ph.D. program in Food Science under the direction of Drs. Chang Yong Lee, Olga Padilla-Zakour, and Andrew Novakovic in the spring of 2011. Given his interest in phytochemicals for eye health, he investigated this subject at the Korea Institute of Science and Technology, Gangneung, South Korea as a research student. Holim Jang received his Ph.D. degree from Cornell University in 2015.

Dedicated To

My Father, Se Kun Jang

My Mother, Kyung Sook Lee

My Brother, Hojun Jang

and My Wife, Jimin Park

ACKNOWLEDGMENTS

I would like to express my appreciation to my advisor, Dr. Chang Yong Lee, who has always provided me with great opportunities and assistance to prepare for my future. He also has given me valuable advice not only about research but also about how to live my life.

I would also like to thank my committee members, Dr. Olga Padillar-Zakour and Dr. Andrew Novokovic. Both have always encouraged me to keep doing my research.

I am grateful to Dr. Sang Hoon Jung at KIST who provided me with generous support and leadership of my research. I would like to thank each member of the Natural Product Research Center at KIST. Without their support, I may not have completed my degree.

I appreciate Dr. Dae-Ok Kim, Dr. Kiwon Lee, Dr. Woo Jung Park, and Dr. Ho Jin Heo for their support and research ideas, which helped greatly while pursuing my doctoral degree.

Lastly, I would like to thank my family for their extensive support throughout my graduate studies. In particular, I appreciate Se Kun Jang and Kyung Sook Lee, for whom I hold dear respect and love.

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CHAPTER 1

COFFEE AND ITS HEALTH BENEFITS

1.1 COFFEE

Beverages are an integral part of our daily diet and an important source of calories and nutrients. Coffee is one of the most consumed beverages worldwide, with about 2.25 billion cups of coffee consumed every day (Ponte, 2002). A wide variety of phytochemicals has been shown to be present in coffee, conferring coffee with numerous health benefits. Because of the popularity and wide consumption of coffee, the health benefits associated with coffee have been researched extensively. However, many metabolites of coffee and their potential physiological mechanisms in the human body have not yet been studied.

The word coffee is thought to be derived from the province of Kaffa in Ethiopia, where coffee was first cultivated. One of the earliest records of coffee appears was found in this province. During the 9th century, a goat herdsman noticed his goats becoming excitable and starting to jump after eating red berries. The goat herdsman ate the berries himself and felt energetic; he gave these berries to a monk at the local monastery. The monk found that consumption of the berries after boiling helped the monk to stay awake during religious services; the berries were then distributed to other monasteries around the world. Coffee beans have also been used as a type of energy food for Ethiopian nomadic mountain warriors (Dicum et al., 1999). Coffee was introduced to North America in 1607 and Europe in 1615 (Hatzold, 2012). In the 17th century, French and British colonists introduced coffee planting to Central and South America. The first modern soluble coffee was created in the 1930s by Nestle in Brazil, and the popularity of freeze-

dried coffee increased dramatically during World War II. In the 2000s, coffee shop chains became increasingly popular, further promoting the consumption of coffee. Recent market studies by Mintel have shown that the market for single-serve coffee machines has increased to 3.1 billon US dollars, demonstrating the popularity and profitability of the coffee industry.

Therefore, coffee is one of the most valuable commodities for trade on the international market. More than 50 countries produce coffee. In crop year 2012/2013, world coffee production reached 145.1 million bags (ICO, 2014). South America continues to be the world's leading coffee-producing region. Indeed, for crop year 2012/2013, total production in South America was estimated to be 67.6 million bags, representing 46.5% of total coffee production worldwide. Asia and Oceania had the second largest coffee production (42.4 million bags) during the 2012/2013 crop year, followed by Central America and Mexico (18.5 million bags) and Africa (16.7 million bags). Moreover, dramatic growth in the production of Robusta coffee has been observed, with production increasing from 18.8 million bags a year during the regulated market period (1990/1991) to 39.3 million bags (2012/2013). This drastic increase in the production of Robusta coffee is primarily attributed to increased coffee production in Vietnam since the 1980s; in Vietnam, only 1 million bags of coffee were produced in the crop year 1989/1990, while an estimated 22 million bags were produced during 2012/2013. Total Robusta coffee production in crop year 2012/2013 was estimated to be 56.5 million bags, representing 38.9% of total coffee production worldwide; this is increased from the 25.9 million bags produced in 1989/1990 (27.5% of total production worldwide). Total Arabica coffee production in crop year 2012/2013 was estimated to be 89 million bags, accounting for 61.3% of the total coffee production worldwide;

this is increased from the 68.3 million bags produced in 1989/1990 (72.5% of total production worldwide).

Coffee is a complex agricultural product, and production of coffee requires careful attention. The first step in coffee production is harvesting. Three to four years after the coffee is planted, flowers grow where the leaves join the branches. Clusters of fruit follow the flowers. Coffee beans, the seeds produced by the coffee plant, are the pits inside the fruits of the coffee tree. Ripe berries are harvested manually or mechanically (Souza, 2008). Manual harvesting is associated with high labor costs; however, selection of ripe berries provides higher quality beverages. Despite this, the use of mechanical harvesting has been increasing due to the high costs of labor. Before the berries undergo dry or wet processing, debris and damaged berries are eliminated in washing channels. For the dry processing method, the washed berries are spread out in the sun and turned several times a day until they have dried evenly. Depending on the weather conditions, the berries are dried for several weeks until the moisture contents drops to 11–12.5% (Souza, 2008). At larger plantations, drying machines are sometimes used to quicken the process. For the wet processing method, harvested berries are passed through a pulping machine with water where the skin and pulp is separated from the bean. The beans are transported to large, water-filled fermentation tanks and remain in the tank for 12–48 h depending on the temperature, climate, altitude, and bean condition (Hatzold, 2012). The purpose of this step (i.e., fermentation) is to remove the slick layer of mucilage, which can cause contamination of the coffee. When the fermentation is complete, the coffee is washed with water using machines. The wet coffee is dried in the sun or using a mechanical dryer to reduce the moisture content. After being dried,

the beans undergo further processing, including hulling, polishing, cleaning, and sorting according to size, density, and color.

Three additional steps in coffee production, i.e., roasting, grinding, and brewing, can affect the composition of coffee. Roasting transforms green coffee beans to aromatic brown beans, enhancing the characteristics of the beans. Most roasting machines heat the beans to 180–240°C for 8–15 min. Several reactions occur during roasting, including the Maillard reaction, degradation of phenolic compounds, and formation of aroma components (Van Boekel, 2006). Since aroma components are volatile and water soluble, the resulting flavor can be reduced by moisture, light, and oxygen. Grinding is needed to get the maximum flavor from the roasted beans. Proper grinding methods depend on how the coffee is to be brewed. The final step in coffee production is brewing, which must be performed at the right temperature for the correct amount of time.

1.2 CONSTITUENTS OF COFFEE

The coffee tree belongs to the Rubiaceae family, genus *Coffea*. More than 80 coffee species have been identified worldwide; however, *Coffea arabica*, known as Arabica coffee, and *C. canephora*, known as Robusta coffee, account for the entire global coffee market (ABIC, 2011; Farah, 2012). These two species differ in terms of their ideal growing climate, physical aspects, beverage characteristics, and chemical composition. The chemical composition of coffee beans changes during coffee production, especially roasting (Table 1.1).

Table 1.1 Chemical compositon of unroasted and roasted *C. arabica.* (Farah, 2012)

Compounds	Concentration (g/100g)	
	Unroasted	**Roasted**
Carbohydrates/fiber		
Sucrose	6.0-9.0	4.2
Reducing sugars	0.1	0.3
Polysaccharides	34-44	31-33
Lignin	3.0	3.0
Pectins	2.0	2.0
Nitrogenous compounds		
Protein	10.0-11.0	7.5-10
Free amino acids	0.5	ND
Caffeine	0.9-1.3	1.1-1.3
Trigonelline	0.6-2.0	0.2-1.2
Nicotinic acid	ND	0.016-0.026
Lipids		
Coffee oil	15.0-17.0	17.0
Diterpene esters	0.5-1.2	0.9
Minerals	3.0-4.2	4.5
Acids and esters		
Chlorogenic acids	4.1-7.9	1.9-2.5
Aliphatic acids	1.0	1.6
Quinic acids	0.4	0.8
Melanoidins	ND	25

Coffee is composed primarily of water, carbohydrates, fiber, proteins, free amino acids, lipids, minerals, organic acids, chlorogenic acids (CGA), trigonelline, and caffeine. During the roasting process, changes in the chemical composition of coffee beans occur from the Maillard reaction, carbohydrate caramelization, and organic compound pyrolysis (Belitz et al., 2009), which result in decreases in the amounts of carbohydrates, proteins, lipids, minerals, and free amino acids in the coffee beans.

Coffee contains a variety of polyphenols, which account for 10% of the dry weight. The main phenolic components are CGA, which are derived primarily from esterification of *trans*-cinnamic acids (e.g., caffeic, ferulic, and *p*-coumaric) with (-)-quinic acid. CGA cause the coffee to taste astringent, bitter, and acidic. The transformation of CGA during roasting and brewing is complex. Although the levels of CGA are greatly reduced during processing with high pressure and heat, coffee is a significant dietary source of CGA; a single serving of espresso coffee supplies about 24–423 mg of CGA (Crozier et al., 2012). Consumption of non-espresso coffee may provide 1–2 g of CGA to many coffee drinkers each day, exceeding intake of CGA from fruits and vegetables (Ludwig et al., 2014). Studies in colostomy patients indicate that about one-third of ingested CGA and 95% of caffeic acid are absorbed intestinally (Olthof et al., 2001). Thus, about two-thirds of ingested CGA reaches the colon, where it is metabolized by the microflora (Olthof et al., 2003). The use of HPLC-MS coupled with available standards, such as hydroxycinnamate sulfates and glucuronides, has enabled elucidation of the CGA metabolic pathway (Figure 1.1).

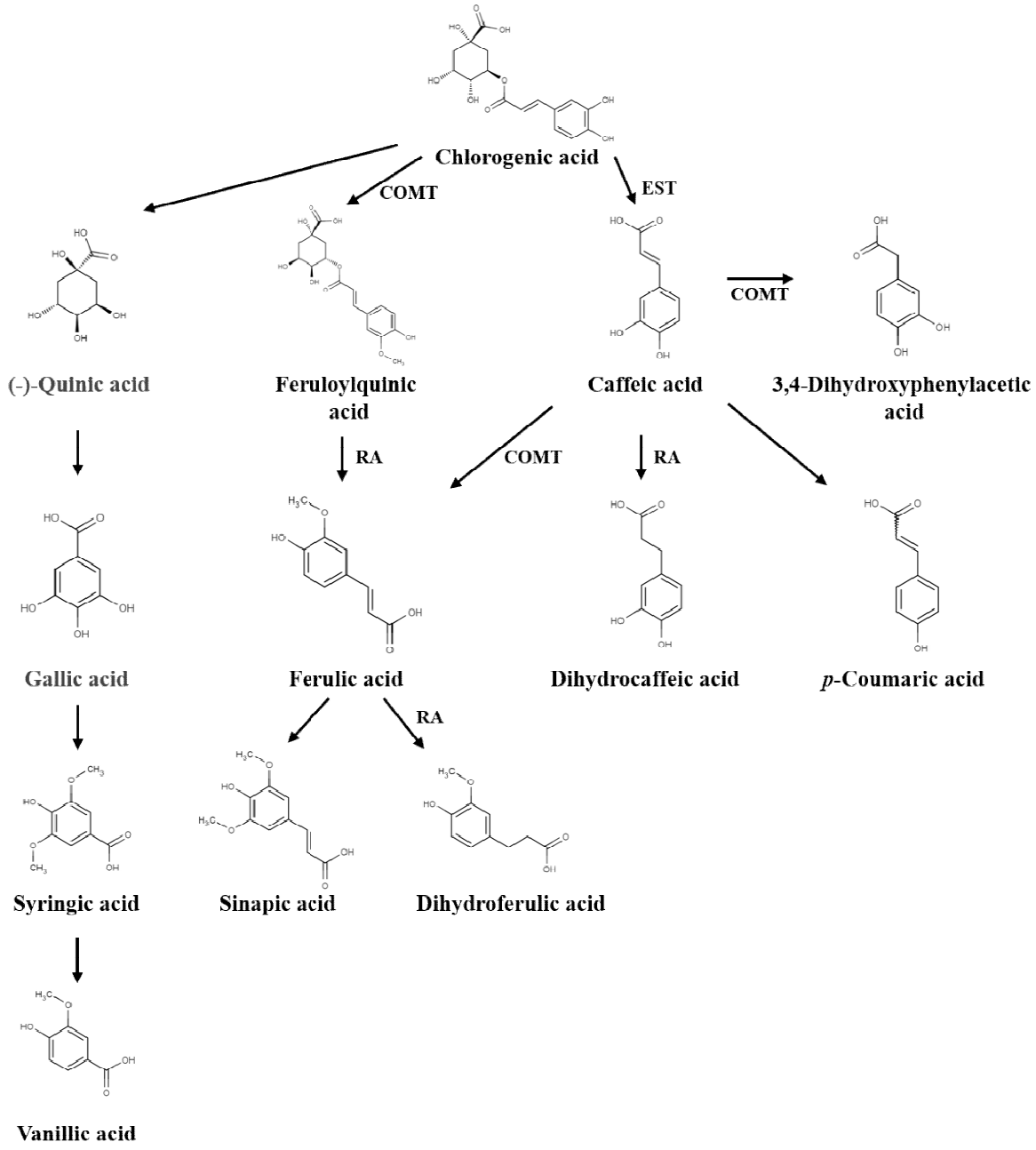

Figure 1.1 Proposed metabolism of chlorogenic acids. COMT, catechol-*O*-methyltransferase; EST, esterase; RA, reductase. (Del Rio et al., 2010; Duarte and Farah, 2011)

In last few years, many *in vitro* and *in vivo* studies have reported that CGA is associated with beneficial properties, such as reducing the risk of type 2 diabetes, neurodegenerative disease, heart disease, and liver cancer (Kwon et al., 2010; Larsson and Wolk, 2007; Van Dam and Feskens, 2002; Vinson et al., 1995). Additionally, because of its high CGA content, coffee is the most important antioxidant source in our diets (Kono et al., 1997; Natella et al., 2002).

Caffeine, the purine alkaloid, is the principal constituent in coffee. The alkaloid is heat stable, and caffeine is not reduced during the roasting process. Typical caffeine levels in a cup of coffee vary between 50 and 100 mg, depending on the coffee species and brewing method (Barone and Roberts, 1996). Caffeine is rapidly absorbed in the stomach and small intestine and stimulates the central nervous system as an adenosine-receptor antagonist. Caffeine metabolism occurs primarily in the liver, where the activity of the cytochrome P450 isoform CYP1A2 accounts for almost 95% of the primary metabolism of caffeine (Higdon and Frei, 2006). Other caffeine metabolites are formulated by cytochrome and enzymes (Figure 1.2).

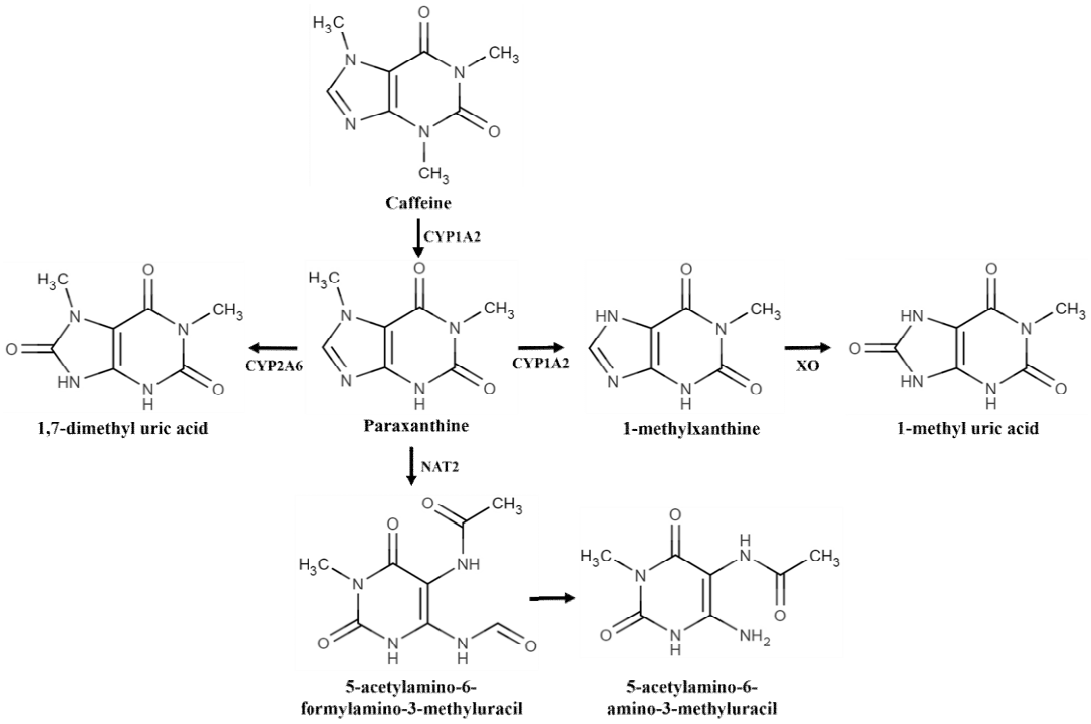

Figure 1.2 Caffeine metabolic pathway. CYP1A2, cytochrome P450 1A2; CYP2A6, cytochrome P450 2A6; NAT2, *N*-acetyl transferase 2; XO, xanthine oxidase. (Crews et al., 2001; Krul and Hageman, 1998)

Although many studies have investigated the properties of caffeine, its effects on health are still controversial (Heckman et al., 2010; Nehlig, 1999). The consumption of caffeine causes acute elevation of blood pressure, increased metabolic rates, and diuresis. In addition, caffeine may exert protective effects against type 2 diabetes, Parkinson's disease, and hepatic injury (Ludwig et al., 2014). However, some studies have revealed the negative effects of caffeine, such as increasing the risk of cancer, osteoporosis, hypertension, and pregnancy complications (Higdon and Frei, 2006). Therefore, decaffeinated coffee may be preferred for some people. Caffeine is extracted from green beans before roasting with three main methods, including use of organic solvents (dichloromethane or methyl acetate), water, or supercritical carbon dioxide (Farah, 2012).

1.3 HEALTH BENEFITS OF COFFEE

In recent years, coffee consumption per capita has increased because of the ability to concentrate and due to its pleasant flavor. Due to its widespread popularity, the potential health benefits or risks associated with coffee intake have important public health implications. Additionally, studies of the health effects of coffee have increased dramatically in the last 25 years. According to these studies, coffee is associated with a variety of potential health benefits, including a reduced incidence of severe chronic and degenerative diseases, such as cancer, cardiovascular disorders, diabetes, liver diseases, Alzheimer's disease, and Parkinson's disease (Bøhn et al., 2012; Higdon and Frei, 2006; Nkondjock, 2009). However, although some experimental evidence has described the biological mechanisms of coffee's effects *in vitro* and *in vivo*, much of the available epidemiological data on coffee consumption are still conflicting (Ludwig et al., 2014). Some epidemiological studies were not designed to focus on coffee consumption.

Moreover, in these studies, considerable variability in the quantity of coffee consumption, such as cup size, was noted. The caffeine in coffee has been shown to exert negative effects on cardiovascular disease (Hakim et al., 1998). Additionally, coffee has been shown to be associated with a lower risk of Parkinson's disease in women who never used postmenopausal hormones, but a greater risk among hormone users (Ascherio et al., 2003). Therefore, further studies on the bioavailability and pharmacokinetics of coffee constituents are needed to elucidate the compounds and mechanisms responsible for these health benefits.

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CHAPTER 2

EFFECTS OF COFFEE ON RETINAL DEGENERATION

2.1 THE EYE AND RETINA

The eyes are the organs of vision that form our perceptions of the brightness and color of an outside image on a photosensitive layer of cells (Jacobson and Marcus, 2011). Eyes are typically spherical and are filled with a transparent gel-like substance called the vitreous gel (Figure 2.1) (Atchison et al., 2000). The structures of the eye are complex and function in concert to facilitate sight. The cornea is the outermost layer that covers the front of the eye, and the iris is a thin, circular structure in the eye that controls the diameter and size of the pupil; relaxing or tightening of the muscles around the iris changes the size of the pupil, regulating the amount of light that enters the eye. Additionally, the ciliary body releases a transparent liquid within the eye and contains the ciliary muscle, which changes the shape of the lens, allowing the formation of a focused image on the back wall of the eye, called the retina. The macula is located in the center of the retina and is concentrated with sensitive part of the retina responsible for detailed central vision. The fovea is the very center-most part of the macula.

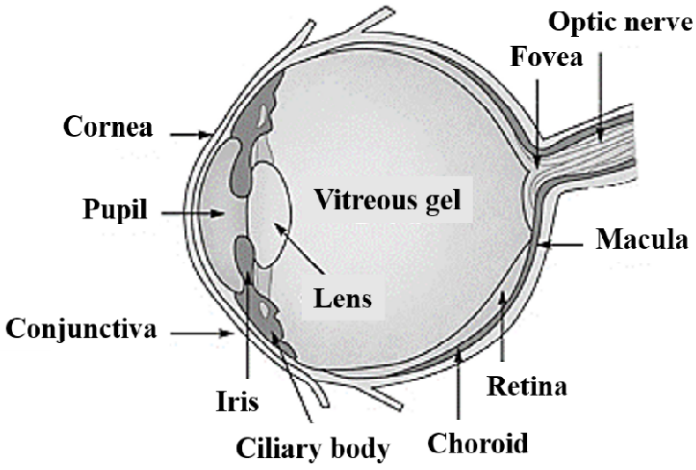


Figure 2.1 Schematic diagram of the anatomy of the eye (NIH, 2015).

The retina is approximately 200 μm thick and contains several classes of neurons, rods, cones, horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells (RGCs) (Figure 2.2) (Wässle, 2004). When light enters the eye and reaches the retina, a cascade of biochemical reactions occurs in photoreceptors. Rods and cones, the photoreceptors of the retina, specialize in transforming light into an electrical signal. The rods function in dim light, transmitting black and white images to the brain, while the cones provide high-resolution color vision. A photon of light activates rhodopsin by causing isomerization of 11-*cis*-retinal attached to all-*trans*-retinal (Baehr et al., 2003). All-*trans*-retinal is reduced by NADPH-dependent all-*trans*-retinol dehydrogenase to all-*trans*-retinol. To continue the visual cycle, the rhodopsin chromophore must be recycled. In retinal pigment epithelium (RPE) cells, all-*trans*-retinol is esterified and then converted to 11-*cis*-retinol. The 11-*cis*-retinol can be oxidized to 11-*cis*-retinal, which is then transferred back into photoreceptor cells to regenerate rhodopsin. At the synaptic terminals of rods and cones, signals are transferred to bipolar and horizontal cells (Wässle, 2004). Horizontal cells provide lateral interactions in the outer plexiform layer. Bipolar cells transfer the light signals to the inner plexiform layer, containing the dendrites of amacrine and ganglion cells. Amacrine cells provide a feedback synapse onto the bipolar cell, while ganglion cells collect the signals of bipolar and amacrine cells, and their axons transmit these signals to the brain through the optic nerve. Recently, the characteristics of ganglion cells have been analyzed using many different *in vivo* techniques. These techniques have been applied to the retinas of non-mammalian organisms, such as the tiger salamander and goldfish (Arnett, 1978; Kim and Rieke, 2001). Furthermore, studies have used intracellular, patch-clamp, multi-electrode recording from slices, and whole mounts of mouse, rabbit, cow, and primate retina to examine the characteristics of ganglion cells (Curcio et al., 1987; Euler et al., 1996).

