Anti-aging Activity of Selected Nutraceuticals in *Drosophila melanogaster*

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Abstract

Aging is a complex biological process involving both genetic and environmental factors. Age-related accumulation of oxidative damage is one of the most widely accepted and well studied mechanisms to underlie aging. Therefore, augmentation of antioxidant defenses shall be conducive to lifespan extension. Dietary manipulations, including calorie restriction and dietary supplementation, have been demonstrated to be able to extend lifespan and ameliorate certain age-related diseases. Nutraceuticals rich in antioxidants have the potentials to be competent anti-aging candidate compounds. The present study was to investigate the anti-aging activity of four nutraceuticals, including black tea extracts (BTE), soybean isoflavones (SIF), apple polyphenols (AP), and blueberry extracts (BBE), in *Drosophila melanogaster,* an aging model.

Consumption of tea, soybean, apple, and blueberry has been associated inversely with cardiovascular diseases, various cancers, and hypercholesterolemia. However, few existing reports have investigated their lifespan prolonging activity or their abilities to ameliorate neurodegenerative diseases in vivo. The present research studied the effect of the above four functional foods or nutraceuticals on the lifespan of fruit fly under both the normal physiological and the oxidative stress conditions.

For the first part, life spans of fruit flies, fed on either control diet or diet with varying doses of supplements, were recorded. At the same time, endogenous antioxidant enzyme activities, lipid peroxidation (LPO) level, expression of genes

related to antioxidant system, longevity determination, and age-related diseases were investigated at selected time points throughout the entire life of flies; As for the second part, similar experiment protocols were conducted, detecting various survival parameters and changes at molecular level, for fruit flies living under certain oxidative stress, such as, 10% lard fatty acid in diet, intensive paraquat (PQ) / hydrogen peroxide (H_2O_2) challenge, or chronic PQ exposure.

Results showed that 10 mg/ml of BTE, SIF, AP or 5 mg/ml of BBE in diet had the anti-aging activity in fruit flies. The mean life spans were increased by at least 10%. On the one hand, those foods or nutraceuticals could in general elevate expressions and activities of endogenous antioxidant enzymes as well as lower LPO level to certain degrees in fruit flies. On the other hand, longevity determined gene MTH was down-regulated by AP or BBE in diet.

The present study also investigated the potentials of selected nutraceuticals in antagonizing exogenous oxidative challenge and alleviating neurodegenerative diseases. Addition of 10% fatty acid derived from lard significantly shortened the maximum lifespan from 74 to 15 days in fruit flies. Administration of 10 mg/ml BTE in diet could prolong it to 28 days, increasing by 87%. Gene expression of CAT was down-regulated significantly with addition of lard fatty acids into diet while BTE supplementation could partially restore it to normal level. A similar changing pattern was observed in CAT activity test. However, SIF did not show a comparable activity in this assay.

In addition, pretreatment of BTE, AP, or BBE could enhance the resistance to

intensive oxidative stress induced by PQ / H_2O_2 in OR flies but not in SOD^{n108} or CAT^{n1} mutant flies. Chronic exposure to PQ could shorten lifespan of fruit flies from more than 73 to less than 35 days while it reduced their climbing ability by more than 60%. At the same time, PQ exposure led to a reduced expression of SOD, CAT, and Rpn11 with accumulation of ubiquitinated proteins. Consumption of AP or BBE could partially and significantly slow or reverse the aging process and locomotive dysfunction as well as elevate expressions and activities of the antioxidant enzymes and Rpn11 in fruit flies.

Furthermore, to exclude the possibility that the anti-aging activity of selected nutraceuticals in fruit flies was actually due to food restriction. Body weight tracking and food intake comparison were conducted in the present study. Results revealed no difference in average body mass and stomach redness index between the flies in control and experimental groups, proving that the anti-aging activity of these foods and nutraceuticals was not mediated by caloric restriction.

In conclusion, the present study demonstrated that BTE, SIF, AP, or BBE in diet could prolong the mean lifespan of fruit flies. Therein, BTE could reduce the high mortality rate caused by a high-fat diet; AP or BBE was able to alleviate PQ-induced mortality rate and restore the decline of locomotive activity in fruit flies. The anti-aging and anti-neurodegenerative activities were at least partially mediated by their interaction with endogenous antioxidant system, Rpn11, and MTH. Furthermore, it has also been proved that this lifespan-prolonging activity was not due to changes in food intake.

摘要

衰老是一個十分複雜的生化過程，其中涉及到基因、環境等各方面因素。隨年齡增長而蓄積的氧化傷害是目前最廣爲接受和深入研究的衰老機理之一。基於該理論，提升抗氧化防禦力有益於壽命延長。對飲食的調控，包括卡路里限制、食物補充等方案已經被證明有延長壽命和緩解相關衰老疾病的功效。具有強抗氧化活性的天然營養添加劑可能具有良好的抗衰老效果。本研究選取了以下四種天然產物：紅茶提取物（BTE）、大豆異黃酮（SIF）、蘋果多酚（AP）、藍莓提取物（BBE），以黑腹果蠅（*Drosophila melanogaster*）爲實驗模型，研究它們在體內的抗衰老活性。

攝入茶、大豆、蘋果、藍莓等能夠降低心血管疾病、多種癌症、高膽固醇血症等的發病率。但是，關於它們在體內的抗衰老和抗神經退行性疾病的活性卻鮮有報導。本項目研究了以上四種功能食品或是說天然營養品對正常生理條件或氧化刺激環境下的果蠅的壽命影響。

第一部分實驗主要是觀察記錄果蠅在基礎培養基和加入了不同劑量添加物的培養基下的壽命，在不同時間點檢測其內源性抗氧化酶的活性，脂質過氧化物水準，抗氧化相關基因、長壽基因、衰老相關疾病基因等的表達水準；第二部分實驗是採用類似的研究方案去記錄和檢測不同氧化刺激環境下果蠅的各項存活指標和內在分子水準上的調控變化。這些氧化壓力環境包括：在培養基中加入 10%的由豬油中提取的脂肪酸，急性百草枯（PQ）或是過氧化氫（H_2O_2）刺激，慢性百草枯刺激等。

研究結果顯示，10mg/ml 的紅茶提取物，大豆異黃酮，蘋果多酚或是 5mg/ml

的藍莓提取物在果蠅體內具有明顯抗衰老功效。在這些實驗中，果蠅平均壽命可以延長至少 10%。一方面來講，這些食品或天然產物能夠提高編碼抗氧化蛋白的基因表達水準，增強抗氧化酶活性並且在不同程度上降低果蠅體內的脂質過氧化物水準。另一方面，長壽基因 MTH 的表達在蘋果多酚和藍莓提取物組中顯著下調。

與此同時，本項目還研究了部分天然提取物對外來氧化壓力的抗性及對神經退行性疾病的緩解作用。在食物中加入 10%的脂肪酸可以將果蠅最長壽命由 74 天縮短至 15 天。而 10mg/ml 的紅茶提取物可以將其延長至 28 天，增長率達 87%。同時，CAT 的基因表達會隨著脂肪酸的攝入而顯著下調，紅茶提取物可部分使其表達水準恢復正常。CAT 抗氧化酶活的實驗也顯示出類似的結果。但是，大豆異黃酮在該實驗中並未顯示出相應活性。