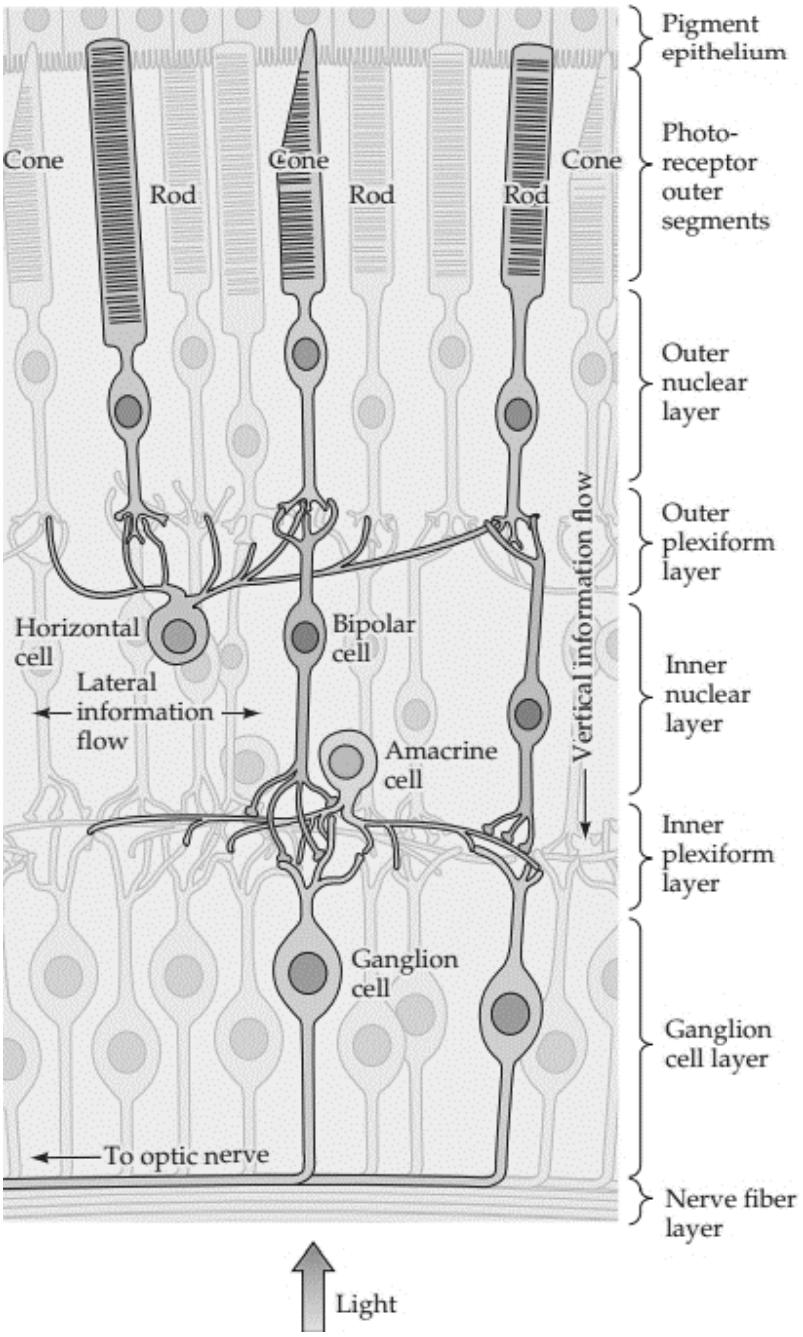


Figure 2.2 Structure of the retina (Purves, 2008)

2.2 RETINAL DEGENERATION

Retinal degeneration is a complex group of diseases with a neuroinflammatory and immune involvement leading to apoptotic cell death (Remé et al., 1998). Retinal degeneration, as occurs in age-related macular degeneration (AMD), diabetic retinopathy, and glaucoma, is a leading cause of blindness worldwide (Pizzarello et al., 2004).

AMD is defined as an abnormality of the RPE that leads to degeneration of the overlying photoreceptors in the macula and consequent loss of central vision (Jager et al., 2008). The disease is characterized by the accumulation of drusen, which are extracellular deposits of lipids, cellular debris, and protein, underneath the RPE basement membrane. Early AMD is identified by drusen of the RPE without loss of vision. Advanced AMD has two forms: geographic atrophy (i.e., dry AMD) and choroidal neovascularization (i.e., wet AMD). An estimated 8 million people in the US suffer from intermediate or advanced forms of AMD, making AMD the leading cause of vision loss in elderly individuals in the developed world.

Diabetic retinopathy begins with microaneurysms and progresses to exudative changes that lead to macular edema, ischemic changes, collateralization and dilatation of venules, and proliferative changes (Antonetti et al., 2012). Diabetic retinopathy causes impaired contrast sensitivity and visual fields, resulting in difficulties with vision during daily living. An increased duration of diabetes and poor glucose control are major risk factors for retinopathy. Approximately 40% of patients with diabetes who are over the age of 40 years have some retinopathy, with 8.2% of these patients having vision-threatening retinopathy. Diabetic retinopathy is a leading cause of

visual loss in the US for patients between the ages of 20 and 74 years, with 12,000 to 24,000 new cases of diabetic retinopathy-induced blindness diagnosed each year (Leonard, 2002).

Glaucoma is a group of progressive optic neuropathies that are characterized by slow, progressive degeneration of RGCs (Kwon et al., 2009). Patients with glaucoma typically lose peripheral vision and may lose all vision if not treated. Intraocular pressure and impaired ocular blood flow are considered to be risk factors for glaucoma. Black race, older age, family history, and myopia are also risk factors for glaucoma. Glaucoma is estimated to affect more than 80 million individuals worldwide by 2020, with at least 6–8 million individuals becoming bilaterally blind (Pizzarello et al., 2004).

2.3 OXIDATIVE STRESS

Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS) (Apel and Hirt, 2004). ROS can be free radicals, defined as oxygen species that have been elevated to a higher energy level or act as strong oxidizing agents. In pathophysiology, hydrogen peroxide, superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide (NO), singlet oxygen ($^1O_2$), lipid peroxyl radicals ($LOO^{\cdot}$), and peroxynitrites ($ONOO^{\cdot}$) are major ROS (Stadtman and Berlett, 1997). These ROS are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes and from a variety of cytosolic enzyme systems, such as lipoxygenases, NADPH oxidase, and cytochrome P450. Exogenous sources, such as ultraviolet light, ionizing radiation, chemotherapeutics, inflammatory cytokines, and environmental toxins, also can trigger ROS production in our bodies. Most of these species of ROS are present at low levels during normal physiological conditions as byproducts of normal aerobic metabolism or as secondary

messengers in signal transduction pathways (Sharma et al., 2012). The excessive production of ROS is largely counteracted by an intricate antioxidant defense system, including enzymatic scavengers such as catalase, superoxide dismutase, and glutathione peroxidase (Finkel and Holbrook, 2000). In addition, nonenzymatic antioxidant defense systems are important for scavenging ROS. These include ascorbate, pyruvate, flavonoids, and carotenoid. However, when ROS production overwhelms the defense system, ROS can cause severe damage to mitochondrial and cellular proteins, lipids, and nucleic acids (Uttara et al., 2009).

2.4 OXIDATIVE STRESS AND RETINAL DEGENERATION

Retinal degeneration is mainly attributed to oxidative stress, which may be a consequence of attenuated antioxidant cell defense systems or augmented levels of ROS in the retina (Jarrett et al., 2008). The retina is vulnerable to and abundant in ROS; oxidative stress can cause extreme damage to the retina through dysregulation of intracellular physiology, leading to ocular neurodegeneration.

The retina is a tissue with high metabolism and has the highest oxygen consumption per unit weight of all human tissues (Yu and Cringle, 2001). Oxygen and nutrients are supplied by two separate circulatory systems, the retinal vasculature and the choroidal vasculature (Yu and Cringle, 2005). The retinal vasculature is supplied by the central retinal artery, which enters the retina with the optic nerve at the optic disc. The artery branches supply blood to the neurons and glial cells of the inner portions of the retina. Choroidal circulation is supplied by the long and short ciliary arteries and the anterior ciliary arteries, which feed the large arteries in the outer portion of the choroid. These artery branches supply blood to meet the high metabolic demands

of the photoreceptors (Newman, 2013). The high partial pressure of oxygen in photoreceptors in the retina promotes generation of ROS via the mitochondria, which may also cause damage to mitochondrial DNA (mtDNA) (Cui et al., 2012).

Importantly, the retina is also exposed to high-energy light. Each day, the human retina absorbs approximately 10^{12}–10^{15} photons (Hunter et al., 2012), which can cause irreversible damage to the retina, including immediate thermal injury by bright light and photochemical damage by exposure to light for an extended period of time (Nowak, 2013). Although radiation is partly absorbed by the cornea and lens, light from 400 to 760 nm reaches the retina. The shorter the wavelength, the greater the energy; therefore, within the visible light range, blue light (400–500 nm) has the highest energy and can cause damage to photoreceptors. According to one study, exposure of the retina to blue light *in vivo* leads to cell proliferation and mitotic alterations in RPE and choroidal cells as well as spots on the RPE cell layer, similar to early signs of AMD (Ham et al., 1978).

In addition, polyunsaturated fatty acids are abundant in the photoreceptor membrane. Docosahexaenoic acid (DHA) absorbed from the diet accounts for more than 30% of total fatty acids in the membrane (SanGiovanni and Chew, 2005). Because of the susceptibility of unsaturated fatty acids to oxidation, photoreceptors are vulnerable to lipid peroxidation (Nowak, 2013). This may produce peroxides and radicals, which may cause functional and structural damage to the cell layer, resulting in the degeneration of photoreceptors.

2.5 TREATMENT OF RETINAL DEGENERATION

While there is no cure for retinal degeneration, treatments have been developed for AMD that may prevent severe vision loss or slow the progression of the disease considerably. In dry AMD, the most common form of AMD, the deterioration of vision can be slowed by diet and nutrition. A combination of beta-carotene, vitamin C, vitamin E, zinc, and copper has been shown to result in a 25% reduction in the risk of developing advanced macular degeneration in patients (Group, 2001). Anti-vascular endothelial growth factor (VEGF) medications and laser surgery are available for the treatment of wet AMD. Anti-VEGF medication has to be injected into the eye and blocks the development of new blood vessels and leakage from abnormal vessels within the eye (Nowak, 2006). Ranibizumab (Lucentis; Genentech/Roche), bevacizumab (Avastin; Genentech/Roche), and aflibercept (Eylea; Regeneron Pharmaceuticals) are currently the most common therapies for wet AMD (Zhang et al., 2012). Photodynamic therapy (PDT), which was developed in the 1990s (Henderson and Dougherty, 1992), involves having a light-sensitive medicine (verteporfin) injected into a vein in the patient's arm. Verteporfin attaches itself to the abnormal blood vessels in the macula. Upon exposure to a low-powered laser, a chemical reaction occurs that seals off the leaky vessels. This method, which reduces harm to other retinal tissues, also halts the progression of macular degeneration. Alternatively, laser photocoagulation can be used to treat wet AMD (Schmidt-Erfurth and Hasan, 2000). Exposure to high-energy laser light can destroy actively growing abnormal blood vessels. However, with this method, some loss of vision may occur because the laser can damage neighboring retinal tissues that are perceived as blind spots. Therefore, laser photocoagulation is only used in patients who are unable to be treated with anti-VEGF medication or PDT.

Treatment for diabetic retinopathy is often very effective in preventing, delaying, or reducing vision loss. No treatment may be required during the early stages of retinopathy; however, patients with advanced retinopathy may require photocoagulation treatment or injection with anti-VEGF or anti-inflammatory medicines into the eye (Porta and Allione, 2004). Surgical removal of the vitreous gel (i.e., vitrectomy) may also help improve vision if the retina has not been severely damaged (Bhagat et al., 2009). Notably, in patients with diabetic retinopathy, controlling blood sugar levels remains the most critical part of the treatment (Fong et al., 2004).

Treatment of glaucoma aims to reduce the pressure in the affected eye (i.e., the intraocular pressure) (Lee and Higginbotham, 2005). Chronic open-angle glaucoma is often treated using eye drops. There are several different types of eye drops available. Prostaglandin analogs increase the flow of fluid out of the eye, which reduces the intraocular pressure. Beta-blockers and carbonic anhydrase inhibitors reduce intraocular pressure by slowing down the production of aqueous humour in the eye. Sympathomimetics are thought to reduce the rate of production of aqueous humour and increase the flow of aqueous humour out of the eyes. The side effects of glaucoma drops may include allergy, redness of the eyes, brief stinging, blurred vision, and irritated eyes (Weinreb and Khaw, 2004). Laser surgery for glaucoma slightly increases the outflow of the fluid from the eye in open-angle glaucoma or eliminates fluid blockage in angle-closure glaucoma. Several types of laser surgeries are available for the treatment of glaucoma; these include trabeculoplasty, during which a laser is used to open the trabecular meshwork drainage area; iridotomy, during which a small hole is made in the iris to permit the fluid to flow more freely; and cyclophotocoagulation, during which a laser beam is used to irradiate the middle layer of the eye, thereby reducing fluid production (Realini, 2008). Alternatively, there

are four types of microsurgeries that can be performed in patients with glaucoma, including trabeculectomy, which is the most common type of glaucoma surgery and involves removing part of the trabecular meshwork to allow fluid to flow through the eye's drainage system; viscocanalostomy, which involves removing part of the sclera, enabling the fluid to filter out of the eye and into the body; deep sclerectomy, in which a tiny device is implanted inside the eye to widen the trabecular meshwork; and aqueous shunt implant, in which a tube device is placed into the eye to increase the drainage of fluid out of the eye (Schwartz and Budenz, 2004).

2.6 PHYTOCHEMICALS AND RETINAL DEGENERATION

Phytochemicals are nutrients and non-nutritive components that possess physiological benefits including antioxidant, anti-inflammatory, antitumor, antiviral, and immunomodulatory properties (Grusak, 2002; Surh, 2003). Since the pathogenesis of retinal degeneration seems to involve numerous factors, phytochemicals having multiple pharmacological properties increase the likelihood of retarding the progression of the disease. Accumulating evidence has shown that phytochemicals may be involved in the prevention and reversal of age-related eye diseases, including AMD, glaucoma, and diabetic retinopathy (A Omara et al., 2010; Rhone and Basu, 2008).

High dietary intake of omega-3 fatty acids and the macular pigments lutein and zeaxanthin are associated with a lower risk of AMD (Group, 2013). The Age-Related Eye Disease study (AREDS) showed a beneficial effect of high doses of vitamins C, E, beta-carotene, zinc, and copper in reducing the rate of progression to advanced AMD in patients with intermediate AMD or with one-sided late AMD (Chew et al., 2013). The AREDS-2 study showed that lutein and

zeaxanthin may be substitutes for beta-carotene because of its potential association with an increased incidence of lung cancer (Group, 2013). Resveratrol, originally isolated from the skin of grapes, has been shown to reduce symptoms of AMD. Treatment with 50 or 100 µM resveratrol significantly reduces the *in vitro* proliferation of RPE cells by 10% and 25%, respectively (King et al., 2005). Research has also indicated that resveratrol protects human retinal pigment epithelium cells from acrolein-induced damage (Sheu et al., 2010).

Ginkgo biloba extract has numerous properties that theoretically should be beneficial in treating non-intraocular pressure-dependent mechanisms in glaucoma (Cybulska-Heinrich et al., 2012). Its multiple beneficial actions include increased ocular blood flow, antioxidant activity, platelet-activating factor inhibitory activity, nitric oxide inhibition, and neuroprotective activity. Based on electroretinogram measurements, epigallocatechin gallate (EGCG), a catechin-based flavonoid from green tea, confers neuroprotection of retinas injured by ischemia/reperfusion (Falsini et al., 2009). Therefore, EGCG supplementation may be beneficial in the treatment of glaucoma.

Mirtogenol is a combination of two herbal extracts, bilberry and French Maritime pine bark. Both extracts have antioxidant properties and are associated with reduced risk for developing symptomatic glaucoma by controlling intraocular pressure and improving ocular blood flow (Steigerwalt et al., 2008). French Maritime pine bark extract has been also shown to strengthen the capillaries in the retina, thereby contributing to protection against retinopathy (Jain et al., 2014). Grape seed extract can protect blood vessels and capillaries from ROS damage (Agarwal et al., 2004). Research has shown that grape seed extract may be effective in protecting blood

vessels and capillaries against symptoms of diabetic retinopathy (Zafra-Stone et al., 2007). The flavonoid quercetin may be beneficial for preventing blood clots because it inhibits inflammation by reducing histamine formation (Guardia et al., 2001). Quercetin also mitigates the formation of insulin-like growth factor, which is significant for the development of diabetes (Vessal et al., 2003). Furthermore, quercetin reduces high blood pressure, which can induce stress on the walls of retina blood vessels (Edwards et al., 2007).

2.7 COFFEE AND OCULAR HEALTH

Coffee contains many phytochemicals and it is thought to be a major source of dietary antioxidants. However, few studies have examined the association between coffee and ocular health, and most of these studies have focused on the effects of caffeine. Interestingly, one study showed that consumption of coffee may prevent dry eye syndrome (Moss et al., 2000). Moreover, according to a 5-year prospective cohort study, coffee consumption is not associated with the development of early age-related maculopathy (Tomany et al.). The effects of caffeine on intraocular pressure, which can induce glaucoma, are still controversial. Caffeinated beverages (over 180 mg of caffeine) may cause ocular hypertension, but they have not been shown to have a clinical impact on glaucoma (Avisar et al., 2002; Jiwani et al., 2012).

In addition to caffeine, chlorogenic acid (CGA) has been reported to exhibit several pharmacological activities, including antioxidant, antimicrobial, hepatoprotective, immunomodulatory, and hypoglycemic activities (Bassoli et al., 2008; Lou et al., 2011b; Wu et al., 2006). Furthermore, CGA exerts neuroprotective effects in scopolamine-induced amnesia in

mice and ischemic stroke in rabbits (Kwon et al., 2010a; Lapchak, 2007). However, the potential protective effects of CGA on retinal health have not been investigated.

Therefore, in this study, we examined the protective effects of coffee and CGA against hypoxia-induced damage to the retina. We found that phenolic acid metabolites, including CGA, caffeic acid (CA), and dihydrocaffeic acid (DHCA), which may form after coffee consumption, could reach the eye and exert protective effects against hypoxia and optic nerve crush (ONC)-induced damage in the retina.

2.8 EFFECTS OF CHLOROGENIC ACID AND COFFEE EXTRACTS ON RETINAL DEGENERATION

2.8.1 Introduction

The retina, a thin layer of tissue on the inside back wall of the eye, contains millions of light-sensitive cells and other nerve cells that receive and organize visual information. Collectively, these send visual information to the brain through the optic nerve. The retina is one of the most metabolically active tissues in the body, consuming oxygen more rapidly than any other tissues, including the brain. Therefore, it is susceptible to a variety of diseases caused by oxidative stress, including age-related macular degeneration, diabetic retinopathy, and glaucoma—all of which can lead to partial or complete blindness (Ames, 1992). One mechanism of retinal degeneration is hypoxia, a reduction in retinal oxygen supply caused by pathologies such as central retinal artery occlusion, ischemic central retinal vein thrombosis, complications of diabetic eye disease, and some types of glaucoma that cause vascular eye diseases (Levin, 2003; Sennlaub et al., 2002). Retinal hypoxia can negatively impact both tissue function and cell viability, and is a potential risk factor for sight-threatening disorders (Kaur et al., 2008).

Unsurprisingly, there is great interest in identifying neuroprotective compounds that inhibit hypoxia. Particularly promising in this capacity are natural products and phytochemicals that act as antioxidants and can be taken regularly without causing significant side effects (Losso and Bawadi, 2005; Lu et al., 2006; Mozaffarieh et al., 2008). One important group of neuroprotectants comprises derivatives of CGA. Collectively, these phenolic phytochemicals are known to have hepatoprotective, antibacterial, anti-inflammatory, DNA protective, and anticancer activities, among others (Belkaid et al., 2006; Bouayed et al., 2007; dos Santos et al., 2006; Henry-Vitrac et al., 2010; Lou et al., 2011a; Xu et al., 2012). Several studies have also

suggested that the antioxidant properties of CGA make it a powerful neuroprotectant (Kim et al., 2012; Lee et al., 2012; Sato et al., 2011). CGA derivatives are found in a variety of edible plants, including tea, fruits, and vegetables. However, the major source of CGA intake in humans is coffee (Clifford, 2000). CGA is 4-12% of raw coffee, while a 200-mL cup of prepared coffee contains 200 mg of total CGA (Farah et al., 2006; Matei et al., 2012). Coffee consumption appears to decrease the risk of developing chronic diseases such as Parkinson's, prostate cancer, and diabetes (Higdon and Frei, 2006; Ross et al., 2000; van Dam and Hu, 2005; Wilson et al., 2011). It also reduces the extent of cognitive declines associated with aging (Chu et al., 2009; Shukitt-Hale et al., 2013; van Gelder et al., 2007). The latter pattern in particular supports the idea that the neuroprotective effects of CGA derivatives stem from their antioxidant activities.

Despite these generally positive results, the effects of coffee consumption on ocular health are not yet clear. In particular, clinical studies on the relationship between coffee consumption and risk of glaucoma have yielded conflicting results (Jiwani et al., 2012; Pasquale et al., 2012). Thus, we designed the current study to investigate whether coffee—and, in particular, its main polyphenol, CGA—has protective effects against the degeneration of retinal ganglion cells (RGC-5), both *in vitro* and *in vivo*.

2.8.2 Materials and methods

Animals

All animal studies were carried out in a pathogen-free barrier zone at the KIST Gangneung Institute and were done in accordance with the procedure outlined in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Procedures used in this study were approved by the Animal Care and Use Committee of KIST. Male ICR mice weighing between 30-35 g (5 weeks of age) were used in the present study, and were acclimated for at least one week, caged in groups of

five or less, and were fed with a diet of animal chow and water *ad lib.* They were housed at 23 ± 0.5 °C and 10% humidity with a 12 h light-dark cycle.

Preparation of the crude extract from coffee powder

The coffee sample was obtained from a local supermarket (Colombian roast and ground coffee, Tesco, UK). Ground coffee (30.0 g) was refluxed with 70% EtOH (600 mL) three times each for 3 h, and the combined solution was filtered and evaporated under vacuum to give a 70% EtOH extract (9.76 g).

HPLC analysis

Coffee extract (CE) was analyzed by HPLC to quantify CGA using an Agilent 1200 series system, equipped with a G1379B vacuum degasser, a G1312A binary pump, a G1329A autosampler, a G1316A column oven, and a G1315B DAD detector (Agilent, Palo Alto, CA, USA). The separation was carried out on a Shiseido MGII C-18 column (250 × 4.6 mm I.D., 5 μm particle size). The mobile phase consisted of 0.1% TFA in water and acetonitrile (93:7) with a flow rate of 1.0 mL/min during for 45 min. The UV detector was set at 330 nm with full spectral scanning from 200 to 400 nm and sample injection volume was 10 μl.

A standard solution of CGA (31.25 – 1000 μg/mL) was prepared in methanol and 10 μL of each was injected into the HPLC column via the auto-injector. The linearity was evaluated by linear regression analysis, which was calculated by the least-squares regression method. The limit of detection (LOD) and the lower limit of quantification (LOQ) were defined as the minimum concentration at the signal-to-noise ratio (S/N) equal to 3.3 and 10, respectively. The precision, accuracy, and recovery were evaluated by analyzing three different concentrations in the same day and on three different days. All the experiments were carried out in triplicate.

Culture of RGC-5 cells and cell viability

RGC-5 cells that are proven to be mice origin are highly relevant to the study of glaucomatous neurodegeneration and have been previously shown to express ganglion cell markers and ganglion cell-like behavior in culture (Takeoka et al., 2001; Van Bergen et al., 2009).

To mimic physiological hypoxic stress, RGC-5 cultures were transferred into a closed hypoxic chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) in which the gas levels (1% O_2, 5.13% CO_2, and 93.87% N_2) and temperature (37 °C) were automatically controlled. After washes with phosphate buffered saline (PBS), cells were treated *S*-nitroso-*N*-acetyl-penicillamine (SNAP), a nitric oxide (NO) donor, with low glucose (5.5 mM) Dulbecco's modified Eagles's medium (DMEM; Gibco, Carlsbad, CA) which mimics physiological normal blood sugar levels *in vivo*. Cells remained in the hypoxic chamber until viability was 50–70% of the control cells, which were not exposed to hypoxic condition. To measure the relative number of living cells, an EZ cytox cell viability assay kit (Daeil Labservice, Seoul, Korea) for WST-1 assay was used. The number of living cells in each well was determined by measuring the optical density (OD) at 460 nm with a microplate reader (BioTek Instruments, Winooski, VT, USA). This quantity was expressed relative to the control value.

Microscopic analysis of cell viability by Hoechst 33342 and PI double staining

The Hoechst 33342 and PI double staining method was used to determine whether cell death occurred as a result of apoptosis or necrosis (Jung et al., 2010). The cells were stained with 8 μM of Hoechst 33342 and 1.5 μM of PI for 30 min at 37 °C. After being washed twice with serum free media, the cells were imaged using a fluorescence microscope (Olympus, Tokyo, Japan).