此外，預先餵食紅茶、蘋果或是藍莓提取物的果蠅對 PQ 或 H_2O_2 誘導的急性氧化刺激的抵抗能力顯著增強。但是這種抗性的提高僅限於 OR 果蠅，在 SOD 或是 CAT 基因敲除的果蠅上無法觀察到。長期的 PQ 環境會將果蠅的壽命從 73 天以上降到 35 天以內，同時，果蠅的攀爬力也會隨之下降 60%以上。此外，PQ 會下調超氧化物歧化酶、過氧化氫酶、Rpn11 的基因表達並誘導泛素化蛋白的蓄積。攝入蘋果多酚或是藍莓提取物的果蠅能夠顯著地減慢衰老效果，緩解運動功能缺失，並且顯著提高 Rpn11、抗氧化蛋白的基因表達以及抗氧化酶的活性。

此外，爲了排除實驗過程中可能存在的食物限制對壽命的影響和對天然產物抗衰老活性的評價，本研究跟蹤記錄了果蠅的體重，並對不同組間的進食量進行比較。結果顯示，對照組和實驗組果蠅的平均體重及腹部紅斑的參數水準沒

有顯著差異，進而證明這些食品或天然營養物的抗衰老功效並非由能量限制實現。

綜上所述，本研究證明紅茶提取物、大豆異黃酮、蘋果多酚和藍莓提取物在果蠅體內具有抗衰老活性。其中，紅茶提取物可以降低高油脂培養基引起的高死亡率；蘋果多酚或藍莓提取物則能夠緩解 PQ 引起的死亡率上升和運動功能下降。這些抗衰老和對抗神經退行性疾病的活性可能跟選取的天然產物與果蠅體內的抗氧化體系、Rpn11 及 MTH 間的反應、調控有關。而且，這種壽命延長效果並非由食物攝取量的變化引起。

Lists of Abbreviations

AD	Alzheimer's disease
ANOVA	Analysis of variance
AP	Apple polyphenols
BBB	Blood brain barrier
BBE	Blueberry extracts
BTE	Black tea extracts
CAT	Catalase
CcO	Cytochrome c oxidase
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CuZnSOD (SOD1)	Copper-Zinc superoxide dismutase
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ETC	Electron transport chain

FDA	Food and Drug Administration
FRTA	Free radical theory of aging
GPCRs	G protein-coupled receptors
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HDL	High density lipoproteins
HPLC	High performance lipid chromatography
HSP	Heat shock protein
LDL	Low density lipoproteins
LOOHs	Lipid hydroperoxides
LPO	Lipid peroxidation
MDTA	Mitochondrial decline theory of aging
MnSOD (SOD2)	Manganese superoxide dismutase
MTH	Methuselah
OD	Optical density
OR	Oregon-R-C
PCR	Polymerase chain reaction
PD	Parkinson's disease
PQ	Paraquat
PTA	Programmed theories of aging

RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SIF	Soybean isoflavones
STA	Stochastic theories of aging
TF_1	Theaflavin-1
TF_2A	Theaflavin-3-gallate
TF_2B	Theaflavin-3'-gallate
TF_3	Theaflavin-3,3'-digallate
UPS	Ubiquitin proteasomal system
UV	Ultraviolet
VSMC	Vascular smooth muscle cells

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Chapter 1

General Introduction

1.1 Introduction

Pursuit of youth is one of the most conserved instincts for mankind. However, during the past centuries, even the most powerful emperor could not get even close to the idea of immortality. To date, our understanding to aging is still quite limited. As a complex biological process, aging involves a variety of factors. On the one hand, the variation of average life span from different regions is believed due to the differences in not only genes, but also environmental conditions and eating habits. On the other hand, most organisms actually die from age-related diseases rather than aging itself. It is estimated that around 100,000 people worldwide die of age-related causes each day (Aubrey et al., 2007). In modern society, neurodegenerative diseases have been a rising lethal threat to human beings. WHO has also promoted the concept of 'healthy life span', aiming to increase the ratio of healthy to total life expectancy.