Protein extraction and western blot analysis

The cells were scraped using a cell scraper and centrifuged at 14,000 × *g* for 10 min. The cell pellets were resuspended in cell lysis buffer [1 M Tris pH 7.4, 2 M NaCl, 1 M EDTA, 10% NP40, 1 × protease inhibitors, 1 mM PMSF] and then incubated on ice for 10 min. The extracted cells

were centrifuged at 14,000 × *g* for 30 min at 4 °C. The supernatant was sonicated, and the total protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis was performed with the primary antibodies including anti-cleaved caspase-3, anti-Bad, anti-Thy-1 (1:1000, Cell Signaling Technology, Beverly, MA, USA), and β-actin (1:3000, Santa Cruz Biotechnology, CA, USA). Secondary antibodies were purchased from Santa Cruz Biotechnology Co. Ltd (1:3000, Santa Cruz Biotechnology, CA, USA). Immunoreactive bands were detected using enhanced chemiluminescence reagents (Amersham Bioscience, GE Healthcare, UK) and were measured via densitometry using a LAS-4000 image reader and Multi Gauge 3.1 software (Fuji Photo Film, Japan)

Histological analysis: Hematoxylin and eosin staining

The enucleated eyes were fixed in 10% formalin for 24 h, embedded in paraffin and sectioned through an equatorial plane at 4 μm thickness using a HM340E microtome (Walldof, Germany). Four sections (n = 4) were used for analysis.

Hematoxylin solution was added to the retina section (0.1% hematoxylin, 10% ammonium) for 8 min. The sections were then washed three times with distilled water. Bluing reagent (0.2% lithium carbonate solution) was added to the section for 1 min. The sections were quickly rinsed in 95% alcohol, and 1% Eosin Y solution was added to the sections for 1 min. Eosin Y was washed off with 95% alcohol three times, and the sections were cover slipped with a mounting medium and observed under a light microscope (Olympus, Tokyo, Japan).

TUNEL staining

Briefly, the sections were submitted to enzymatic digested with 20 μg/mL of proteinase K for 15 min, then washed with PBS and incubated with 3% hydrogen peroxide in PBS for 5 min at room

temperature and twice rinsed in PBS. They were then immersed, incubated with a stock solution of terminal deoxynucleotidyl transferase (TdT) reaction enzyme in a humidified chamber at 37 ℃ for 1 h, then washed three times in PBS for 1 min. The sections were subsequently incubated with anti-digoxygenin peroxidase conjugate and peroxidase substrate. Histological analysis was performed with a light microscope (Olympus, Tokyo, Japan), allowing us to look for signs of apoptosis (brown staining).

Optic nerve crush

Five-week-old ICR mice with a body weight of 30-35 g were used in the study. Animals were anaesthetized by intraperitoneal injection with a mixture of Zoletil (1.6 μg/g, Verbac Laboratories 06515, France) and Rompun (0.05 μL/g, Bayer), and retinal damage was induced using ONC (Li et al., 1999). The optic nerve of the left eye in all treatment groups was exposed by opening the meninges of the optic nerve with the sharp tips of a forceps, followed by blunt dissection. Then the exposed optic nerve was partially crushed 2 mm behind the globe for 10 sec with a cross-action calibrated forceps. In each case, a “sham” operation was performed in the same way on the partner eye, but without closing the forceps, to check for any falsifying influence of surgery on the treatment effects. In all cases the retinal blood supply remained grossly intact, as judged on the basis of a direct ophthalmoscopic inspection. The animals were killed 12 days after the ONC. All eyeballs were enucleated immediately after mice were sacrificed, then fixed for retrograde labeling of RGCs.

RGC labeling and retinal flat mount preparation

Mice were anesthetized by intraperitoneal or intramuscular administration of a mixture of Zoletil (1.6 μg/g, Verbac Laboratories 06515, France) and Rompun (0.05 μL/g, Bayer). The skin over the cranium was incised, and the scalp was exposed. Holes approximately 2 mm in diameter were drilled in the skull 4 mm posterior to the bregma, 1 mm lateral to the midline, with a

dentist's drill on both sides of the midline raphe. These positions correspond to the superior colliculi as determined from a stereotactic mouse brain atlas.

The superior colliculi were exposed by gentle aspiration of the overlying occipital cortex. A piece of Gelfoam soaked in a 5% solution of the neurotracer dye Fluorogold (Invitrogen, NY, USA) was directly applied to each superior colliculus. Skull openings were then sealed with a petrolatum-based antibiotic ointment. The overlying skin was sutured and antibiotic ointment applied externally.

Five to 7 days after the application of Fluorogold (enough time to allow retrograde uptake of the dye and labeling of the RGC somata), mice were sacrificed by transcardial perfusion with 4% buffered paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) while under the same anesthesia as that used for RGC labeling. Eyes were immediately enucleated, and the retinas were detached at the ora serrata and cut with a trephine around the optic nerve head. Four radial relaxing incisions were made and the retinas prepared as flattened whole mounts on silane-coated microscope slides (Danias et al., 2003).

Statistical analysis

The data are expressed as a mean percentage of the control value plus standard error of the mean (SEM). Statistical comparisons were made using a one-way ANOVA followed by a Dunnett's test. Statistical analyses were conducted using GraphPad Prism (version 4.0) (GraphPad, San Diego, CA, USA). Significance was defined as $p < 0.05$.

2.8.3 Results and discussion

Cell viability following treatment with SNAP and/or hypoxia

Under normoxic conditions, we observed no changes in cell viability when RGC-5 cells were treated with SNAP (data not shown). Likewise, the two lowest concentrations of SNAP did not

have any noticeable effects on the viability of RGC-5 cells exposed to hypoxic conditions (Figure 2.3). However, both the 50- and 100-μM treatments resulted in highly significant reductions in viability ($p < 0.001$). The 100-μM treatment had a particularly noticeable effect, causing a 41% reduction of viability. Thus, this is the concentration that was used in the *in vitro* experiments (Figure 2.3). The negative effects of SNAP observed here are similar to those previously reported by Sato et al. (Sato et al., 2010). They found that treatment with SNAP under hypoxic conditions resulted from activation of caspase and the formation of superoxide anions and peroxynitrite (Sato et al., 2010).

Cell viability following treatment with SNAP and CGA

CGA significantly blunted the negative effect of SNAP on RGC-5 cells (Figure 2.4A, B). This effect was dose-dependent, with cell viability increased by 72.6%, 92.7%, and 101.6% in response to 25, 50, and 100 μM CGA pretreatments, respectively (all $p < 0.001$ in comparisons with the control; Figure 2.4A). The antioxidant N-acetylcysteine (NAC) and the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) were used as positive controls. Both treatments also significantly reduced the negative impacts of SNAP observed in the untreated controls

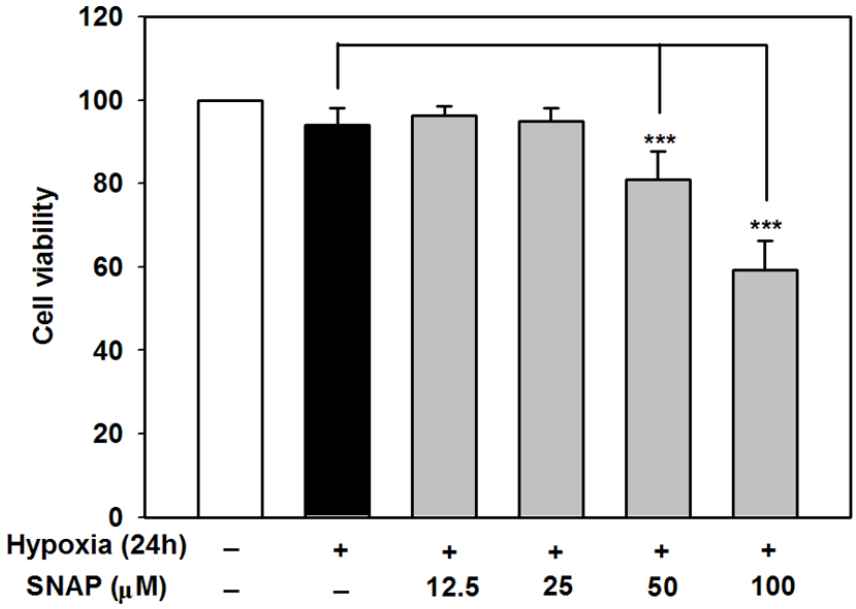
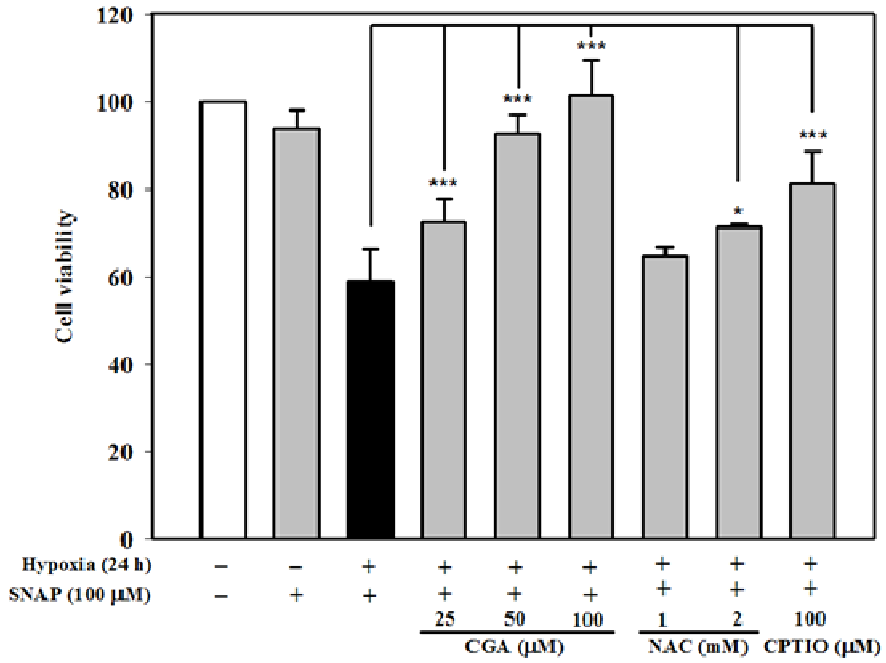


Figure 2.3 Results of the WST-1 assay measuring the effects of *S*-nitroso-*N*-acetyl-penicillamine (SNAP) on the viability of retinal ganglion (RGC-5) cells cultured under hypoxic conditions for 24 h. Values are presented as means (n = 6 independent experiments per treatment); error bars indicate SEM. Triple asterisks (***) indicate $p < 0.001$.

Figure 2.4 Effects of the chlorogenic acid (CGA) treatment on RGC-5 cells exposed to SNAP under hypoxic conditions. (A) Results of the WST-1 assay. Values are presented as means (n = 6 independent experiments per treatment); error bars indicate SEM. Single asterisks (*) indicate $p < 0.05$; triple asterisks (***) indicate $p < 0.001$. (B) Representative fluorescence microscopy of PI (red) and Hoechst 33342 (blue) staining. (a) Control RGC-5 cells under normoxic conditions. (b) RGC-5 cells under hypoxic conditions. (c) RGC-5 cells treated with 100 µM SNAP under hypoxic conditions. (d–f) RGC-5 cells pre-treated with CGA (25, 50, and 100 µM concentrations, respectively) prior to being exposed to 100 µM SNAP under hypoxic conditions. Scale bar = 50 µm.

A


120
100
80
60
40
20
0
Cell viability
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***
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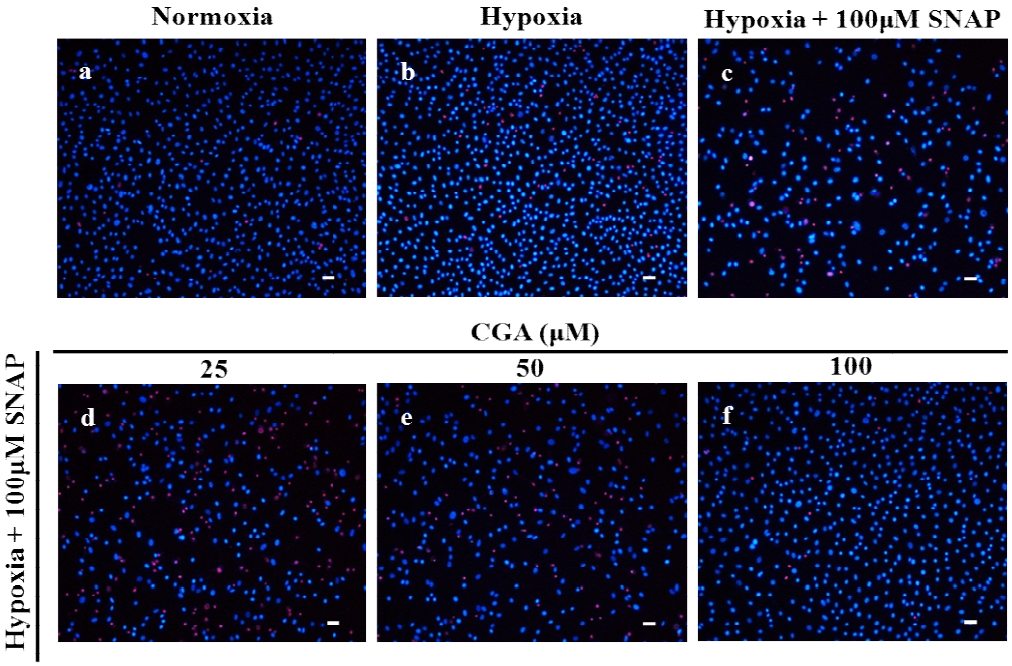
*
Hypoxia (24 h) – – + + + + + + +
SNAP (100 μM) – + + + + + + + +
25 50 100 1 2 100
CGA (μM)
NAC (mM)
CPTIO (μM)

B


Normoxia
Hypoxia
Hypoxia + 100μM SNAP
a
b
c
CGA (μM)
25
50
100
Hypoxia + 100μM SNAP
d
e
f

($p < 0.05$ for the 2 mM NAC treatment, and $p < 0.001$ for the 100 μM CPTIO treatment; Figure 2.4A). Previous work has shown that the benefits of CPTIO stem from its suppression of both caspase activation and NO production (T. Sato et al., 2010). The similar viabilities observed in cells treated with CPTIO and CGA treatments suggest that CGA may protect cells by scavenging NO that might otherwise lead to neurodegeneration of RGC-5 cells under hypoxic conditions. Fluorescence microscopy of Hoechst 33342- and PI-stained cells revealed similar patterns to those produced by the WST-1 assay (Figure 2.4B). More apoptotic/necrotic cells were observed in cultures treated with SNAP than in control cultures. However, lower levels of cell death were observed in cultures that had been pre-treated with CGA; this was particularly noticeable in response to the 100-μM CGA treatment.

Our western blot analysis showed upregulation of Bad and cleaved caspase-3 in cells that had been exposed to SNAP under hypoxic conditions (Figure 2.5). However, this response was noticeably suppressed in cells given a 100-μM CGA pretreatment. A similar pattern was observed in cells treated with CPTIO as a positive control. Bad (BCL-2 antagonist of cell death), a pro-apoptotic protein in the BH3 domain-only group (D. C. Huang & Strasser, 2000), is the key regulator of apoptosis and is, itself, regulated primarily by phosphorylation (Franke & Cantley, 1997; Gross, McDonnell, & Korsmeyer, 1999). Bad can be cleaved into a shortened form by caspases, which are cysteine proteases that play an important role in apoptosis (Cohen, 1997; Condorelli et al., 2001; Fujita et al., 1998). When this cleavage occurs, Bad can be a more potent inducer of apoptosis (Condorelli et al., 2001; Fujita et al., 1998). Apoptosis is also facilitated by the activity of caspase-3, which is known to promote neuronal apoptosis (Keane et al., 1997; Kuida et al., 1996).

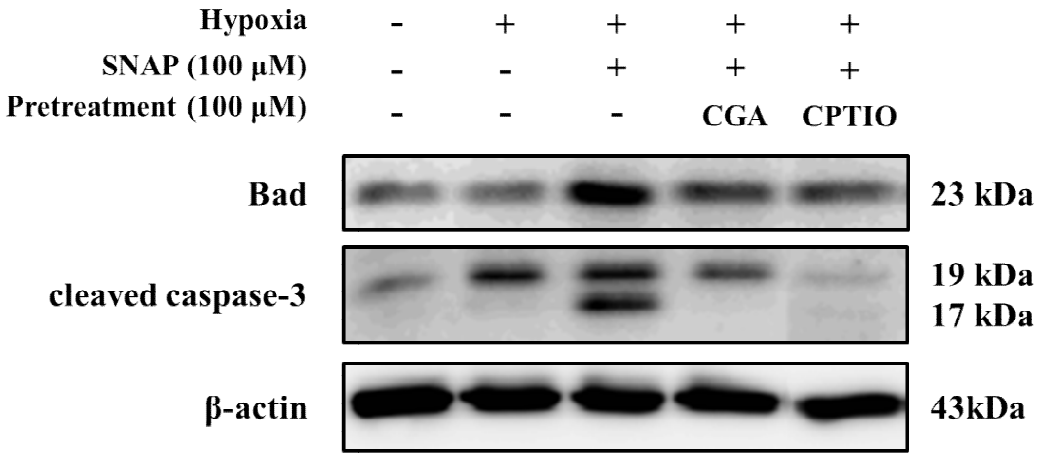


Figure 2.5 Results of a western blot investigating the expression of apoptotic proteins in RGC-5 cells subjected to 100 μM SNAP for 24 h under hypoxic conditions. The nitric oxide (NO) scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) was used as a positive control. Pretreatment with CGA appeared to have anti-apoptotic effects on hypoxia and NO-induced down-regulation of both Bad and cleaved caspase-3.

Effects of CGA on optic nerve crush-induced retinal damage in mouse

ONC is a well-known experimental model for chronic glaucoma. During the procedure, the surgically exposed optic nerve is clamped for several seconds, resulting in both primary RGC death due to the optic nerve injury and secondary death of surrounding, uninjured RGCs (Levkovitch-Verbin et al., 2001). Results from our histological hematoxylin and eosin staining assay can be seen in Figure 2.6. Relative to tissues that underwent no ONC (Figure 2.6, a), those that were crushed but received no chemical treatment exhibited a clear thinning of the inner plexiform layer (IPL) (Figure 2.6, b). However, this thinning appears to have been inhibited in tissues treated with CGA (Figure 2.6, c and d).

In addition to investigating these morphological patterns, we also used TUNEL staining to explore whether CGA could also reduce ganglion cell apoptosis associated with ONC (Figure 2.7). Control tissues (Figure 2.7, a) had fewer TUNEL-positive cells than those subjected to ONC (Figure 2.7, b). As with the hematoxylin and eosin assay, however, we found that the negative effects of ONC were mitigated by treatment with CGA; in this case, there were notably fewer TUNEL-positive cells in the ganglion cell layer (GCL) of tissues treated with CGA (Figure 2.7, c and d).

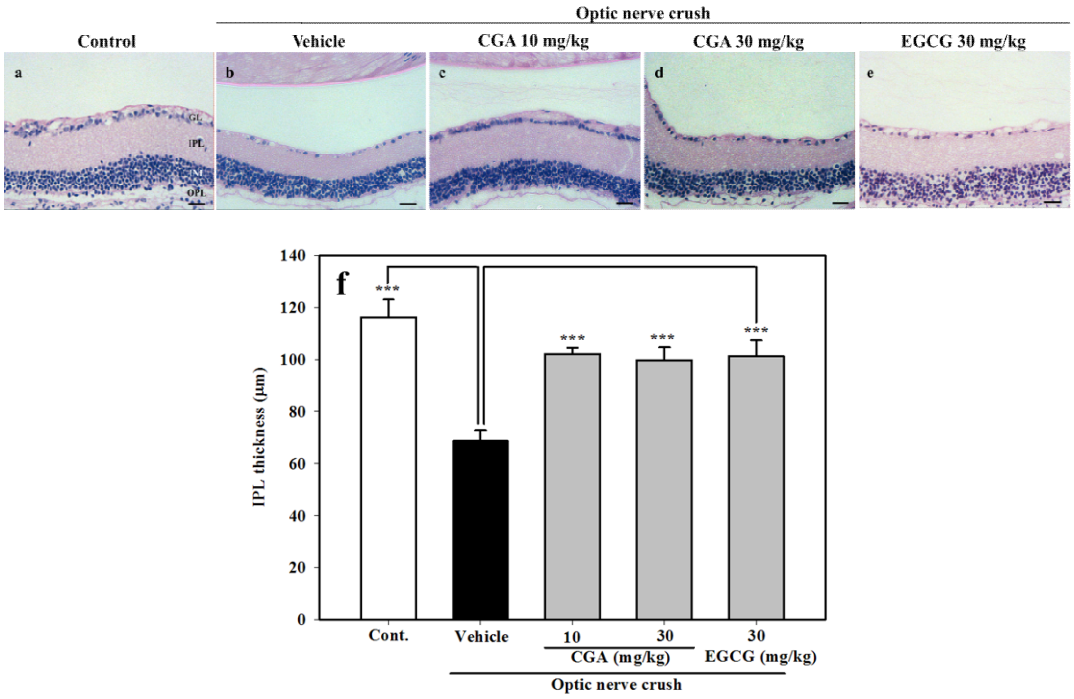


Figure 2.6 Optic nerve crush (ONC) experiments on the impacts of a CGA treatment on retinal tissues of mice. Representative photomicrographs of tissues stained with hematoxylin and eosin. Tissues from (a) a control, (b) a mouse that underwent ONC only, (c) a mouse that underwent ONC and was treated with 10 mg/kg of CGA, (d) a mouse that underwent ONC and was treated with 30 mg/kg, and (e) a mouse that underwent ONC and was treated with 30 mg/kg of epigallocatechin gallate (EGCG). The thickness of IPL (f). Results are mean values with error bar indicating mean SEM; Triple asterisks (***) indicate $p < 0.001$.

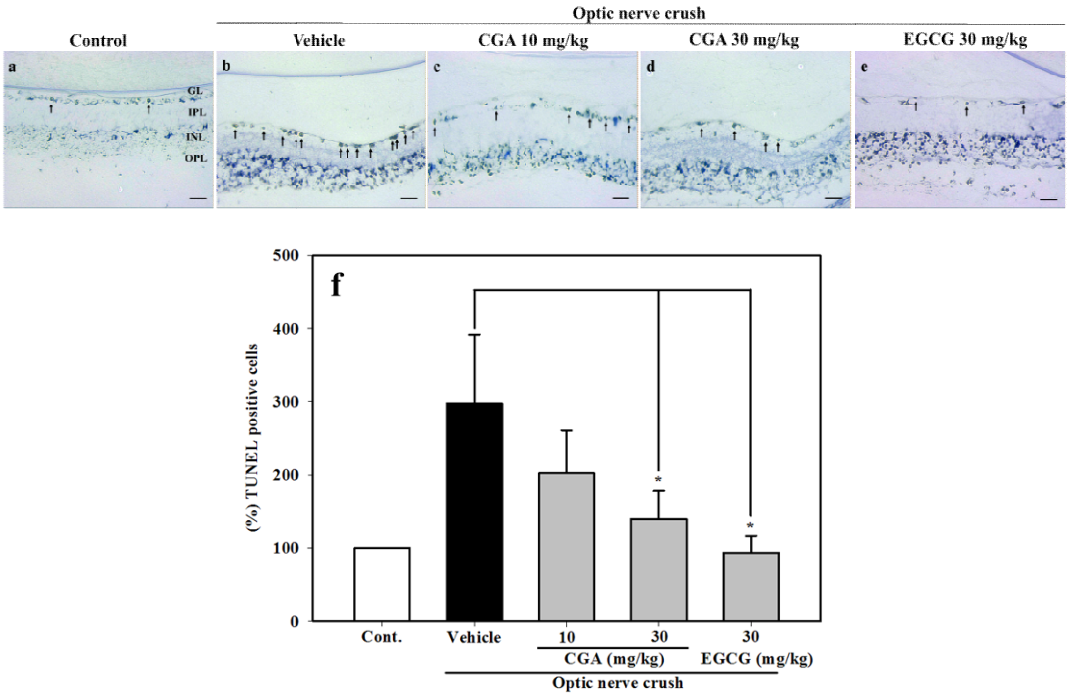


Figure 2.7 Representative photomicrographs of TUNEL-stained cells; (a-b) correspond to the same treatments as those from images a-b in Figure 2.6. Arrows indicate TUNEL-positive cells (brown stain). Scale bar = 50 µm. The quantification of TUNEL positive cells (f). Results are mean values with error bar indicating mean SEM; Single asterisk (*) indicate $p < 0.05$.

RGC survival was much lower in tissues that had undergone ONC than in controls (Figure 2.8, a-d). In tissues that had been treated with CGA, however, RGC survival was markedly higher (Figure 2.8, e-h). This was particularly true in tissues that had been dosed with 30 mg/kg of CGA (Figure 2.8, g and h). Further, both CGA treatments appeared to prevent the dramatic decrease (52.7%) in Thy-1 protein that occurred in tissues that had undergone ONC (Figure 2.9). Thy-1 is a surface glycoprotein expressed uniquely in RGCs; it serves as an early marker of RGC loss in models of retinal damage (Huang et al., 2006).

Indeed, 30 mg/kg CGA was similar or more effective for the neuroprotective effects on retinal degeneration than the same concentration of epigallocatechin gallate (EGCG) used as positive control, thus, the patterns reported here strongly suggest that CGA is a powerful neuroprotectant that can prevent retinal cell death. Previously reported mechanisms for the degeneration by ONC include blockade of axonal transport resulting in inadequate supply of neurotrophic factors, disturbances of intracellular calcium homeostasis, activation of cell death genes, and local excitotoxicity due to uncontrolled activation of NMDA receptors (Kreutz et al., 1999; Schuettauf et al., 2006). ONC induces apoptotic cascades during which cytochrome *c* is released and both caspases 9 and 3 are subsequently activated (Kermer et al., 2000; Kermer et al., 1999). Injury of the optic nerve also causes an inflammatory response involving the release of cytotoxic compounds such as free radicals, NO, and glutamate (Magharious et al., 2011). Administration of SNAP under hypoxic conditions has been associated with NO release, causing RGC-5 cell death by decreasing mitochondrial membrane potential, increasing the formation of superoxides, and activating caspases (Sato et al., 2010). Thus, our current data suggest that the beneficial

effects of CGA on retinal cells, both *in vitro* and *in vivo*, are partly due to its NO scavenging activity.

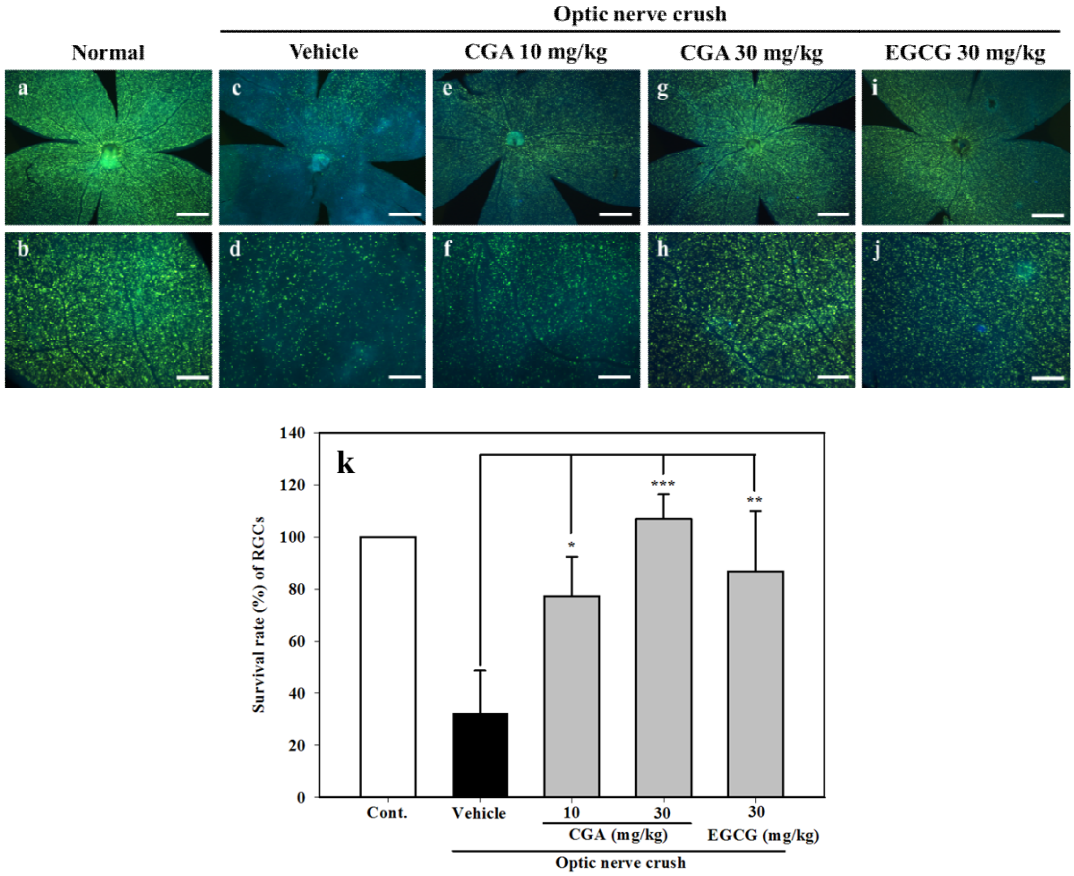


Figure 2.8 Representative micrographs showing Fluorogold-labeled RGCs harvested from mice 14 days after ONC. Retina from (a) an untreated control mouse, (c) a mouse that underwent ONC only, (e) a mouse that underwent ONC and was treated with 10 mg/kg of CGA, (g) a mouse that underwent ONC and was treated with 30 mg/kg of CGA, and (i) a mouse that underwent ONC and was treated with 30 mg/kg EGCG. Images in the lower row are 100 × magnifications of the images above them; scale bars in the upper row represent 500 μm, while scale bars in the lower row represent 100 μm. (k) Quantitative analysis of the survival (%) rate of RGCs 14 days after ONC. Experimental values are expressed as mean SEM; Single asterisks (*) indicate $p < 0.05$; double asterisks (**) indicate $p < 0.01$; triple asterisks (***) indicate $p < 0.001$.

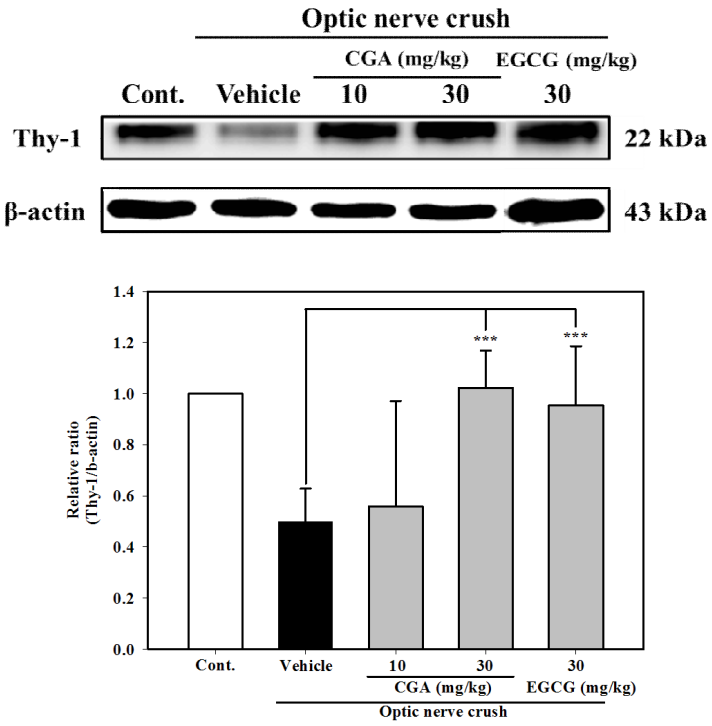


Figure 2.9 Results of western blot analysis investigating Thy-1 expression in RGCs 14 days after the ONC procedure. Densitometric analysis of Thy-1 levels, and data are shown as mean SEM; Single asterisks (*) indicate $p < 0.05$.

HPLC chromatograms of CE and standardization of CGA in CE

HPLC chromatograms of CE showed a CGA peak retention time at 28.5 min (Figure 2.10). We observed no interference from other materials in the extract, indicating that the specificity of the method was reliable. The limits of detection (LOD) and limits of quantification (LOQ) for CGA were 7.67 μg/mL and 23.26 μg/mL, respectively (Table 2.1). Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions and expressed as the % relative standard deviation (%RSD) of each analyte concentration. Our analyses were associated with very low levels of relative standard deviation (RSD; intraday: >2.57%; interday: >2.60), indicating that our CGA measurements were highly precise (Table 2.2). The accuracy by mean recovery (%) for CGA ranged from 98.56-103.07% (Table 2.2), thus it can be accurately determined by this method from any small changes in the standard sample concentration (Lim et al., 2011).

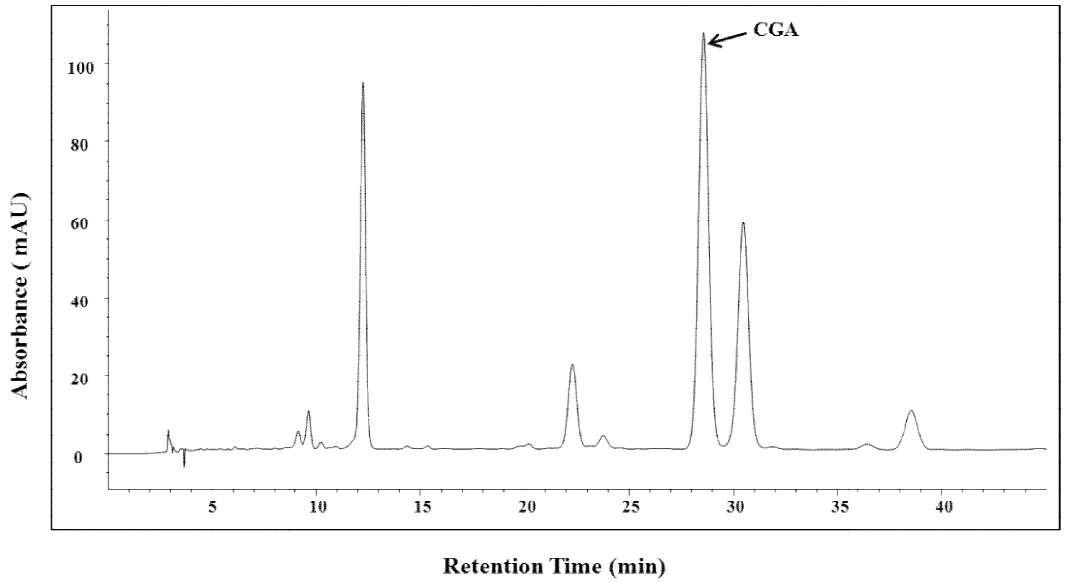


Figure 2.10 High-performance liquid chromatography analysis of coffee extract (prepared in 70% EtOH); ultraviolet detection was performed at 330 nm. The arrow indicates the peak corresponding to CGA.

Table 2.1 Calibration curve, correlation coefficients, limits of detection (LOD), and limits of quantification (LOQ) of CGA.

Standard sample	**Range (µg/mL)**	**slope**	**intercept**	**R^2**	**LOD[α] (µg/mL)**	**LOQ[α] (µg/mL)**
CGA	31.25-1000	24.226	167.56	1.0	7.67	23.26

[α] n=3 replicates

Table 2.2 Intra and interday precision and accuracy for the determination of CGA

Standard sample	Concentration (µg/mL)	Intraday (n=3)			Interday (n=3)		
		Found concentration (µg/mL) ± SD	Recovery (%)	% RSD[α]	Found concentration (µg/mL) ± SD	Recovery (%)	% RSD[α]
CGA	61.25	64.42 ± 0.94	103.07	1.46	61.60 ± 1.60	98.56	2.60
	125	126.88 ± 3.06	101.50	2.41	125.28 ± 0.45	100.22	0.36
	250	253.45 ± 6.51	101.38	2.57	246.47 ± 0.40	98.59	0.16

[α] RSD refers to the relative standard deviation

Effects of CE on optic nerve crush-induced retinal damage in mouse

In addition to investigating the effects of pure CGA on retinal degeneration, we also explored whether CE, a major source of CGA, has the similar benefits. As we observed in the CGA activity, lower levels of RGC death were found in control tissues (Figure 2.11, a and b) than in those that were crushed but not treated with exogenous compounds (Figure 2.11, c and d). In tissues treated with 50-mg/kg of CE, however, cell death was markedly reduced (Figure 2.11, i and j). The protective effects of CE in a dose-dependent manner (Figure 2.11, e-j). Likewise, while the 50-mg/kg CE treatment was associated with a preservation of Thy-1 expression, the 10- and 30-mg/kg CE treatments were less effective than the group of 50-mg/kg CE treatment (Figure 2.12).

Based on the HPLC chromatogram of CE, we found 14.7 mg/g of CGA in CE. Although the amount of CGA in CE is less than the reported amount, the group of CE treatment shows similar protective effects with the CGA treatments (Figure 2.10 and 2.11) (Farah et al., 2006). Since significant amounts of CGA are metabolized in the human intestine and some phenolic metabolites of coffee are known to possess high antioxidant activity (Gomez-Ruiz et al., 2007), we expect that the additional bioactivities are originated from those metabolites. Therefore, the research on the protective effects of CGA and coffee metabolites on retina is being conducted.

In conclusion, this study shows that CGA and coffee extract are responsible for reduction of the RGC apoptosis induced by hypoxia and NO. Therefore, coffee consumption may provide additional health benefits by preventing retinal degeneration.

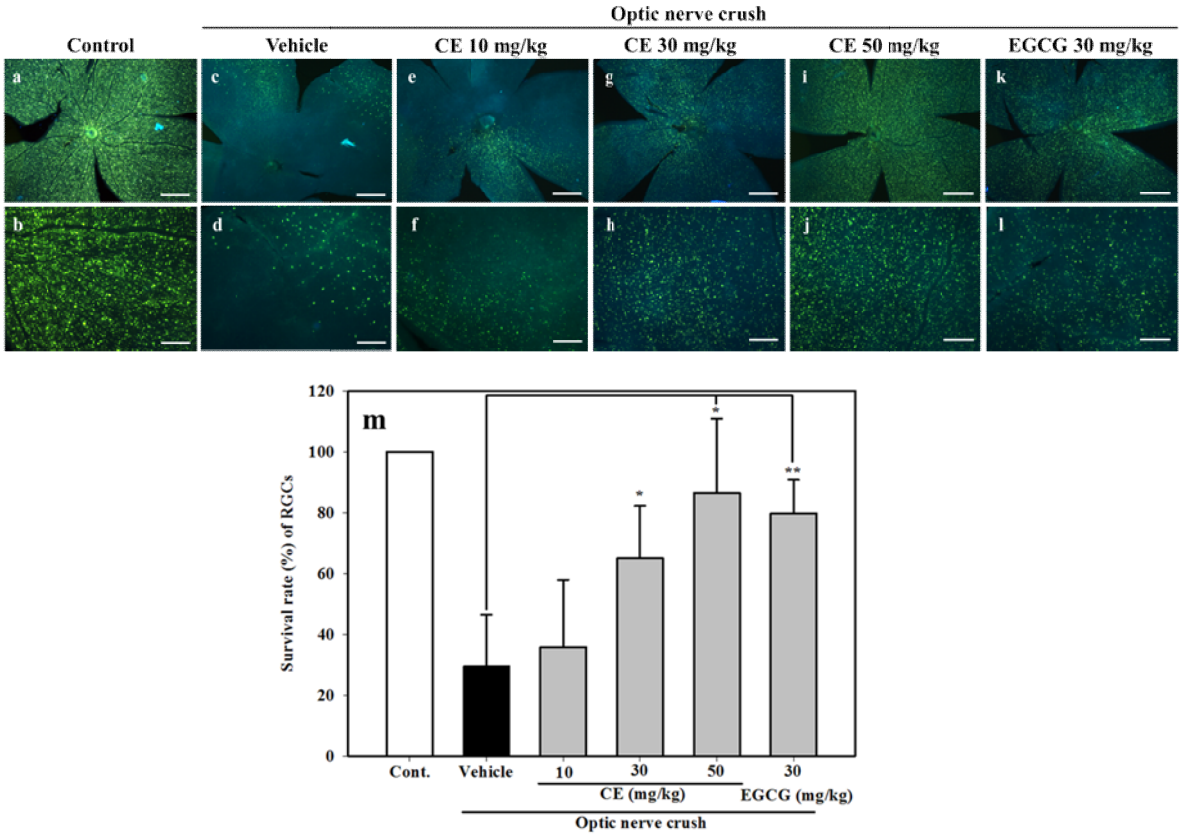


Figure 2.11 ONC experiments on the impacts of CE treatment on retinal tissues of mice. (A) Representative micrographs of Fluorogold-labeled tissues from (a) a control, (c) a mouse that underwent ONC, (d) a mouse that underwent ONC and was treated with 10 mg/kg of CE, (g) a mouse that underwent ONC and was treated with 30 mg/kg of CE, (i) a mouse that underwent ONC and was treated with 50 mg/kg of CE, and (k) a mouse that underwent ONC and was treated with 30 mg/kg EGCG. Images in the lower row are 100 × magnifications of the images in the row above them; scale bars in the upper row represent 500 μm, while scale bars in the lower row represent 100 μm. (m) Quantitative analysis of the survival (%) rate of RGCs 14 days after ONC. Experimental values are expressed as mean SEM; Single asterisks (*) indicate $p < 0.05$; double asterisks (**) indicate $p < 0.01$.

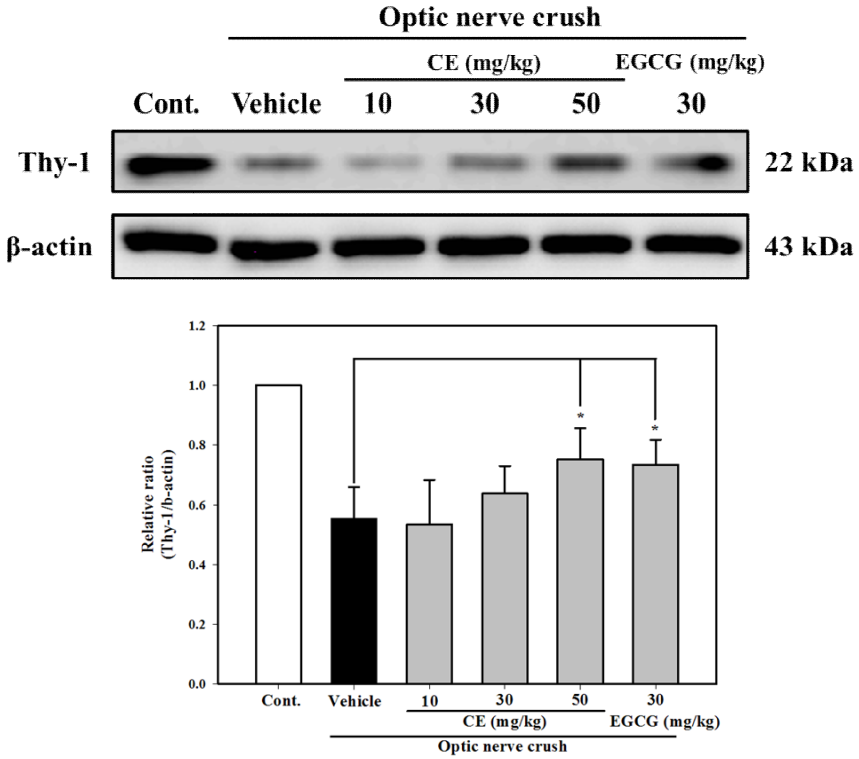


Figure 2.12 Results of western blot analysis investigating Thy-1 expression in RGCs 7 days after the ONC procedure. Densitometric analysis of Thy-1 levels, and data are shown as mean SEM; Single asterisks (*) indicate $p < 0.05$.

2.9 EFFECTS OF PHENOLIC ACID METABOLITES FORMED AFTER COFFEE CONSUMPTION ON RETINAL DEGENERATION *IN VIVO*

2.9.1 Introduction

Coffee is one of the most consumed beverages worldwide, and laboratory studies have shown that coffee and its phenolic constituents have a variety of physiological actions, including beneficial effects on blood circulation and in patients with diabetes, cancer, and Alzheimer's disease (Higdon and Frei, 2006). Coffee contains relatively large amounts of phenolic compounds such as CGA, which *in vitro* and *in vivo* studies show might be responsible for some of the health benefits of coffee (Kempf et al., 2010; Sato et al., 2011; Somoza et al., 2003). Daily intake of CGA as a result of coffee consumption is in the range of approximately 0.5–1.0 g (Thom, 2007). CGA is an ester of CA and quinic acid that has been investigated for its antibacterial, DNA protective, anticancer, and anti-inflammatory effects, which might be explained by its anti-oxidative activity (dos Santos et al., 2006; Lou et al., 2011c; Shibata et al., 1999; Weng and Yen, 2012). In addition, several studies have reported that CGA has neuroprotective effects (Kim et al., 2012; Kwon et al., 2010b; Nakajima et al., 2007). In a previous study, we showed that oral administration of coffee extract and CGA protects against retinal degeneration *in vivo* (Jang et al., 2013).

Phenolic phytochemicals derived from coffee are transformed by digestive enzymes and microbiota in the colon (Gonthier et al., 2003; Monteiro et al., 2007), and phenolic phytochemicals from coffee have shown good bioaccessibility and bioavailability in experimental models (Ferruzzi, 2010; Saura-Calixto et al., 2007). After hydrolysis of the ester bond and further metabolism, phenolic phytochemicals from coffee are absorbed mostly in the

colon, but also in the small intestine (Williamson et al., 2011). Recently, specific metabolites were detected in human plasma after oral ingestion of coffee, including CGA, CA, DHCA, dihydroferulic acid (DHFA), ferulic acid (FA), and isoferulic acid (IFA) (Renouf et al., 2014). However, transportation of coffee metabolites to the eye and their local effects have not been studied.

The retina contains RGCs, photosensitive cells known as rods and cones, and other nerve cells, and receives visual information that it sends to the superior colliculus of the brain through the optic nerve. The retina is subject to high levels of cumulative irradiation because of the abundance of photosensitizers (Beatty et al., 2000), and it requires a constant and abundant oxygen supply from the blood to support its high metabolic rate (Wanek et al., 2013). Therefore, the retina is at a relatively increased risk of damage induced by oxidative stressors such as reactive oxygen species (ROS) and nitric oxide species, in comparison with other parts of the body. Excessive oxidative stress is thought to be a key contributing factor in the pathophysiology of retinal disorders such as age-related macular degeneration, glaucoma, and retinopathy (Beatty et al., 2000; Gurler et al., 2000; Tezel, 2006). Clinical studies have shown that the progression of retinal diseases can be slowed by dietary treatment with antioxidant compounds such as vitamins, carotenoids, zinc, and phenolic phytochemicals (Barker et al., 2011; Moustafa, 2004; Rhone and Basu, 2008; Seddon et al., 1994; Snodderly, 1995). It is now generally believed that hypoxia, in which a part of the body is deprived of adequate oxygen, plays an important role in the pathogenesis of retinal degeneration, which induces overproduction of nitric oxide (NO) in blood vessels and generates reactive nitrogen species such as peroxynitrite (El-Asrar et al., 2001).

Traumatic optic nerve injury causes axonal degeneration and loss of RGCs by inflammatory and oxidative stress in an *in vivo* ONC model (Levkovitch–Verbin et al., 2000).

Although humans are exposed to high amount of phenolic phytochemicals due to coffee intake, and such compounds show promising neuroprotective effects that are mediated by anti-oxidative activity, the effects of coffee metabolites against oxidative stress in the context of retinal degeneration have not been studied. Thus, the purpose of this study was to determine which coffee metabolites reach the retina after coffee ingestion, and which metabolites most effectively protect the retina from hypoxia-induced degeneration.