The first documented study on aging issue was conducted in 1532 by Muhammad in his book 'Ainul Hayat' (Ainul et al., 2007). Almost half a century has passed, the mechanism and cause of aging are still not clear. In order to increase both average and maximum life spans as well as decrease the occurrence of age-related

diseases, aging needs to be explored at molecular level more thoroughly. Scientists have established different theories of aging during past decades. Among them, free radical and mitochondrial theories are now most widely accepted. Generally speaking, these theories state that aging is due to the accumulation of oxidative damages caused by free radicals, as well as the subsequent dysfunction of mitochondria electron transport chain. Based on this hypothesis, if free radicals are scavenged more effectively, then life span can be prolonged, at least to a certain degree. At the same time, with lower degree of oxidative damages in neurons, age-related diseases might also be ameliorated.

1.2 Overview of current aging theories

There are dozens of theories established to illustrate aging process. Arking (1991) suggests that the causes of each theory are supposed to arise from programmed or stochastic processes. Specifically speaking, if the effects postulated by one theory are conjectured to take place accidentally, then it will be classified into the category of stochastic theories; on the contrary, if the properties of the theory are someway built into the basic biology of the species and supposed to happen in certain fixed sub-cellular locations, then it will be in the group of programmed theories.

1.2.1 Stochastic theories of aging (STA)

Basically, this category of theories share a common feature stating that aging is due to the failure of repairing stochastic damages in cells.

1.2.1.1 General development of STA

The precursor of this concept is the wear and tear theory, initially raised by Weismann in 1890s, who believed that all organisms were constantly exposed to wounds, infections, injuries; and also from time to time, consuming excessive fat, sugar; receiving undue UV lights or out-sourced stresses. Those accumulated damages would cause minor damages to cells and tissues, contributing to the age-related decline of organ functional efficiency. However, this theory's practical value is far less than its conceptual contribution. It has been revealed that animals raised in protected environment, who does not suffer from those minor exogenous insults, still age (Edward, 1999). Later on, scientists try to modify and even re-formulate the failure of repair hypothesis. Somatic mutation theory suggested by Jacobs and Court (1966) postulates that aging is due to alterations of chromosome number or formations of lesions in existing chromosomes, caused by accumulation of stochastic genetic mutations. Almost 10 years later, further evidences gathered by Hart and Setlow (1974) help to develop the theory of DNA damage and repair,

claiming that DNA damage contributes to aging process and there is a positive correlation between DNA repair capacity and life span. However, nowadays those theories are no longer regarded to be the sole potential candidates for the explanation of aging. As a promising modified successor, free radical theory has been becoming one of the most widely accepted aging mechanism hypotheses.

1.2.1.2 Free Radical Theory of Aging (FRTA)

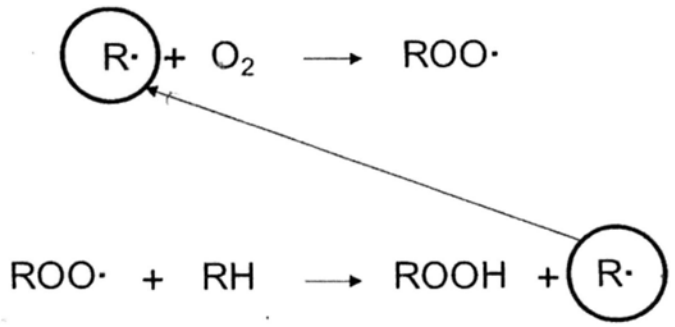
FRTA was first proposed by Harman (1956), stating that aging is due to accumulation of oxidative damages to tissues and organs caused by free radicals. It has been considered as one of the major theories providing a testable biological mechanism for aging process.

1.2.1.3 Free radicals and their deleterious effects

Oxygen is the essential element that keeps organisms alive. Most plants and animals require oxygen for energy production. However, there are highly reactive, naturally occurring chemicals induced to form in the presence of oxygen. Free radicals are any substances with unpaired electrons in the outer shells and ready to react with healthy molecules in a destructive way. They can be produced in large quantities in cells by different mechanisms, such as, exposure to oxygen, radiation, or environmental toxins, for example, pesticide and herbicide. The three major

stages of free radical reactions are: initiation, propagation, and termination. No matter how it is initiated, once formed, the free radicals can propagate itself indefinitely with the help of oxygen until those radicals reach a high concentration to react with each other and produce a non-radical species (Gutteridge and Halliwell, 2000) (Figure 1).