2.9.2 Materials and methods

Detection of coffee metabolites in the eye using UPLC MS/MS analysis

Thirty-three rats were used for this experiment. The rats were fasted overnight during the study. Each rat was fed a suspension of CGA (500 mg/kg) in 0.3 mL of phosphate buffered saline (PBS) once per day for 2 days. On the second day, rats were sacrificed 1 hour after CGA administration and the eyes were enucleated. Each eye was weighed and homogenized with liquid nitrogen using an autoclaved mortar and pestle. Tissues were thoroughly collected with 1.5 mL of acetonitrile with 1% formic acid. After centrifugation at 6000 *g* for 5 min at 4 °C, the supernatant was evaporated in a SpeedVac system (Thermo Savant SPD 1010, Holbrook, NY, USA). The residue was then reconstituted with 150 µL of 50% methanol containing 0.1% formic acid. After sample preparation, aliquots (2 µL) of tissue samples were injected directly onto an Acquity BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters Corporation, Milford, MA, USA) at 40 °C and chromatographic separation was achieved using a mobile phase consisting of 0.1% formic acid

(solvent A) and 95% acetonitrile/0.1% formic acid (solvent B). The gradient program was 5–40% B over 5.5 min at a flow rate of 0.3 mL/min, and negative ion electrospray tandem mass spectra were acquired using an AB Sciex QTrap 6500 coupled with an Eksigent UltraLC-100XL pump and Analyst (version 2.1) operating software (AB Sciex, Concord, ON, Canada). The electrospray voltage was set to 4000 V and the probe temperature was set to 600 °C. Nitrogen was used as the collision gas, and the nebulizer, curtain, and collision gases were used. The selective monitoring parameters for CGA and coffee metabolites are listed in Table 2.3. Dwell times were set to 30 ms for each transition.

Vitamin C equivalent antioxidant capacity (VCEAC) assay using ABTS and DPPH radicals

A modified ABTS assay was used to measure *in vitro* antioxidant activity (Kim et al., 2002). Briefly, 1.0 mM AAPH was mixed with 2.5 mM ABTS as a diammonium salt in PBS solution. The mixture was heated in a water bath at 68 °C for 15 min. The concentration of the ABTS solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm. Twenty µL of coffee metabolite solution was added to 980 µL of the adjusted ABTS solution. The decrease in absorbance was measured after 10 min using a spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). For a control solution, 50% ethanol was used. DPPH radical scavenging was also used to evaluate the antioxidant capacity of the coffee metabolites (Shang et al., 2014). A working DPPH solution was prepared in ethanol (0.15 mg/mL). A serial dilution of coffee metabolites solution (100 µL) was added to 100 µL of DPPH working solution. After incubation in the dark at room temperature for 30 min, the absorbance at 515 nm was measured with a spectrophotometer. The decrease in sample absorbance was calculated by comparing the measured value to that of the 50% ethanol blank sample. The median inhibitory concentrations

(IC_{50}) of the metabolites for scavenging ABTS and DPPH radicals were determined by measuring the effects a series of concentrations of coffee metabolites. Ascorbic acid was used as a positive control. The antioxidant activities of the coffee metabolites were expressed as VCEAC.

Table 2.3 The MS parameters of target compounds[a)].

Compounds	Transition (m/z)	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
CGA	353 → 191, 85	-160, -45	-10, -10	-22, -56	-17, -19
CA	179 → 135, 134	-120, -35	-10, -10	-22, -34	-11, -15
DHCA	181 → 137, 109	-150, -35	-10, -10	-16, -22	-11, -13
DHFA	195 → 136, 121	-45, -120	-10, -10	-22, -34	-13, -13
FA	193 → 134, 178	-15, -15	-10, -10	-24, -18	-15, -15
IFA	193 → 178, 134	-55, -55	-10, -10	-16, -22	-15, -11

a) DP declustering potential (Volt); EP, entrance potential (Volt); CE (Volt); CXP, collision cell exit potential (Volt).

RGC-5 cell culture

The transformed neuronal precursor cells (RGC-5) were grown in normal growth medium (Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin/streptomycin in a humidified incubator with a 5% CO_2 atmosphere at 37 °C.

To induce hypoxic stress, RGC-5 cultures were treated with 100 µM *S*-nitroso-*N*-acetyl-penicillamine (SNAP), a NO donor, and transferred into a closed hypoxic chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) with an atmosphere of 1% O_2, 5.13% CO_2, and 93.87% N_2. The chamber was maintained at 37 °C. After 24 h, RGC-5 cells in 96-well plates were subjected to the MTT cell viability assay and microscopic analysis, and cells in 60-mm^2 dishes were subjected to western blot analysis.

Cell viability

Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After 1 h of pretreatment with coffee metabolite samples dissolved in DMSO, RGC-5 cultures in 96-well plates were subjected to SNAP treatment and hypoxic stress for 24 h. The medium was removed and MTT solution (0.5 mg/mL) was added for 1 h at 37 °C. DMSO solution was added to each well and the plate was shaken for 10 min. The number of living RGC-5 cells in each well was determined by measuring the optical density (570 nm test wavelength and 690 nm reference wavelength) using a spectrophotometer (BioTek Instruments, Winooski, VT, USA). The NO scavenger CPTIO was used as a positive control treatment. Cell viability was expressed relative to the control value.

Microscopic analysis of cell viability by Hoechst 33342/PI double staining

Apoptotic and necrotic cells were analyzed using the Hoechst 33342/ PI double staining method (Jung et al., 2010). The cells were stained with 8 μM of Hoechst 33342 and 1.5 μM of PI for 30 min at 37 °C. After being washed twice with serum free media, the stained cells were imaged with a fluorescence microscope (Olympus, Tokyo, Japan).

Protein extraction and western blot analysis

Cells in 60-mm^2 dishes were washed with warm PBS and scraped with a cell scraper using cell lysis buffer. The collected cells were gently mixed 3 times with a vortexer and incubated on ice for 10 min between each mixing. The extracted cells were centrifuged at 14,000 × *g* for 30 min at 4 °C. The supernatant was collected and the total protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was performed with the following primary antibodies: anti-cleaved caspase-3, anti-Bad, anti-Bcl-X_L, anti-phospho JNK, anti-phospho ERK, anti-PARP, (1:3000 dilution, Cell Signaling Technology, Beverly, MA, USA), and β-actin (1:5000 dilution, Santa Cruz Biotechnology, CA, USA). Secondary antibodies were purchased from Santa Cruz Biotechnology Co. Ltd (1:3000 dilution, Santa Cruz Biotechnology, CA, USA). Immunoreactive bands were detected using enhanced chemiluminescence reagents (Amersham Bioscience, GE Healthcare, UK) and measured via densitometry using a LAS-4000 image reader and Multi Gauge 3.1 software (Fuji Photo Film, Japan).

Optic nerve crush

The ONC-induced retinal damage procedure was slightly modified from that used in our previous report (Jang et al., 2013). Five-week-old ICR mice with a body weight of 30–35 g were anaesthetized by intraperitoneal injection with a mixture of Zoletil (1.6 μg/g, Virbac Laboratories, Carros, France) and Rompun (0.05 μL/g, Bayer, Kiel, Germany). The canthus and the tissue around the optic nerve were dissected using surgical scissors and forceps. The exposed optic nerve was crushed about 2 mm posterior to the eye for 1 sec with a calibrated cross-action forceps under direct visualization. The skin was closed and antibiotic ointment was applied to the wound. Intravitreal injections were performed 1 day before and 1 hour after the surgery. Randomly selected mice were undergone a sham procedure as a control group. Samples were dissolved in PBS and 2 μL of the solution was injected using a 33-gauge needle attached to a Hamilton syringe (Hamilton, Reno, NV, USA). Seven days after the surgery, the animals were sacrificed and the eyes were enucleated and fixed for flat-mounting of the retina.

RGC labeling and retinal flat mount preparation

Two days prior to the scheduled ONC surgery, each mouse was anesthetized by intraperitoneal administration of a mixture of Zoletil and Rompun. The fur on the head was shaved and the mice were placed in a stereotactic apparatus. After disinfection of the operation area with povidone-iodine solution followed by 70% alcohol, the skin of the head was cut to expose coronal, sagittal, and transverse sutures. Two small holes (approximately 2 mm in diameter) were drilled 0.5 mm from the sagittal and transverse sutures using a dentist's drill on both sides of the sagittal suture. The superior colliculus was carefully exposed using forceps and a vacuum pump. A thin layer of cotton soaked in a 5% solution of Fluorogold^{TM} (Invitrogen, NY, USA) was placed on the surface of the superior colliculus. The holes on the skull were filled with cotton and the skin

wound was closed with suture clips. Nine days after the application of Fluorogold^{TM}, mice were sacrificed and the eyes were immediately enucleated and transferred to 4% paraformaldehyde for 10 min. After each eye was fixed, it was transferred to cold PBS for dissection of the retina. Four radial cuts were made on the removed retina, after which it was spread on a glass slide with mounting solution. For each flat-mounted retina, 1 medial image was captured per quadrant using a 40× objective. The densities of Fluorogold^{TM}-positive RGCs were determined using a fluorescence microscope, and the average number of living RGCs was calculated for each retina.

Statistical analysis

The data in this study are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made using one-way ANOVA followed by a Dunnett's test. Statistical analyses were conducted using SigmaPlot (Systat Software, Inc., San Jose, CA, USA). Significance was defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.9.3 Results

Identification of metabolites in the rat eye

In order to identify coffee metabolites in the rat eye after oral treatment (once per day for 2 days) with CGA, a UPLC MS/MS selected reaction monitoring (SRM) method was developed. Standard compounds were used for the identification of each metabolite. As shown in Figure 2.13, 5 metabolites of coffee were detected in the rat eye, and the SRM peaks at 2.84 min, 3.07 min, 3.17 min, 4.24 min, and 4.39 min were identified as CGA, DHCA, CA, DHFA, and FA, respectively. A kinetic study was not performed.

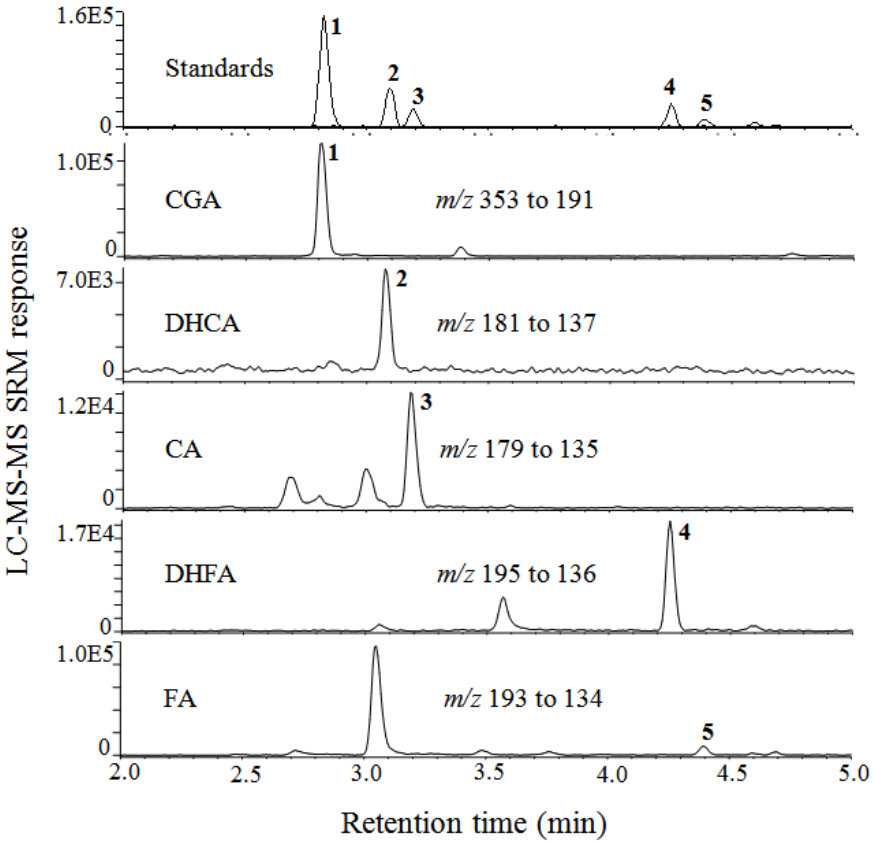


Figure 2.13 Representative UPLC-MS-MS SRM chromatograms showing the detection of chlorogenic acid (CGA), dihydrocaffeic acid (DHCA), caffeic acid (CA), dihydroferulic acid (DHFA), and ferulic acid (FA) in the rat eye after oral treatment with CGA.

Radical scavenging activity of coffee metabolites

To determine which coffee metabolites have antioxidative activity, radical scavenging activities were evaluated using the ABTS and DPPH methods. The value for antioxidative activity was expressed as the median effective dose and VCEAC. As shown in Table 3.2, the relative VCEAC value in the ABTS assay were as follows: DHCA (2.85 ± 0.12) > DHFA (1.06 ± 0.04) > CA (1 ± 0.05) > CGA (0.71 ± 0.03). Similar to the ABTS assay results, DHCA showed the highest scavenging activity value (1.53 ± 0.21) for DPPH radicals among the metabolites, but CA (1.03 ± 0.07) had better DPPH radical scavenging activity than DHFA (0.64 ± 0.07). These results suggested that DHCA has stronger scavenging capacity than the other coffee metabolites.

Table 2.4 Determination of coffee metabolites antioxidant activity evaluated by ABTS and DPPH radical scavenging assay.

Sample	ABTS		DPPH	
	Median effective dose (μg/mL)	**Relative VCEAC**	**Median effective dose (μg/mL)**	**Relative VCEAC**
CGA	328.33 ± 1.07	0.71 ± 0.03	328.33 ± 1.07	0.54 ± 0.04
CA	234.69 ± 0.77	1 ± 0.05	281.25 ± 5.51	1.03 ± 0.07
DHCA	81.86 ± 0.17	2.85 ± 0.12	192.86 ± 16.54	1.53 ± 0.21
DHFA	220.64 ± 1.32	1.06 ± 0.04	454.38 ± 27.99	0.64 ± 0.07
L-ascorbic acid	233.54 ± 9.89	1	287.60 ± 14.34	1

Effects of coffee metabolites on cell viability decreased by SNAP and hypoxia

Under hypoxia and SNAP-induced stress, the cell viability of RGC-5 cells was reduced by 43.3% (Figure 2.14). However, phenolic metabolites derived from coffee such as CGA, CA, and DHCA significantly protected RGC-5 cells from death caused by NO and hypoxic stress in a concentration-dependent manner. Pretreatment with 50 and 100 μM CGA increased cell viability by 55.8% ($p < 0.05$) and 84.5% ($p < 0.001$), respectively, in comparison with the control treatment. Pretreatment with 10, 50, and 100 μM CA increased RGC-5 viability by 56.7% ($p < 0.05$), 74.1% ($p < 0.001$), and 98.0% ($p < 0.001$), respectively, in comparison with the control treatment. Moreover, cell viability was increased by 72.2% ($p < 0.001$) and 74.2% ($p < 0.001$) by pretreatment with 50 and 100 μM DHCA, respectively, in comparison with the control group. The positive control treatment CPTIO (100 μM), a NO scavenger, significantly reduced the negative effect of SNAP and hypoxia ($p < 0.01$). To confirm the protective effects of coffee metabolites, Hoechst 33342/PI double staining was performed. As illustrated in the fluorescence microscopic histogram (Figure 2.15), apoptotic and necrotic cells were observed in the SNAP-treated and hypoxia induced cultures. However, pretreatment with CGA, CA, and DHCA dose-dependently decreased cell death. Similar to the results obtained with the MTT assay, treatment of RGC-5 cells with DHFA did not protect them against NO and hypoxia stress, (Figure 2.14).

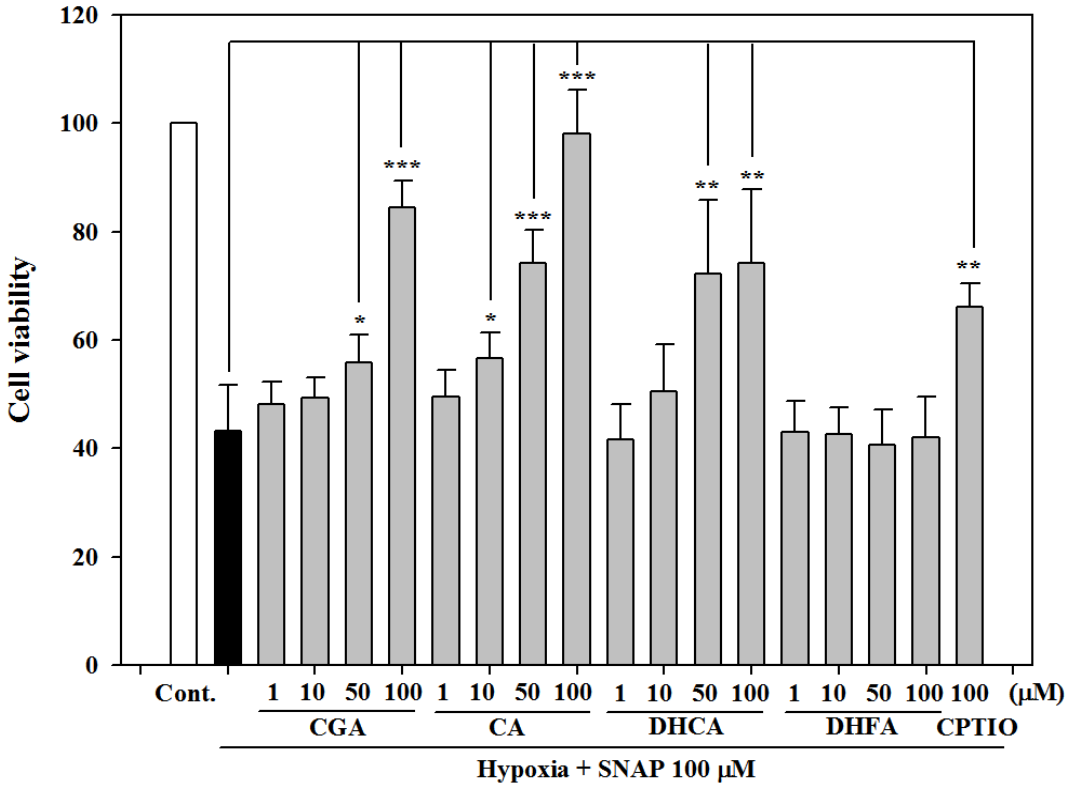


Figure 2.14 Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay measuring the effects of *S*-nitroso-*N*-acetyl-penicillamine (SNAP) on the viability of retinal ganglion (RGC-5) cells cultured under hypoxic conditions for 24 h. The nitric oxide (NO) scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) was used as a positive control. Values are presented as means (n = 3 independent experiments per treatment); error bars indicate SEM. Single asterisks (*) indicate $p < 0.05$; double asterisks (**) indicate $p < 0.01$; triple asterisks (***) indicate $p < 0.001$.

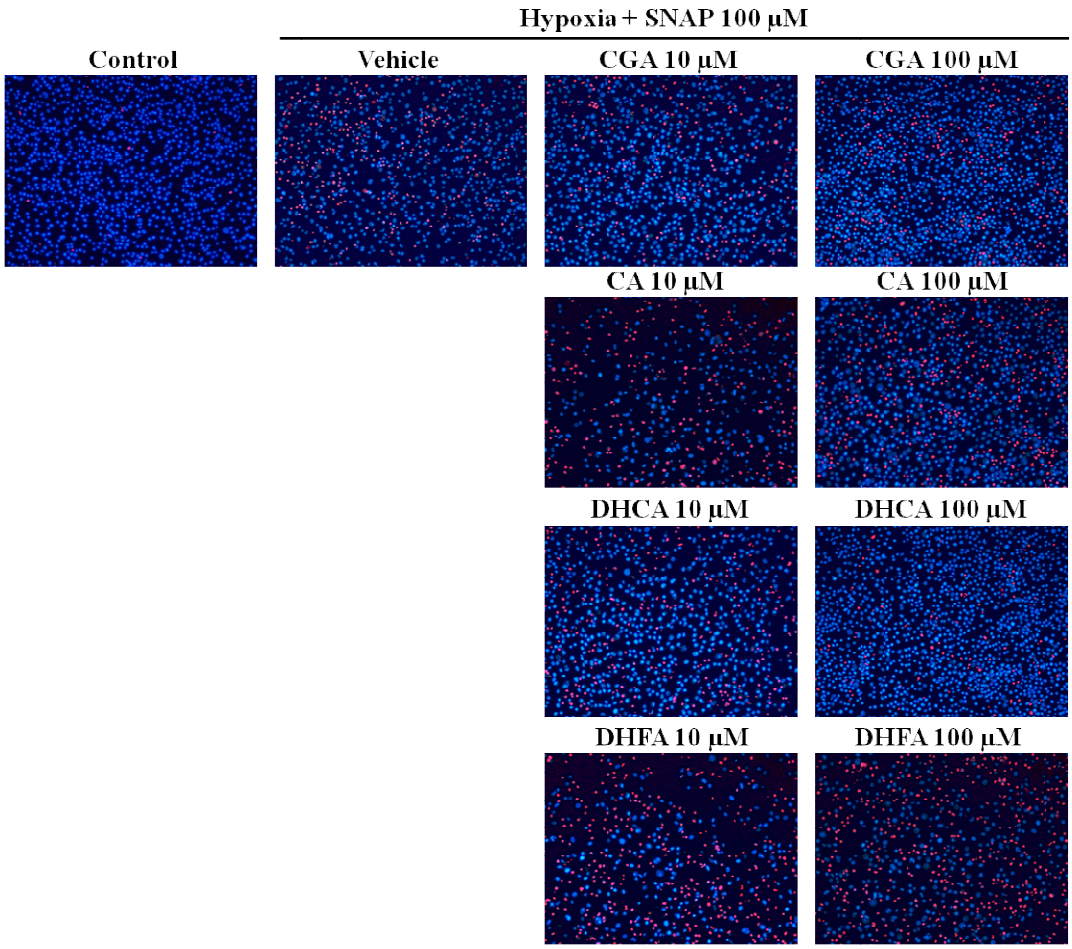


Figure 2.15 Representative fluorescence microscopy of PI (red) and Hoechst 33342 (blue) staining. (a) Control RGC-5 cells under normoxic conditions. (b) RGC-5 cells treated with 100 µM SNAP under hypoxic conditions. (c–d) RGC-5 cells pre-treated with CGA (10 and 100 µM concentrations) prior to being exposed to 100 µM SNAP under hypoxic conditions. (e-f) RGC-5 cells pre-treated with CA (10 and 100 µM concentrations). Scale bar = 50 µm.

Effects of coffee metabolites on Bad, Bcl-X_L, PARP, cleaved caspase 3, phosphorylated ERK, and JNK

To understand the intracellular mechanism of action of coffee metabolites, western blot analysis was performed to evaluate the effect of selected metabolites on apoptosis-related proteins (Figure 2.16 and 2.17). Hypoxia and SNAP treatment increased expression of Bad and cleaved caspase 3 protein, which are markers of cells undergoing apoptosis, and reduced expression of Bcl-XL protein, which have anti-apoptotic functions. Immunoblot analysis showed that CGA, CA, and DHCA treatment dose-dependently down-regulated expression of Bad and cleaved caspase 3. Moreover, CGA, CA, and DHCA significantly down-regulated expression of Bcl-XL.

Because activation of JNK and down-regulation of ERK expression could be intracellular mediators of the effects of oxidative stressors such as NO, peroxynitrite, and hypoxia, we monitored the effects of coffee metabolites on hypoxia-induced activation of ERK and JNK (Figure 2.16 and 2.17). JNK protein is activated by phosphorylation under stressful conditions. We found that JNK phosphorylation was down-regulated by pretreatment with CGA, CA, and DHCA. Previous reports show that activation of ERK signaling can protect retinal ganglion cells from degeneration (Johnson et al., 2009; Kilic et al., 2005). We found that ERK phosphorylation was increased after treatment with coffee metabolites (Figure 2.16 and 2.17). Therefore, our results confirm that specific coffee metabolites protect RGC-5 cells by activating pro-survival signaling pathways.

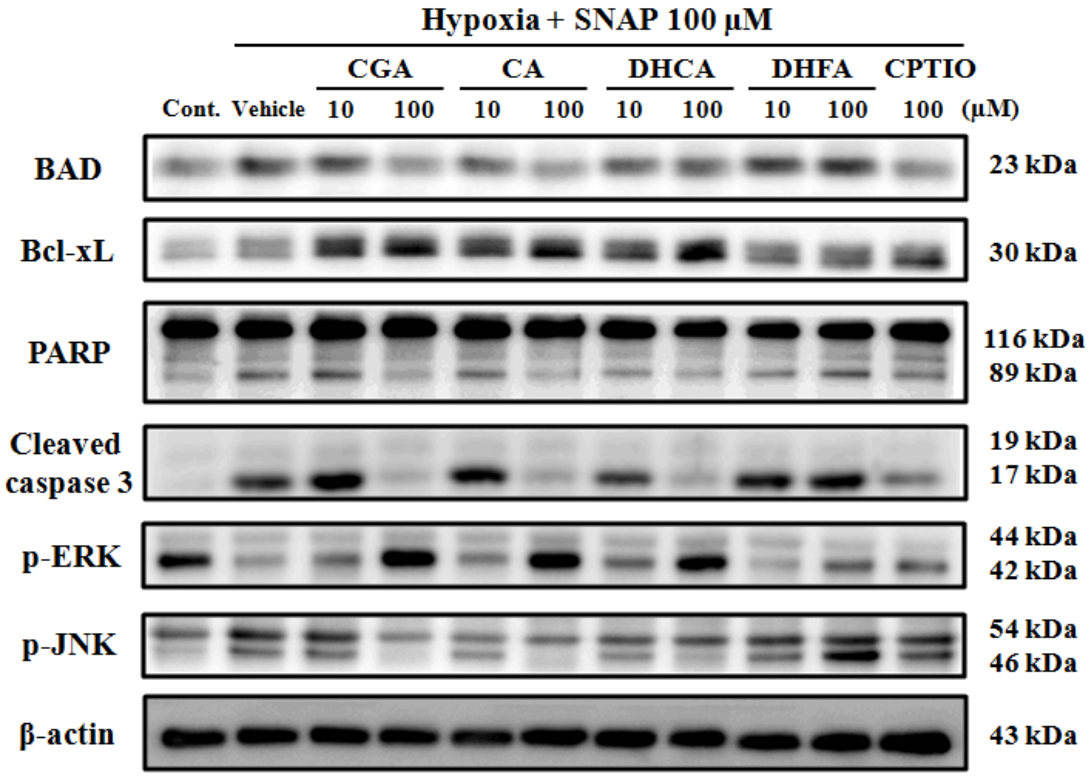


Figure 2.16 Results of a western blot investigating the expression of apoptotic proteins (Bad, Bcl-X_L, PARP, cleaved caspase 3, p-ERK, and p-JNK) in RGC-5 cells subjected to 100 μM SNAP for 24 h under hypoxic conditions. Pretreatment with CGA, CA, and DHCA appeared to have anti-apoptotic effects on hypoxia and NO-induced down-regulation of BAD, PARP, cleaved caspase-3 and p-JNK expression and up-regulation of Bcl-X_L and p-ERK expression.

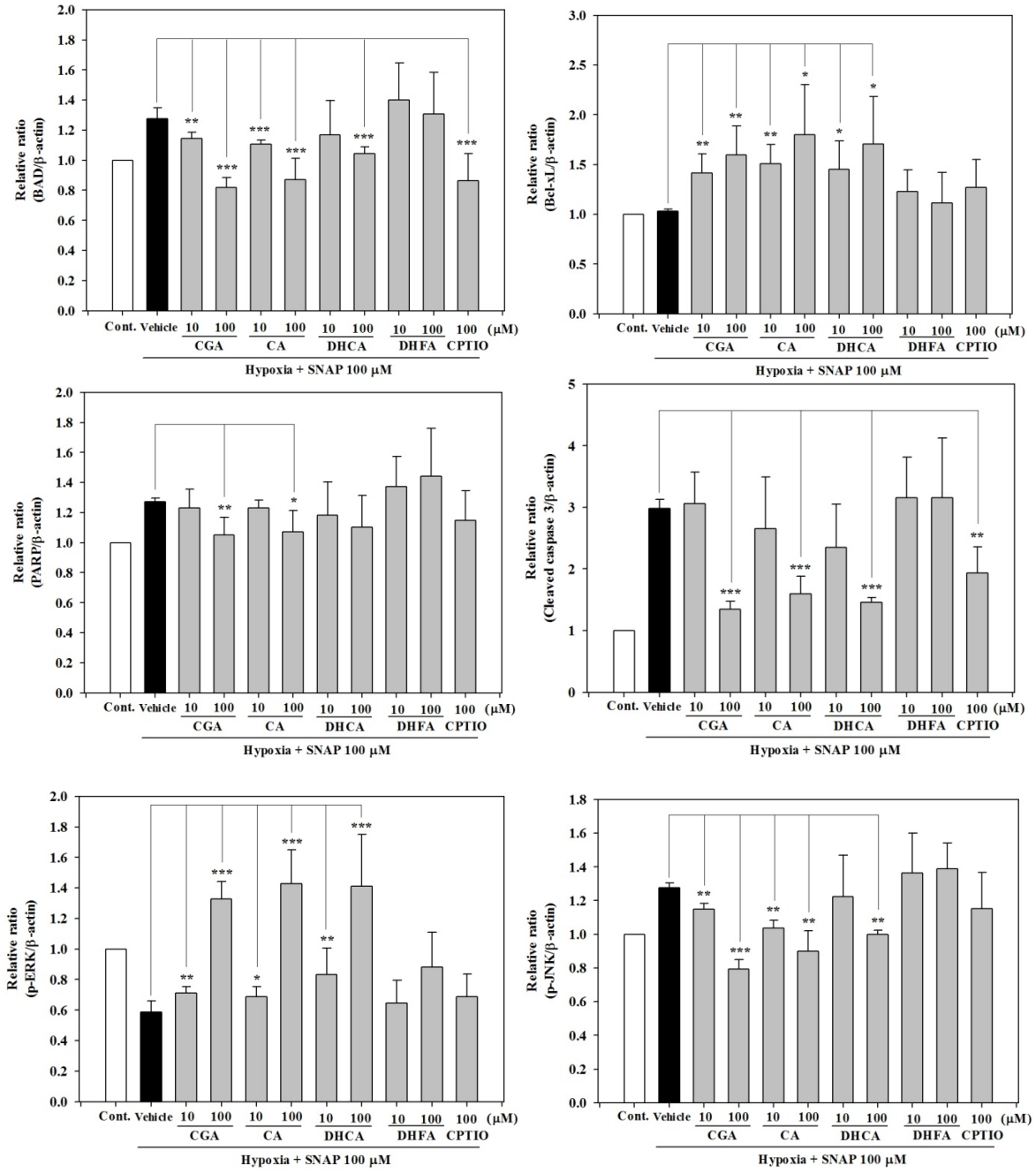


Figure 2.17 Results of western blot analysis investigating the expression of apoptotic proteins in RGC-5 cells subjected to 100 μM SNAP for 24 h under hypoxic conditions. Densitometric analysis of BAD, Bcl-X_L, PARP, cleaved caspase-3, p-ERK, and p-JNK expression levels, and data are shown as mean SEM; Single asterisks (*) indicate $p < 0.05$; double asterisks (**) indicate $p < 0.01$; triple asterisks (***) indicate $p < 0.001$.

Effects of CGA on ONC-induced retinal damage in mice

To evaluate cell survival, Fluorogold^{TM} was applied to the surface of the superior colliculus 10 days before the animals were sacrificed. Representative sections from the evaluation of RGC survival are shown in Figure 2.18. In eyes subjected to a sham crush procedure, the density of Fluorogold^{TM}-labeled RGCs was 1893.8 ± 325.9 RGCs/mm^2 (mean ± SD, n = 8). The density of RGCs subjected to ONC was 724.3 ± 149.6 RGCs/mm^2 (n = 8), which was a 38.2% decrease in density in comparison to RGCs subjected to the sham procedure (Figure 2.19). However, the survival rates of RGCs in the retina that had been subjected to ONC and treated with 0.1, 1, and 10 μM CGA were higher by 51.4%, 60.6%, and 67.8%, respectively, than the ONC procedure group. The survival rates of RGCs treated with 0.1, 1, and 10 μM CA were higher by 55.4%, 56.5%, and 59.6%, respectively, and the survival rates of RGCs treated with 0.1, 1, and 10 μM DHCA were higher by 51.7, 64.6, and 68.6%, respectively, than the ONC procedure group. In all cases, the concentrations of coffee metabolites used were based on the concentrations measured in human plasma after oral consumption of coffee and the blood flow rate in the human eye (Garcia Jr et al., 2002; Renouf et al., 2014).

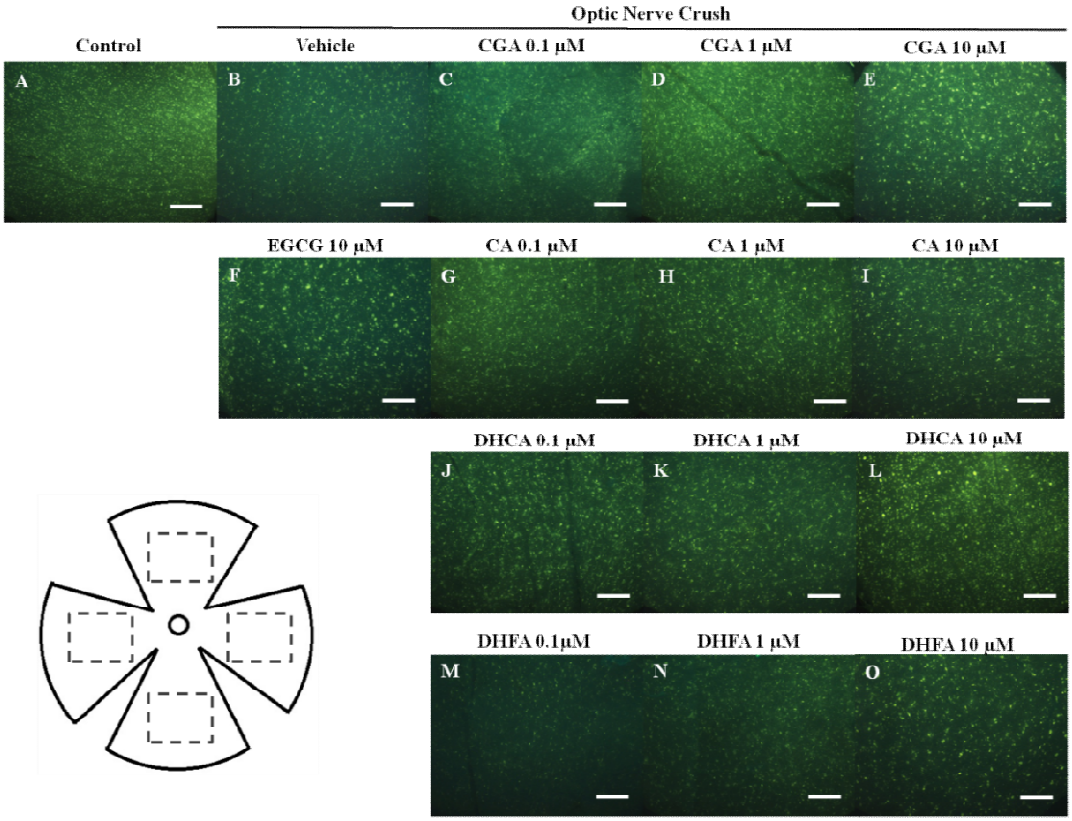


Figure 2.18 Results of *in vivo* optic nerve crush (ONC) experiments investigating the impacts of coffee metabolite treatment on retinal tissues of mice. Representative micrographs of Fluorogold^{TM}-labeled tissues from (a) a control mouse, (b) a mouse that underwent ONC, (c-e) a mouse that underwent ONC and was treated with 0.1, 1, and 10 µM of CGA, respectively, (g-i), a mouse that underwent ONC and was treated with 0.1, 1, and 10 µM of CA, respectively, (j-l) a mouse that underwent ONC and was treated with 0.1, 1, and 10 µM of DHCA, respectively, and (m-o) a mouse that underwent ONC and was treated with 0.1, 1, and 10 µM of DHFA, respectively. (f) a mouse that underwent ONC and was treated with 10 µM of EGCG as a positive control. Images were taken at 400× magnification and the scale bar represents 100 µm.

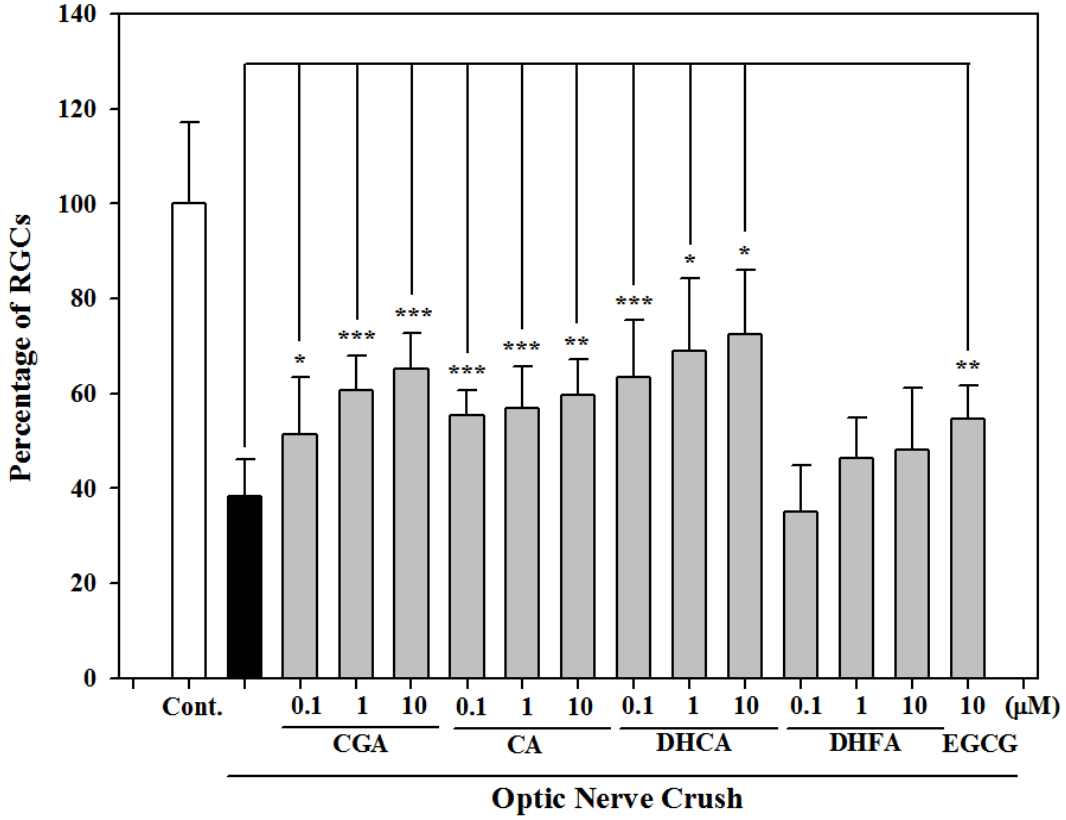


Figure 2.19 Quantification of the number of living RGCs by labeling with Fluorogold^{TM}. Data were expressed as mean ± SEM. Single asterisks (*) indicate $p < 0.05$; double asterisks (**) indicate $p < 0.01$; triple asterisks (***) indicate $p < 0.001$.

2.9.4 Discussion

RGCs are the last neurons to receive visual information from photoreceptors via other neurons such as horizontal and amacrine cells, and the loss of RGCs can occur in many ocular and systemic diseases such as glaucoma, diabetic retinopathy, and inflammatory disorders (Barber et al., 1998; Quigley et al., 1995; Sivakumar et al., 2011). In particular, glaucoma has been characterized as the progressive death of RGCs leading to optic nerve degeneration, and glaucoma eventually results in permanent visual loss (Harwerth and Quigley, 2006; Kuehn et al., 2005). Several investigations have focused on protecting RGCs with neurotrophins such as ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and inhibitors of glutamatergic excitotoxicity such as memantine (Zhang et al., 2012). Recently, it has been reported that natural products such as *Ginkgo biloba* exhibited a protective effect on RGCs (Ma et al., 2008; Ma et al., 2010).

Coffee is one of the most consumed beverages in the world, and CGA is known to be a major ingredient in coffee, with 20–423 mg contained in a single serving of espresso coffee (Ludwig et al., 2014). A previous study on the overall plasma bioavailability of coffee metabolites showed that a single coffee metabolite absorbed in plasma could be detected at concentrations up to 100 μM for 24 h after consuming 2 g of coffee (Renouf et al., 2014).

In our previous study, we showed that coffee and its major component, CGA, have protective effects against retinal degeneration caused by hypoxia and NO. The primary purpose of this study was to determine which metabolites produced after coffee consumption play a major role in the beneficial effects of coffee on retinal degeneration (Jang et al., 2013).

Because the quantity of CGA in the coffee extract (4%, data not shown) was too low to detect metabolites in the eye of the rat with certainty, and because the high dose of coffee extract necessary to provide a suitable amount of CGA could be toxic to the rat, oral administration of CGA was used to determine the delivery of metabolites to the eye. CA, DHCA, DHFA, FA, and IFA, all of which are major metabolites in human plasma after coffee consumption, are metabolized from CGA by catechol-*O*-methyltransferase, esterase, and reductase (Del Rio et al., 2010; Nardini et al., 2002; Williamson et al., 2011). In our study, however, peaks indicating FA and IFA in the eye tissues were negligible (Figure 2.13).

The development of ophthalmic drugs has been focused on topical treatment and intravitreal injection instead of oral treatment, because of a number of obstacles, including the blood-ocular barrier and bioavailability. The blood-ocular barrier formed by the blood-aqueous barrier and the blood-retinal barrier is considered similar to the blood-brain barrier because of their structural components and functions. In practice, drug delivery through the blood-brain barrier involves 4 factors of Lipinski's "rule of five": molecular weight, H-bond donors, H-bond acceptors, and logP (Pavan et al., 2008). According to this rule, masses above the 400 to 600 Da threshold do not cross the blood-brain barrier (Fischer et al., 1998). However, it is known that peptides and proteins with molecular weight in excess of 600 Da can cross the blood-brain barrier (Banks, 2009). Phenolic phytochemicals with molecular weights over 400 Da, including (-)-epicatechin gallate and (-)-epigallocatechin gallate from green tea extracts and anthocyanins from blackcurrant, have been shown to pass thorough the blood-ocular barrier in *in vivo* studies by oral administration (Chu et al., 2010; Matsumoto et al., 2006; Prior and Wu, 2006). Most coffee

metabolites have molecular weights of less than 200 Da, and our study shows that some of these coffee metabolites can pass the blood-ocular barrier.

Hypoxia is implicated in retinal neovascularization such as diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity, which is the one of most important risk factors for vision loss in the United States (Aiello et al., 1995). Hypoxia can also generate oxidative stress and induce neuronal cell death by mitochondria through energy depletion, altered ionic homeostasis, and O_2- sensing molecules, such as HIF-1α and NF-κB (Banasiak et al., 2000; Halterman et al., 1999).

Oxidative stress generated by free radicals is highly associated with neurodegeneration and inflammation. The neuroprotective and anti-inflammation effects of coffee metabolites have been studied along with their anti-oxidative effects. The protective effect of CGA on RGCs with damage induced by L-buthionine-(*S,R*)-sulfoximine (BSO) and glutamate has been reported (Nakajima et al., 2007). In addition, it has been shown that CA extracted from *E. annuus* leaf protects neuronal cells against H_2O_2 toxicity (Jeong et al., 2011). In our study, free radical scavenging effects were tested using ABTS and DPPH, and it was determined that DHCA was the most effective radical scavenger among the coffee metabolites. Furthermore, DHCA showed the strongest protective effect against hypoxia-induced RGC death as determined by MTT and the PI/Hoechst double staining method, followed by CGA and CA. Our results suggest that consumption of coffee could protect the retina against neurodegeneration, with DHCA as the most effective compound.

Moreover, to clarify the effects of coffee metabolites on hypoxia-induced RGC-5 cell apoptosis, we evaluated changes in levels of apoptosis-related proteins, including Bcl-XL, Bad, and PARP. The Bcl-2 family of proteins is predominantly situated upstream of irreversible cellular damage in the intrinsic apoptosis pathway (Danial and Korsmeyer, 2004). More than 20 members of this family have been identified, including proteins that suppress apoptosis (e.g., Bcl-2 and Bcl-XL) and proteins that promote apoptosis (e.g., Bad and Bax). The ratio between pro-apoptotic and anti-apoptotic protein expression, rather than the level of each protein separately, is an important factor in the regulation of apoptosis. In this study, Figure 2.16 and 2.17 shows that hypoxia-induced elevation of the pro-apoptotic protein (Bad)/anti-apoptotic proteins (Bcl-XL) ratio was significantly reduced by treatment with CGA, CA, and DHCA.

Caspase-3, a member of the caspase family, has been shown to play a key role in apoptosis induced by a variety of stimuli (Lakhani et al., 2006). In addition, activation of caspase-3 leads to the cleavage of PARP, resulting in the accumulation of an 89-kDa cleavage product. Both activation of caspases-3 and cleavage of PARP were blocked by pretreatment with coffee metabolites (Figure 2.16 and 2.17).

We next examined whether coffee metabolites affect upstream molecular signaling pathways because activation of MAPKs plays a fundamental role in cellular survival. The activation of JNK and inactivation of ERK occurred prior to caspase 3 activation. In our study, 1 h of treatment with 10 μM and 100 μM of CGA, CA, and DHCA caused significant down-regulation of phosphorylation of JNK, but up-regulation of phosphorylation of ERK. Furthermore,

treatment with coffee metabolites was more effective on protein expression than the NO scavenger CPTIO (Figure 2.16 and 2.17).

ONC is a well-known model used to model indirect or secondary degeneration of RGCs in glaucoma and other optic neuropathies (Chen and Weber, 2001; Kalesnykas et al., 2012; Schwartz and Yoles, 1999). To maintain stable conditions in the *in vivo* test, intravitreal injections were limited to two, given that more than two intravitreal injections might induce leakage of aqueous humor and additional inflammation. To establish a method for morphometric analysis of retrograde-labeled mouse RGCs, Fluorogold^{TM} fluorescent dye was applied to the superior colliculus. Quantification of the RGC level in retinal tissue is essential to better understand the mechanisms of neuroprotection. Compared to Nissl staining with cresyl violet, which stains amacrine cells and endothelial cells in the RGC layer in addition to RGCs, Fluorogold^{TM} application to the superior colliculus is more specific to the RGC (Chiu et al., 2008).

EGCG is one of the most active major polyphenols of green tea and it is the constituent primarily responsible for the health benefits of drinking green tea. Recent research has shown that EGCG protects the retina against NMDA-induced excitotoxicity and reduces retina ischemia/reperfusion injury by attenuating nitric oxide synthase activity (Chen et al., 2012; Peng et al., 2008). According to our data, the intravitreal treatment of coffee metabolites CGA, CA, and DHCA had a stronger protective effect on RGCs than EGCG at the same dose (Figure 2.19).

The most important potential limitation of our experimental study is the effectiveness and bioavailability of coffee metabolites in clinical situations. To minimize this limitation, metabolite doses were calculated to be similar to concentrations found in human plasma after drinking coffee, and the retinal blood flow rate in the human eye was used (Garcia Jr et al., 2002; Renouf et al., 2014). Our study confirmed that coffee metabolites have neuroprotective effects at concentrations as low as 0.1 µM when injected intravitreally, which is the equivalent of 6 g of coffee orally.

In conclusion, DHCA protects hypoxia-induced RGCs from apoptosis most effectively, followed by CGA and CA. Furthermore, intravitreal injections of coffee metabolites given after ONC in mice were associated with a higher survival rate of RGCs. This may suggest a positive effect of coffee consumption on the protection of RGCs.

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CHAPTER 3

PHYLLANTHUS EMBLICA L. (INDIAN GOOSEBERRY) AND ITS HEALTH BENEFITS

3.1 *PHYLLANTHUS EMBLICA* L. (INDIAN GOOSEBERRY)

Phyllanthus emblica Linn (also known as *Emblica officinalis* Gaertn.) belongs to the family Euphorbiaceae. The plant species, which was originally native to India, is now found growing in Pakistan, Uzbekistan, Sri Lanka, South East Asia, China, and Malaysia and is known as emblic myrobalans or the Malacca tree in English, Amla in Hindi, and Amlaki in Sankrit. However, the plant is commonly called the Indian gooseberry.

The Indian gooseberry is a medium-sized tree that grows up to 8–18 meters in height (Mirunalini et al., 2013). The bark of the Indian gooseberry tree is thick (up to 12 mm), is a light grayish-brown or grayish-green color, and peels off in conchoidal flakes (Scartezzini and Speroni, 2000). The Indian gooseberry tree has an asymmetrical shape with 10–20-cm-long spreading branches and bears linear-oblong-shaped, blunt, small leaves, with 100 or more on each branchlet arranged in two ranks to give the appearance of a pinnate leaf. The leaves grow up to a length of 8–10 mm or more long and are 2–3 mm wide. The unisexual, 0.5–1.5 cm long, greenish-yellow flowers appear in compact clusters in the axils of the lower leaves. Indian gooseberry fruits ripen to be nearly spherical or globular in shape and measure 1–2.5 cm in diameter with smooth textured skin containing six trigonous seeds. Each fruit has 6–8 pale vertical bands that appear as ridges extending from the base to the apex. The unripe fruits are light green and turn yellow or brick red at maturity. The fruit is acidulous when fresh and acidulous astringent when dried. The seeds are acrid and sweet and have a dark brown color (Preedy et al., 2011).

The Indian gooseberry is commonly cultivated in home gardens, but is also a commercial crop in countries with tropical and subtropical climates. This plant is propagated by seeds, and the trees start to bear fruit about 5–6 years after planting; they may continue to bear fruit for over 50 years (Orwa et al., 2009). The fruit yield per tree has been reported to be 19.6–20.2 kg for fruit harvested from the wild in India. Cultivated trees can yield 187–299 kg of fruit per tree annually, and 200 kg of fruit per tree can be obtained from grafted trees (Preedy et al., 2011).

3.2 CONSTITUENTS OF THE INDIAN GOOSEBERRY

Chemical composition of Indian gooseberry fruit has been extensively studies and shown to contain tannins, alkaloids, and phenolic compounds. The approximate composition of the Indian gooseberry fruit based on fresh weight is shown in Table 3.1.

Table 3.1 Nutritional value of the Indian gooseberry (Paul and Shaha, 2004).

Nutritional Value	**Amounts (per 100g)**
Carbohydrates	13.1 g
Fiber	3.3 g
Fat	0.1 g
Protein	0.4 g
Water	81.8 g
Thiamine (vit.B_1)	0.03 mg
Riboflavin (vit. B_2)	0.01 mg
β-Carotene	8 mg
Vitamin C	590 mg
Calcium	27 mg
Copper	0.14 mg
Iron	0.21 mg
Magnesium	24.5 mg
Phosphorus	22.8 mg
Potassium	185 mg
Sodium	3.2 mg
Zinc	0.32 mg

The Indian gooseberry is an important source of vitamin C (containing 412-900 mg/100 g), containing more vitamin C than oranges, tangerines, and lemons (Jain and Khurdiya, 2004; Khan, 2009). The consumption of one average-sized fruit (10 g) is expected to meet the recommended dietary allowance of 60 mg vitamin C for humans (Barthakur and Arnold, 1991). Indian gooseberry contains several amino acids, including glutamic acid (29.6%), proline (14.6%), aspartic acid (8.1%), alanine (5.4%), and lysine (5.3%; values are the percentage of the total amino acids) (Barthakur and Arnold, 1991). Moreover, Indian gooseberry fruit is rich in minerals, such as phosphorus, potassium, calcium, magnesium, selenium, iron, manganese, zinc, copper, and sodium (D'Souza et al., 2014). The fruit contains a high concentration of ellagitannins, such as emblicanin A, emblicanin B, punigluconin, and pedunculagin, which contribute to the antioxidant activity of the fruit (Baliga et al., 2013). Additionally, Indian gooseberry contains punicafolin, phyllanemblinin A, phyllanemblin, and various polyphenols, such as gallic acid, quercetin, kaempferol, and ellagic acid (Mirunalini and Krishnaveni, 2010).

Indian gooseberry seeds contain linolenic, linoleic, oleic, stearic, palmitic, and meristic acid (Kapoor, 1989). The leaves contain gallic acids, ellagic acids, chebulic acids, chebulagic acids, chebulinic acids, gallotannin, phyllantidine, and phyllantine (Kapoor, 1989; Asmawi et al., 1993). The bark contain leukodelphinidin, tannin, and proanthocyanidin, and the roots contain ellagic acid and lupeol (Ramakrishna et al., 2012).

3.3 HEALTH BENEFITS OF THE INDIAN GOOSEBERRY

From a dietary perspective, the ripe fruits of Indian gooseberry should be eaten raw and can be used to prepare delicacies, such as fresh juice, pickled fruits, and curries (Thilakchand et al.,

2013). Many dietary products made from the Indian gooseberry are on the market, including ready-to-serve beverages, candies, jams, powders, and fruit bars. Indian gooseberry fruits are also an important ingredient in the pharmaceutical industry and are used in herbal health care products, including hair oil, hair dye, shampoo, face creams, and tooth powder (Kumar et al., 2012).

In addition to its dietary and pharmaceutical use, the Indian gooseberry is also an important medicinal plant in traditional Indian medicine (the Ayurveda) and in various other traditional medicine systems practiced in Southeast Asia (Baliga and Dsouza, 2011; Mirunalini and Krishnaveni, 2010). People still use the fruit to make medicines to treat hypercholesterolemia, atherosclerosis, diabetes, pancreatitis, cancer, stomachache, eye problems, joint pain, diarrhea, obesity, and dysentery. Indian gooseberry fruits can also be used as anti-inflammatory agents.

Preclinical and clinical studies have been carried out to validate the medicinal effects of the Indian gooseberry. According to various studies, Indian gooseberry fruits possess antibacterial, antifungal, antiviral, antidiabetic, hypolipidemic, antiulcerogenic, antioxidant, antimutagenic, anti-inflammatory, immunomodulatory, antipyretic, analgesic, antitussive, antiatherogenic, adaptogenic, gastroprotective, anti-anemic, antihypercholesterolemic, antidiarrheal, anti-atherosclerotic, hepatoprotective, and neuroprotective activities (Khan, 2009; Mirunalini and Krishnaveni, 2010).

In vitro and *in vivo* studies have shown that Indian gooseberry extracts and phytochemicals isolated from Indian gooseberry plants possess free-radical scavenging effects, with emblicanin

A exhibiting the highest antioxidant effects, followed by emblicanin, gallic acid, ellagic acid, and ascorbic acid (Pozharitskaya et al., 2007). In addition, preclinical studies have shown that the Indian gooseberry inhibits oxidative stress, mutagenesis, and carcinogenesis by increasing the levels of antioxidant enzymes (Baliga and Dsouza, 2011). The antioxidant activity of the Indian gooseberry prevents lipid peroxidation induced by various stimuli, including radiation, ethanol, and ochratoxin (Chakraborty and Verma, 2010; Jagetia et al., 2002; Reddy et al., 2009).

The Indian gooseberry has been shown to have hepatoprotective effects against various hepatotoxic agents, including ethanol, heavy metals, carbon tetrachloride, and antitubercular drugs. Furthermore, the Indian gooseberry has been reported to have beneficial effects on liver function and to mitigate hyperlipidemia and metabolic syndrome (Thilakchand et al., 2013). Indian gooseberry plants also have the potential to prevent dental caries by inhibiting virulence factors of *Streptococcus mutans* and *Lactobacillus*, with higher efficiency than chlorhexidine, a common antibacterial agent (Hasan et al., 2012).

The constituents of the Indian gooseberry, e.g., gallic acid, gallotannin, ellagic acid, and corilagin, have been shown to possess antidiabetic effects. Recent studies have shown that extracts of Indian gooseberry fruit ameliorate alloxan-, streptozotocin-, and high-fat diet-induced diabetes *in vivo* (D'Souza et al., 2014). In addition, the extracts of leaves and seeds also possess antihyperglycemic effects in diabetic rats. In a clinical study, regular intake of Indian gooseberry powder was effective in decreasing fasting and 2-h post-prandial blood glucose levels in both normal individuals and patients with diabetes (Akhtar et al., 2011).

Although the Indian gooseberry has high amounts of vitamin C and phytochemicals, a long history of use in traditional medicine, and various health benefits, few studies have examined the bioactive mechanisms through which this plant exerts its beneficial effects. Therefore, further studies on the Indian gooseberry are needed to broaden its dietary, pharmaceutical, and medicinal usage.

3.4 EFFECTS OF *PHYLLANTHUS EMBLICA* L. (INDIAN GOOSEBERRY) EXTRACTS ON RETINAL DEGENERATION IN A MOUSE MODEL OF AMYLOID BETA-INDUCED ALZHEIMER'S DISEASE

3.4.1 Introduction

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder that results in progressive loss of cognitive function (Wyss-Coray, 2006). According to the Alzheimer's Association, an estimated 5.2 million Americans have AD, and AD is the seventh leading cause of death in the United States. Cognitive deficits caused by AD progress affect intellect capacity, mood, behavior, personality, and the patient's ability to carry out activities associated with daily life. Visual disturbances are a common symptom of AD (Mendez et al., 1990). Visual disturbances in AD patients consist of impairment in spatial contrast sensitivity, motion perception, and color discrimination, as well as blurred vision (Rizzo et al., 2000a; Rizzo et al., 2000b). Recent studies have demonstrated that visual impairment in AD patients may arise from retinal abnormalities, in addition to damage to the primary visual cortex and degeneration of cortical areas (Berisha et al., 2007; Guo et al., 2010). Several studies have demonstrated the formation of cataracts, loss of retinal neurons, retinal neuronal filament layer thinning, and impaired axonal outgrowth in AD patients (Chiu et al., 2012; Curcio and Drucker, 1993; Sadun and Bassi, 1990). Although recent histological studies using optical coherence tomography and scanning laser polarimetry have shown retinal damage in AD patients, controversy remains because little is known of the mechanisms underlying this effect (Lu et al., 2010). One of the proposed mechanisms of retinal damage in AD is the accumulation of amyloid-beta (Aβ) peptide, which plays a prominent role in the pathology of AD. Aβ aggregates to form oligomers, protofibrils, and fibrils, which are deposited as amyloid plaque. Several lines of evidence

indicate that amyloid plaque is involved in neuronal dysfunction and neurodegeneration, which is accompanied by increased levels of reactive oxygen species (Roher et al., 1993). Furthermore, Aβ deposits have been reported in glaucomatous optic nerve heads, drusen associated with age-related macular degeneration, and cataractous lenses (Dutescu et al., 2009). However, there are limited data demonstrating Aβ accumulation in the retinas of human AD patients.

Phyllanthus emblica L., commonly known as Indian gooseberry, is widely distributed in China, India, Indonesia, Malaysia, and Thailand, and its fruit has been used in many traditional medicines for atherosclerosis, diabetes, upset stomach, diarrhea, and skin problems (Barthakur and Arnold, 1991). Due to its high levels of vitamin C (412–900 mg/100 g) and minerals, Indian gooseberry fruit is used in juices, jams, and cosmetics (Jain and Khurdiya, 2004). Phytochemical investigations have reported that Indian gooseberry fruit extract contains tannins and various phenolic compounds, including ellagic and gallic acids and corilagin (Poltanov et al., 2009). In addition, Indian gooseberry has been shown to have antimicrobial, adaptogenic, antiatherogenic, antitussive, hepatoprotective, immunomodulatory, and chemoprotective activities (Liu et al., 2008; Mayachiew and Devahastin, 2008). Interestingly, Indian gooseberry is used as a tonic and collyrium for eye disorders, and such use improves sight, reduces cataracts, and minimizes infections (Kumar et al., 2012; Srivasuki, 2012). A recent report showed that beta-glucogallin, a compound isolated from Indian gooseberry, had inhibitory effects on inflammatory eye-diseases such as uveitis by inhibiting aldose reductase (Chang et al., 2013). Other berries, such as blueberries, blackberries, and goji berries, have been considered beneficial to eye health because their constituent flavonoids, including anthocyanins, interact directly with rhodopsin and protect the retina from oxidative stress (Kalt et al., 2010). Berry fruits also have neuroprotective effects

on the brain, and have been shown to enhance neuroplasticity, neurotransmission, and calcium buffering (Miller and Shukitt-Hale, 2012). However, there is a lack of experimental studies regarding the efficacy of the retinal protective effect of Indian gooseberry.

Therefore, we hypothesized that oral administration of Indian gooseberry extract (IGE) would attenuate retinal degeneration caused by intracerebroventricular (ICV) injection of Aβ in an Alzheimer's disease mouse model. This is the first report of the protective effect of a berry fruit on retinal degeneration caused by direct injection of Aβ into the brain.

3.4.2 Materials and methods

Preparation of Indian gooseberry extract

Ripe Indian gooseberry fruit from Thailand (brix 10.0-12.0, pH 2.3-2.5, total acidity 2.1-2.8%) was freeze-dried and ground. The extract of Indian gooseberry fruit contained seed but its skin were removed. Ten grams of the freeze dried powder was mixed with 100 mL of deionized distilled water (DDW), and the resulting mixture was homogenized at 15,000 rpm for 2 min using a Polytron homogenizer (PT 10/35, Kinematica, Kriens-Luzern, Switzerland), filtered through Whatman #2 filter paper (Whatman International Ltd., Kent, U.K.), and rinsed with 50 mL of DDW. The homogenization procedure was repeated twice and the total volume of the mixture was adjusted to 500 mL. Liquid-liquid extraction was applied to the final mixture with equal volumes of the four organic solvents in the sequence of *n*-hexane, chloroform, ethyl acetate, and *n*-butanol to separate phenolics (Son et al., 2014). The ethyl acetate fraction which is considered to have the highest phenolics contents was evaporated to dryness and used for the present study (Kumaran and Karunakaran, 2006).

Animals

ICR mice (male, 4 weeks old) were obtained from Samtako Co. (Osan, Korea) and housed 2 per cage in a room maintained with a 12-h light-dark cycle, 55% humidity, and a temperature of 23–25 °C. All animals and experimental procedures were approved by the guidelines established by the Institutional Animal Care and Use Committee (IACUC) of Gyeongsang National University (certificate: GNU-120409-M0009) and in accordance with the Ethical Committee of Ministry of Health and Welfare of Korea.

IGE was dissolved in water and administered orally at a dose of 5, 10, or 20 mg/kg once per day for 3 weeks. The mice were divided into 5 groups: (I) normal control, (II) Aβ (negative control), (III) Aβ + 5 mg/kg IGE, (IV) Aβ + 10 mg/kg IGE and (V) Aβ + 20 mg/kg IGE (n = 8 in each group). The mice were fed with a diet of animal chow and water *ad libitum*. $A\beta_{25-35}$ was administered by intracerebroventricular (ICV) injection to induce memory impairment. Aβ was dissolved in 0.85% (v/v) sodium chloride solution and each mouse was injected with a Hamilton microsyringe (depth, 2.5 mm; injection volume, 5 μL; dose, 410 pmol per mouse) (Jung Choi et al., 2009).

Culture of RGC-5 cells

The transformed retinal ganglion cell line (RGC-5) was derived by transforming postnatal day 1 rat retinal cells with the Ψ_2 EA1 virus (Van Bergen et al., 2009). The RGC-5 line is used widely in glaucoma research because it has certain characteristics of RGCs, including expression of Thy-1 and brain derived neurotrophic factor (BDNF) (Jang et al., 2013). RGC-5 cells were

grown in normal growth medium (Dulbecco's modified Eagle medium (DMEM) containing 5 mM glucose and supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin/streptomycin) in a humidified incubator with 5% CO_2 at 37 °C. Cultured RGC-5 cells in 96-well plates were used 24 h later for determination of cell viability and microscopic analysis.

Cell viability

Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Jung et al., 2010). In the experiments, 1×10^4 cells were plated in each well of 96-well plates and allowed to attach to the substrate for a 24 h period. The cells were exposed to lead for an additional 24 h in the absence or presence of different concentrations of IGE. The medium was removed and MTT solution (0.5 mg/mL) was added for 1 h at 37 °C. DMSO solution was added to each well and the plate was shaken for 10 min. The number of living RGC-5 cells in each well was determined by measuring the optical density (570 nm test wavelength and 690 nm reference wavelength) using a spectrophotometer (BioTek Instruments, Winooski, VT, USA), and the percentage viability was calculated.

Measurement of intracellular radical scavenging activity of IGE

Intracellular radical activation within RGC-5 cells was determined using DCFH-DA (Kim et al., 2011). The cells were treated with different concentrations of IGE for 1 h and then treated with DCFH-DA (final concentration, 10 μM) for 15 min at 37 °C. After 15 min, the medium was replaced with fresh medium containing the same compounds without DCFH-DA. The 96-well plates were loaded into a plate-holder fluorescence spectrophotometer and monitored at a 485 nm excitation wavelength and a 535 nm emission wavelength. After each well had been

monitored for 30 s as a baseline measurement, the reaction was initiated by adding 1 mM hydrogen peroxide, 1 mM hydrogen peroxide plus 100 μM of iron (II) perchlorate hexahydrate, or 1 mM potassium dioxide, and fluorescence was monitored for 20 min at 37 °C.

Histological analysis of the mouse retina

Enucleated eyes were fixed with 10% formalin for 24 h and embedded in paraffin. The samples were sectioned equatorially at a thickness of 4 μm using an HM340E microtome (Thermo Fisher Scientific, Walldorf, Germany). Each retinal section was exposed to hematoxylin solution (0.1% hematoxylin, 10% ammonium) for 8 min. The sections were washed 3 times with distilled water and exposed to bluing reagent (0.2% lithium carbonate solution) for 1 min. The sections were rinsed in 95% alcohol, exposed to 1% Eosin Y solution for 1 min, and then washed 3 times with 95% alcohol. The sections were cover slipped with a mounting medium and observed under a light microscope (Olympus, Tokyo, Japan). Light-microscope images were taken, and the thickness of the inner nuclear layer (INL) was measured by a single observer at several points in the photographs. Observations were restricted to the orientated central zone, and the fovea and peripheral region were avoided. Data from 3 sections were averaged for each eye and used to evaluate the thickness of the INL.

Protein extraction and western blot analysis of the mice retina

The retina was separated from the eye, lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 1x protease inhibitors), and incubated for 30 min at 4 °C. After the incubation, the retina was sonicated and centrifuged at 14,000 × g for 30 min at 4 °C. The supernatant was transferred to a new vessel and the protein

concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was performed with anti-NF-L, anti-GAPDH, anti-SIRT1, and anti-Thy-1 primary antibodies (1:3000 dilution; Cell Signaling Technology, Beverly, MA, USA). Secondary antibodies were purchased from Santa Cruz Biotechnology Co. Ltd (1:3000 dilution; Santa Cruz Biotechnology, CA, USA). Immuno-reactive bands were detected using enhanced chemiluminescence reagents (Amersham Bioscience, GE Healthcare, UK) and measured via densitometry using an LAS-4000 image reader and Multi Gauge 3.1 software (Fuji Photo Film, Japan)

Statistical analysis

The data in this study are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made using one-way ANOVA followed by Dunnett's test. Statistical analyses were conducted using SigmaPlot (Systat Software, Inc., San Jose, CA, USA) and significance was determined using the p-value of the results (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4.3 Results

Effect of IGE on cell viability

IGE was used at concentrations of 1–100 µg/mL in the *in vitro* experiments, and IGE at these concentrations had no effect on the viability of RGC-5 cells (Figure 3.1).

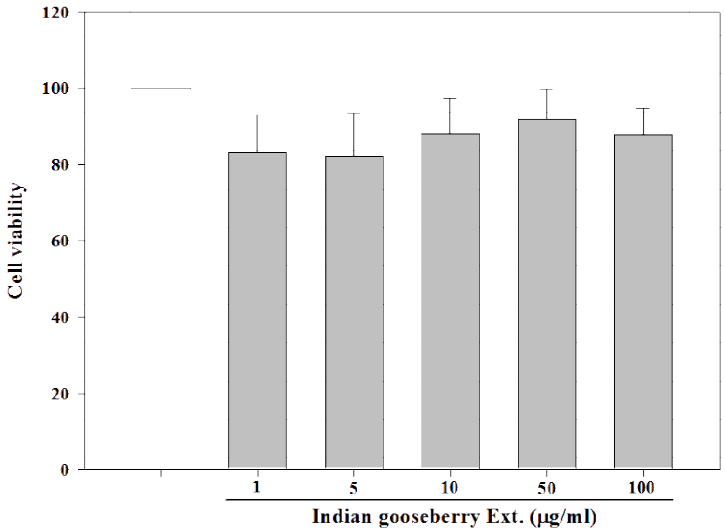


Figure 3.1 The effect of Indian gooseberry on the viability of RGC-5 cells. The cells were treated with the indicated concentrations of Indian gooseberry extracts (IGE) for 24 h, and cell viability was determined by the MTT assay as described in section 2. Data are presented as the mean ± SEM of 3 independent experiments.

Effect of IGE on intracellular ROS level

Following oxidative insult by H_2O_2, O_2, or OH, intracellular ROS levels were increased by 383.35%, 162.12%, and 615.03%, respectively, relative to the control cells (Figure 3.2). However, IGE treatment at 1, 5, 10, 50, and 100 µg/mL significantly inhibited radical activity in RGC-5 cells and decreased intracellular radical formation in a dose-dependent manner.

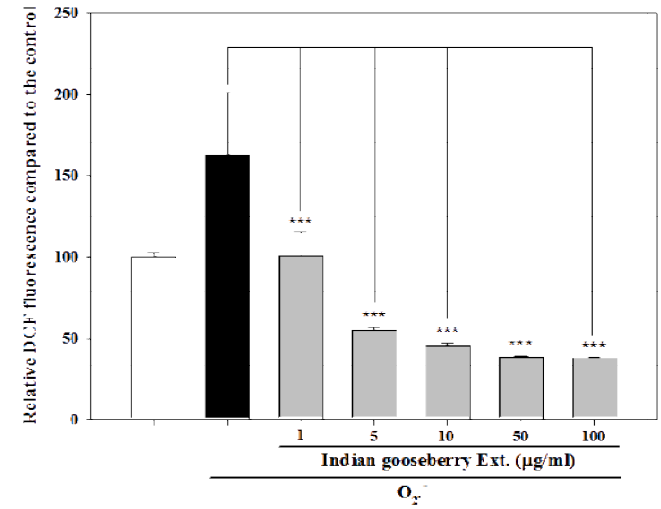
Histological findings from Aβ-induced retinal damage in vivo

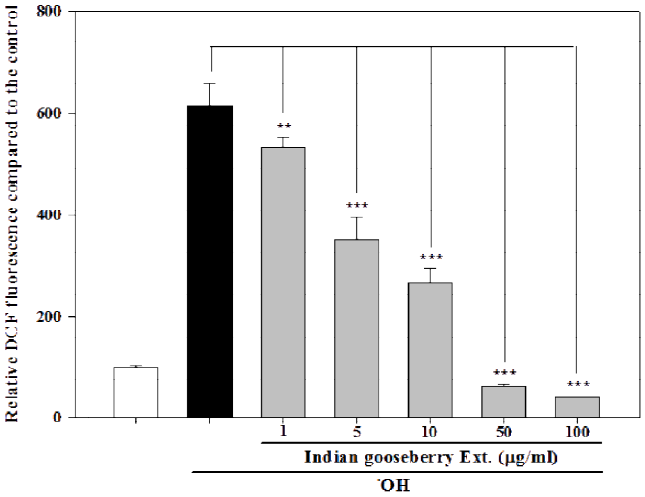
The results from the histological hematoxylin and eosin staining assay are shown in Figure 3.3. Aβ administration changed the cell density of the ganglion cell layer and the thickness of the inner plexiform layer, or outer nuclear layer. However, relative to normal tissue (Figure 3.3, a), samples from mice that were injected Aβ exhibited a significant thinning of the inner nuclear layer (INL) (Figure 3.3, b), which appeared to have been inhibited in tissues treated with IGE (Figure 3.3, c, d, and e).

The average thickness of the inner nuclear layer in Aβ-injected mice was reduced in comparison with that of the control group at several points of measurement (200 µm superior: 25.59 ± 1.46 versus 40.30 ± 2.51 µm, $p < 0.001$; 200 µm inferior: 23.97 ± 0.24 versus 42.71 ± 5.69 µm, $p < 0.001$; 600 µm superior: 25.79 ± 2.60 versus 40.57 ± 1.01 µm, $p < 0.01$; 600 µm inferior: 26.28 ± 2.41 versus 37.81 ± 3.11 µm, $p < 0.01$). However, the average thickness of the INL of the mice that were orally treated with 20 mg/kg IGE were significantly protected from the effect of the Aβ administration (200 µm superior: 44.95 ± 3.01 versus 25.59 ± 1.46 µm, $p < 0.001$; 200 µm inferior: 42.37 ± 2.19 versus 23.97 ± 0.24 µm, $p < 0.001$; 600 µm superior: 41.83 ± 0.58 versus 25.79 ± 2.60 µm, $p < 0.001$; 600 µm inferior: 44.10 ± 0.82 versus 26.28 ± 2.41 µm, $p < 0.001$)

(Figure 3.3B). Oral treatment with 5 and 10 mg/kg IGE also protected against the Aβ-induced reduction in INL thickness (200 µm superior: 20.03% and 44.21%, respectively; 200 µm inferior: 30.25% and 43.93%, respectively; 600 µm superior: 33.82% and 33.06%, respectively; 600 µm inferior: 10.61% and 32.18%, respectively) in comparison with the negative control treatment (Figure 3.3 f).

Figure 3.2 The effects of Indian gooseberry on ROS production induced by H_2O_2, O_2, and OH. Intracellular ROS levels were determined by measuring the fluorescence of DCFH-DA (485 nm excitation wavelength and 535 nm emission wavelength). Data are presented as the mean ± SEM of 3 independent experiments. Double asterisks (**) indicate $p < 0.01$; triple asterisks (***) indicate $p < 0.001$.


Relative DCF fluorescence compared to the control
250
200
150
100
50
0
***
***
***
***
***
1
5
10
50
100
Indian gooseberry Ext. (μg/ml)
O_2^-


Relative DCF fluorescence compared to the control
800
600
400
200
0
**
***
***
***
***
1
5
10
50
100
Indian gooseberry Ext. (μg/ml)
·OH

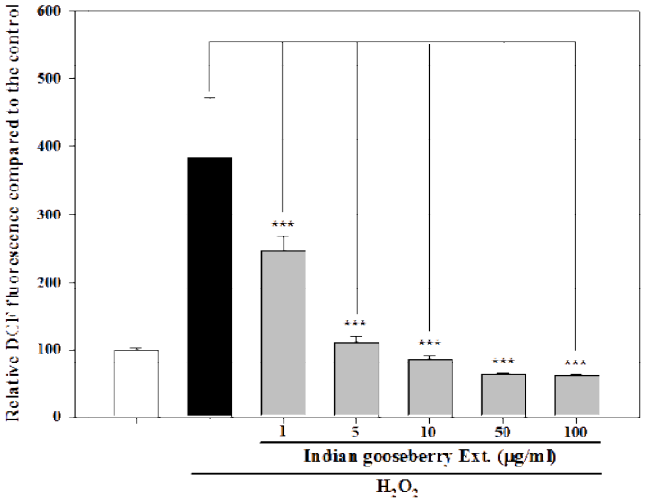
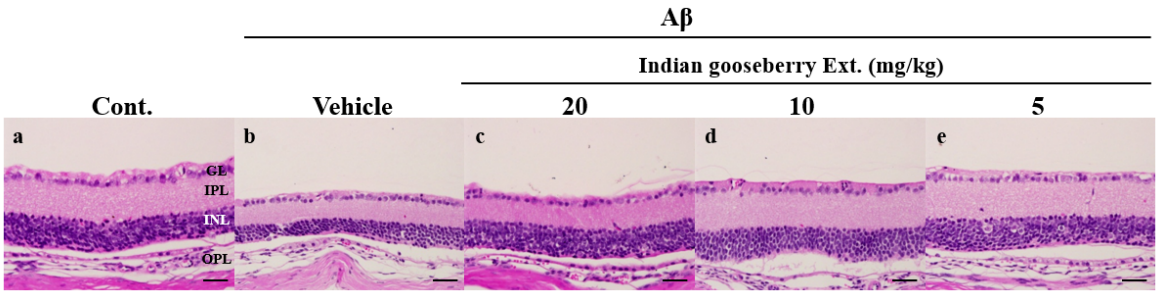
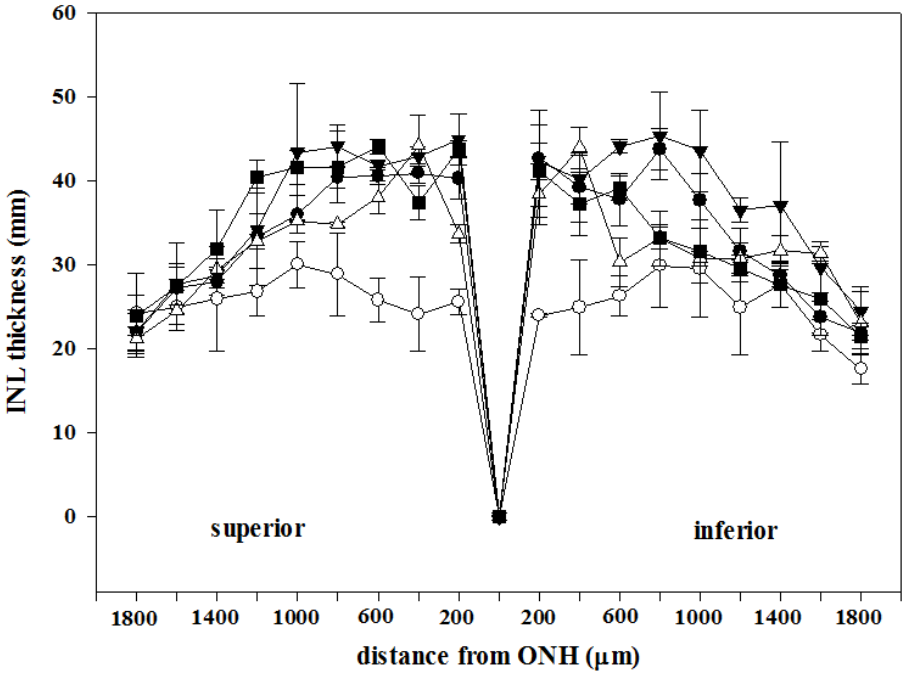

Relative DCF fluorescence compared to the control
600
500
400
300
200
100
0
***
***
***
***
***
1
5
10
50
100
Indian gooseberry Ext. (μg/ml)
H_2O_2

Figure 3.3 Aβ damage in ICR mice. Hematoxylin and eosin (H&E) staining showed obvious disintegration of the inner nuclear layers of the retina after Aβ administration. The thicknesses of the inner nuclear layers from selected groups were measured. Data are presented as mean ± SEM (n = 3 for each group). Oral treatment with IGE at 5, 10, and 20 mg/kg significantly protected the inner nuclear layer from Aβ damage. Scale bar = 50 μm.


Aβ
Indian gooseberry Ext. (mg/kg)
Cont.
Vehicle
20
10
5
a
b
c
d
e
GE
IPL
INL
OPL


INL thickness (mm)
60
50
40
30
20
10
0
superior
inferior
1800
1400
1000
600
200
200
600
1000
1400
1800
distance from ONH (μm)
Control
Vehicle (Ab)
Aβ + Indian gooseberry Ext. 20 mg/kg
Aβ + Indian gooseberry Ext. 10 mg/kg
Aβ + Indian gooseberry Ext. 5 mg/kg

(f)

Effects of IGE on Aβ -induced retinal damage in mice

Measurement of Thy-1 and NF-L levels is a reliable method of assessing RGC injury (Chidlow and Osborne, 2003). ICV administration of 410 pmol Aβ decreased expression of Thy-1 and NF-L proteins in the mouse retina. Three days after the Aβ injection, Thy-1 expression in the retina was significantly decreased to 69.78% of that of the normal control group. However, as shown in Figure 3.4, oral administration of 5, 10, and 20 mg/kg IGE significantly recovered Thy-1 expression to 90.08%, 119.47%, and 114.22%, respectively, of that of the normal control group. Three days after the Aβ injection, NF-L expression in the retina was significantly decreased to 72.26% of that of the normal control group. However, oral administration of 5, 10, and 20 mg/kg IGE significantly recovered expression of NF-L to 103.03%, 109.50%, and 133.42%, respectively, of that of the normal control group. To further clarify our findings and explore the effect of Indian gooseberry extract on the retina under Aβ-induced stress, SIRT1 expression levels were evaluated. Aβ administration decreased SIRT1 expression to 81.11% of that of the normal control retinas. However, 20 mg/kg IGE significantly recovered expression of SIRT1 to 124.94% of that of the normal control group ($p < 0.05$) (Figure 3.5).

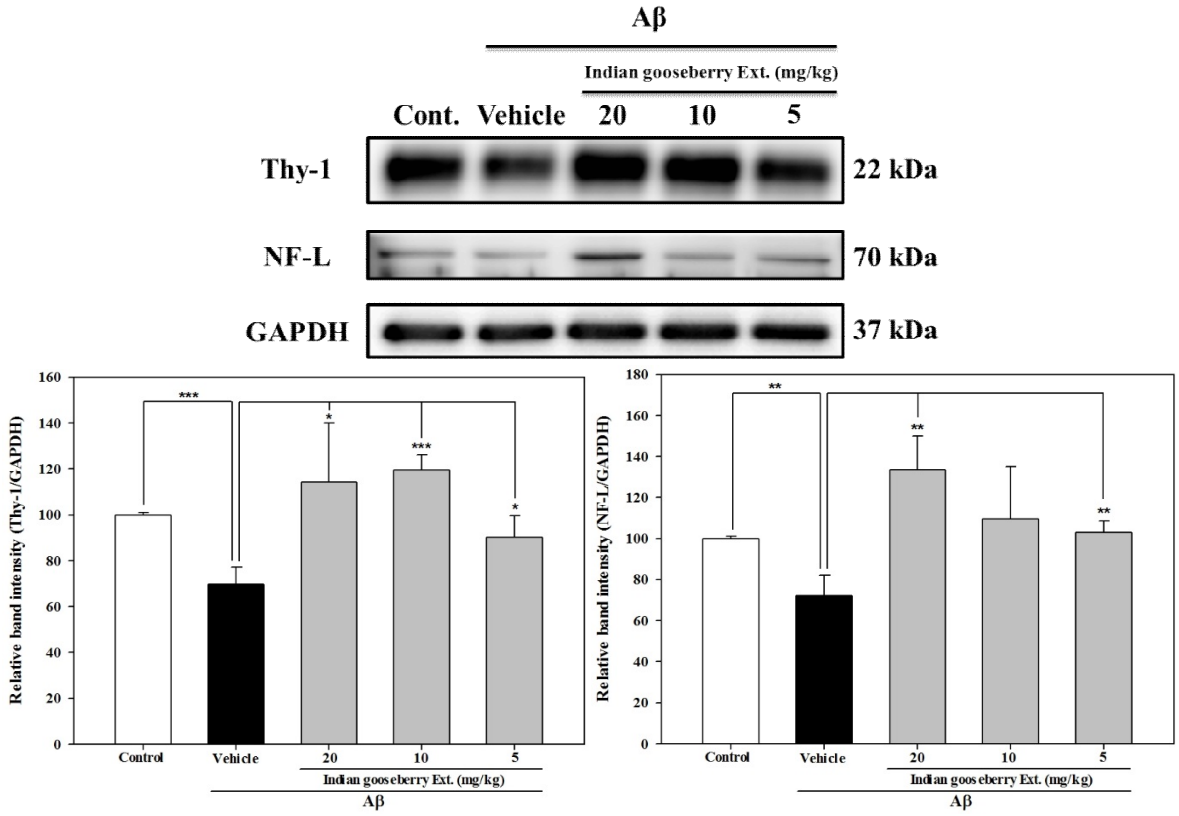


Figure 3.4 Results of western blot analysis investigating Thy-1 and NF-L expression in the retina 3 days after intracerebroventricular Aβ injection. RGCs were collected from an untreated control mouse, a mouse that underwent Aβ injection only, and mice that underwent Aβ injection and treatment with 5, 10, or 20 mg/kg of IGE. Single asterisks (*) indicate $p < 0.05$; double asterisks (**) indicate $p < 0.01$; triple asterisks (***) indicate $p < 0.001$.

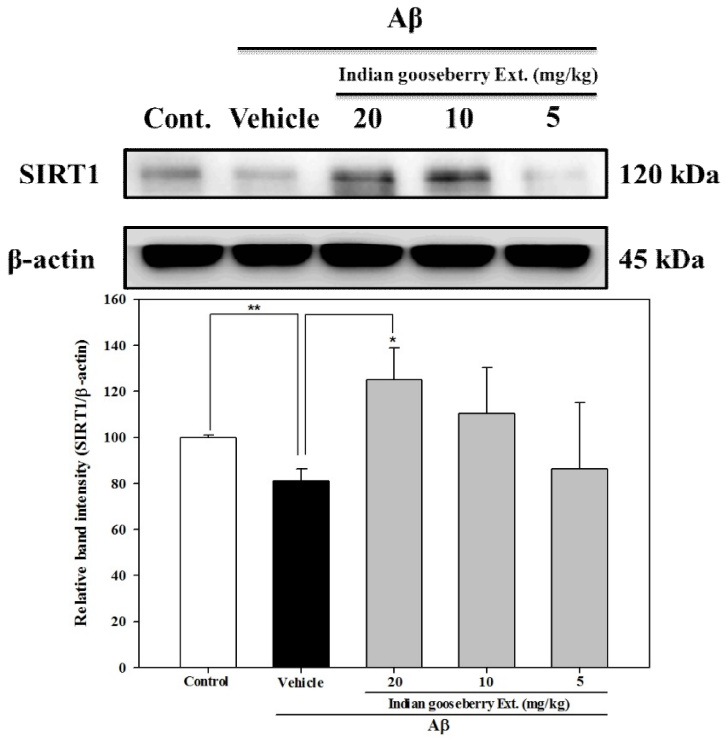


Figure 3.5 Results of western blot analysis investigating SIRT1 expression in the retina 3 days after intracerebroventricular Aβ injection. Single asterisks (*) indicate $p < 0.05$; double asterisks (**) indicate $p < 0.01$.

3.4.4 Discussion

Our results show that ICV administration of Aβ causes retinal damage, and IGE protects the retina against Aβ-induced toxicity. This is the first demonstration of the capacity of ICV Aβ to induce retinal degeneration *in vivo*, as well as the first demonstration of the protective effect of a berry against retinal degeneration induced by Aβ toxicity. Moreover, our results show that IGE inhibits retinal degeneration caused by Aβ peptides by scavenging free radicals and increasing protein abundance of genes such as SIRT1.

Aβ peptides are cleaved from amyloid precursor protein by β- and γ-secretases. The monomer of Aβ is not toxic; however, Aβ peptide aggregation damages neurons (Kadowaki et al., 2005). Moreover, Aβ is a major component of AD plaques and is also present in drusen in patients with age-related macular degeneration (Guo et al., 2010). Laboratory studies have shown that oxidative stress, including protein oxidation, lipid peroxidation, and ROS generation, is a possible mechanism underlying Aβ-mediated neurotoxicity (Yatin et al., 2000), and this finding is supported by the evidence that Aβ-induced neuronal damage is inhibited by antioxidants such as vitamin E, folate, acetyl-L-carnitine, and *Ginkgo biloba* constituents (Dhitavat et al., 2005; Yao et al., 2001).

Indian gooseberry has antioxidant activity and neuroprotective effects. The antioxidative effects of Indian gooseberry might be due to the presence of antioxidant phenolic compounds and tannins (Mayachiew and Devahastin, 2008b). In our study, the tested concentrations of IGE did not show toxicity in RGC-5 cells, showing that it was suitable for our study (Jang et al., 2013; Jo et al., 2013). DCFH-DA was used as a radical probe to quantify intracellular ROS. DCFH-DA

penetrates cell membranes and is hydrolyzed by intracellular esterases to form nonfluorescent DCFH (LeBel et al., 1992). In the presence of ROS such as H_2O_2, O_2, or OH, DCFH is rapidly oxidized to form highly fluorescent DCF within the cell. The physiological oxidants H_2O_2, O_2, and OH significantly increased intracellular ROS levels, but ROS levels were significantly reduced after 1 h of incubation with IGE. Our results indicate that antioxidant constituents of Indian gooseberry are able to permeate through the cell membrane.

Histologic retinal studies in AD patients with optical coherence tomography show retinal nuclear filament layer thinning (Kirbas et al., 2013). Retinal nuclear filament layer thinning in AD is caused by apoptosis of RGCs and atrophy of photoreceptors, which are induced by Aβ, as demonstrated by *in vivo* studies using APP transgenic mice (Tg 2576), APP/PS-1 double transgenic mice, and APPswe/PS1ΔE9 transgenic mice (Guo et al., 2010). In our study, IGE significantly reduced INL thickness in Aβ-treated mice in comparison with controls. The thickness of the INL is linearly associated with the sensitivity of rod and cone cells (Birch et al., 2011). Therefore, Indian gooseberry might protect against visual impairment caused by Aβ-induced neurodegeneration.

To determine the protective effect of IGE on the retina, western blot analysis was performed to evaluate the expression of retina-related proteins. Thy-1 is a cell surface glycoprotein that is expressed during RGC differentiation (Lindsey et al., 2013). NF-L is expressed by RGCs in the optic nerve and is therefore used as a measure of RGC viability (Zhang et al., 2007). ICV injection of Aβ decreased Thy-1 and NF-L protein abundance; however, oral IGE treatment recovered Thy-1 and NF-L expression to normal levels.

To better understand the intracellular mechanism of action of IGE, SIRT1 protein abundance was measured. SIRT1 is an NAD^+-dependent deacetylase that is involved in DNA repair, metabolic regulation, cell stress responses, and cell survival (Donmez and Guarente, 2010; Porcu and Chiarugi, 2005). SIRT1 is activated by the polyphenol resveratrol and has been shown to increase mitochondrial function and promote RGC survival after optic nerve crush (Price et al., 2012; Zuo et al., 2013). In this study, oral administration of 20 mg/kg IGE significantly up-regulated SIRT1 expression in comparison with that of the control group. This result suggests that Indian gooseberry contains SIRT1 activating compounds that may play a significant role in its protective effect against Aβ-induced retinal degeneration.

In this study, we found that IGE protects the retina against Aβ-induced toxicity in mice, and these effects may be primarily related to the antioxidant properties of its phenolic constituents. Therefore, Indian gooseberry consumption may provide health benefits by preventing retinal degeneration.

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CHAPTER 4

CONCLUSIONS

Traditionally, eye health has been viewed as an issue isolated to seniors. Age-related macular degeneration (AMD), which gradually destroys the macula, is a leading cause of vision loss in older adults that generally affects people aged 50 years and older, according to the National Eye Institute (NEI). Moreover, by age 80, more than half of all Americans either currently have a cataract, a clouding of the lens in the eye that affects vision, or have had cataract surgery. However, the increase in the number of frequent computer and mobile smart phone users is expected to impact the eye health of the younger generations.

Other trends, including an aging population, unhealthy diet, and rising healthcare costs, may also result in the development of dietary supplements for eye health to help eye fatigue or stress. According to the analysts Frost & Sullivan (Frost & Sullivan, 2009), the market size of eye health ingredients in the US was estimated at $138 million US dollars in 2008, with a compound annual growth rate of 5.3% from 2008 to 2015. The European market was valued at $43.4 million US dollars in 2007 with a compound annual growth rate of 10.5% from 2007 to 2014.

In anticipation of these social health issues and market trends, my research aimed to identify whether phytochemicals, as antioxidants and anti-inflammatory agents, may help to prevent or inhibit the progression of eye diseases, especially retinal degeneration.

In the first and second studies, we investigated the protective effect of coffee against hypoxia-induced retinal degeneration. One mechanism of retinal degeneration is hypoxia, a reduction in retinal oxygen supply caused by pathologies such as central retinal artery occlusion, ischemic central retinal vein thrombosis, complications of diabetic eye disease, and some types of

glaucoma that cause vascular eye diseases (Levin, 2003). Retinal hypoxia can negatively impact both tissue function and cell viability, and is a potential risk factor for sight-threatening disorders (Kaur et al., 2008). Unsurprisingly, there is great interest in identifying neuroprotective compounds that inhibit hypoxia. Particularly promising in this capacity are natural products and phytochemicals that act as antioxidants and can be taken regularly without causing significant side effects (Mozaffarieh et al., 2008).

Coffee is second only to water as the most widely consumed beverage in the world. In the United States, approximately two-thirds of adults drink coffee on a daily basis, which translates into over 400 million cups of coffee being consumed each day (NCA, 2012). Furthermore, the *per capita* consumption in Northern European countries such as Finland, Denmark, and Sweden may reach 7.31-12 kg/year, which is more than two or three times that of the United States (ICO, 2014). Coffee consumption appears to decrease the risk of developing chronic diseases such as Parkinson's, prostate cancer, and diabetes (Higdon and Frei, 2006). It also reduces the extent of cognitive decline associated with aging. This latter pattern in particular supports the idea that the neuroprotective effects of chlorogenic acid (CGA) derivatives stem from their antioxidant activities. Despite these generally positive results, the effects of coffee consumption on ocular health are not yet clear. In particular, clinical studies on the relationship between coffee consumption and risk of glaucoma have yielded conflicting results (Jiwani et al., 2012; Pasquale et al., 2012).

Therefore, the aim of the first study was to determine whether coffee extracts and CGA, the major phenolic compound in coffee, have protective effects against ischemic damage to the mouse retina *in situ* and hypoxia-induced degeneration in transformed retinal ganglion cells (RGC-5) *in vitro*. To accomplish this, RGC-5 cultures were transferred to a closed hypoxic

chamber filled with nitrogen gas. RGC-5 cells were treated with *S*-nitroso-*N*-acetyl-penicillamine (SNAP), a nitric oxide generator, for 24 h. Under hypoxic conditions, 100 μM SNAP caused a significant reduction in cell viability. However, pretreatment of RGC-5 cells with varying concentrations of CGA (25, 50, and 100 μM) significantly attenuated cell death in a concentration-dependent manner. Pretreatment with CGA reduced the up-regulation of apoptotic proteins such as Bax, Bad, and cleaved caspase-3. To investigate the effects of CGA and coffee extract *in vivo*, we used optic nerve crush (ONC) as an ischemic insult and an inflammation inducer on the retina of mice. Oral administration of CGA or coffee extract attenuated the loss of RGCs and production of apoptotic proteins in the retina.

In the second study, we determined which coffee metabolites reach the retina and protect against retinal degeneration. Rats were administered a solution of CGA and the eyes were analyzed with UPLC-MS/MS to detect the metabolites that reached the eye. The methyl thiazolyl tetrazolium assay and double staining with Hoechst and propidium iodide showed that CGA, caffeic acid (CA), and dihydrocaffeic acid (DHCA) reach the eye to protect retinal ganglion cells (RGCs) from hypoxia-induced damage. Western blotting showed that treatment with coffee metabolites up-regulated anti-apoptotic proteins such as Bcl-2 and Bcl-X_L, and down-regulated pro-apoptotic proteins such as Bad, PARP, and cleaved caspase 3. Adult ICR mice were subjected to ONC-induced RGCs death with intravitreal administration of coffee metabolites 1 day before and 1 hour after the procedure. Retrograde Fluorogold^{TM} labeling showed severe RGCs loss after ONC, and coffee metabolites significantly reduced damage to RGCs. According to this study, we conclude that CGA and coffee metabolites, especially CA and DHCA, reach the eye, where they can significantly reduce apoptosis induced by hypoxia and ONC stress, and thus prevent retinal degeneration.

In the third study, we investigated whether Indian gooseberry extracts have protective effects against the degeneration of the retina in an Aβ-induced *in vivo* model. The retina is considered an extension of the central nervous system (CNS). Indeed, several ocular disorders have been detected in patients with CNS diseases such as stroke, multiple sclerosis, Parkinson's disease, and Alzheimer's disease (AD) (London et al., 2013). In particular, AD has many parallel characteristics with AMD, the most common cause of irreversible blindness in the world (Ohno-Matsui, 2011). Aβ is the main constituent of the amyloid plaques found in the brains of patients with AD, and is also deposited in the drusen of eyes of patients with AMD.

Four-week-old male ICR mice were subjected to retinal degeneration caused by intracerebroventricular injection of Aβ. Oral administration of Indian gooseberry extracts to mice for 3 weeks significantly protected against retinal degeneration caused by Aβ. The down-regulation of Thy-1 and neurofilament light (NF-L) in Aβ-induced retinal damage was significantly attenuated by Indian gooseberry administration. Moreover, sirtuin (SIRT) 1 was significantly up-regulated by treatment with Indian gooseberry. Histological analysis revealed that Indian gooseberry extracts protect against thinning of the thickness of the inner nuclear layer (INL). Our study clearly demonstrates that Indian gooseberry is able to protect against Aβ-induced retinal degeneration, and may be a promising neuroprotective agent to prevent glaucoma or neurodegenerative diseases.

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