- **Propagation**



- **Termination**

$$R\cdot + R\cdot \longrightarrow R:R$$

$$ROO\cdot + ROO\cdot \longrightarrow ROOR + O_2$$

$$R\cdot + ROO\cdot \longrightarrow ROOR$$

Figure 1 Free radical reactions: self propagation and termination.

Reactive Oxygen Species (ROS) cover a wider range. Generally speaking, any highly reactive molecules containing oxygen can be classified into this category. ROS are unavoidable products during normal intracellular metabolism. They actually play essential role in cell differentiation, proliferation, host defense response, etc (Sroka and Madeja, 2009). However, their bad reputations are definitely overwhelming. Various cell components are believed to be damaged by oxygen-derived free radicals, of which lipid peroxidation, DNA damage, and protein oxidation are probably the most critical.

1.2.1.3.1 Lipid peorxidation

Polyunsaturated fatty acids, the main component of cell membranes, are vulnerable to free radical attack; because they contain such multiple double bonds, which possess extremely reactive hydrogens. As a result, the structure is susceptible to free radicals, especially hydroxyl radicals, which will lead to the destruction of cell membrane permeability, and eventually the cellular dysfunction (Douglas et al., 2003).

1.2.1.3.2 DNA damage

DNA damages caused by free radicals mainly include strand break, cross-linking, base hydroxylations, and base excision. The induction of those oxidatively induced

DNA damages will result in mutagenesis and consequently transformation, especially if combined with a deficient apoptotic pathway (Liu et al., 2002; Kryston et al., 2011).

1.2.1.3.3 Protein oxidation

Proteins are also believed to be the main targets of free radicals, or rather, ROS in cells. Aromatic amino acids, cysteine, and disulphide bonds are susceptible to free radicals, which will lead to protein denaturation and enzyme inactivation (Douglas et al., 2003). Furthermore, the reactive protein derivatives generated might act as intermediates to induce propagation of oxidative damages to other cell components (Gebicki et al., 2010).

1.2.1.4 Antioxidant systems

Based on FRTA, if excess amount of free radicals can be efficiently scavenged, the damages caused by accumulated oxidative stress will be ameliorated, thus leading to extended life span. There are two main antioxidant systems, enzymatic antioxidants and non-enzymatic ones. More importantly, they work systematically together, forming an effective antioxidant defense system (Matés et al., 1999).

1.2.1.4.1 Enzymatic antioxidants

This is the main defense system against reactive oxygen species in vivo, mainly

including superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPx), and Glutathione reductase (GR) (Figure 2).

There are two major types of SOD. One is CuZnSOD (SOD1), which mainly exist in cytoplasm, with Copper and Zinc in the active site. The other one is MnSOD (SOD2), locating in mitochondrial matrix, with Manganese in the active site. They can catalyze the reaction to decompose superoxide anion radicals into H_2O_2, which will then be converted to water and oxygen by CAT or GPx (Lippman, 1983).

CAT is one of the most efficient redox enzymes, with Iron in its active site, mainly found in peroxisome (Chelikani et al., 2004). It can catalyze the conversion of H_2O_2 into water and oxygen, which otherwise would be changed into hydroxyl radicals, one of the most active and harmful radicals to living cells.

GPx is a selenium-containing enzyme, protecting cells and tissues from oxidative damage by removing H_2O_2 with the oxidization of glutathione. On the other hand, GR can convert oxidized glutathione to its reduced form. However, the contribution of GPx in insects is relatively low (Helfand et al., 2003). So in fruit flies, SOD and CAT are of more research interests.

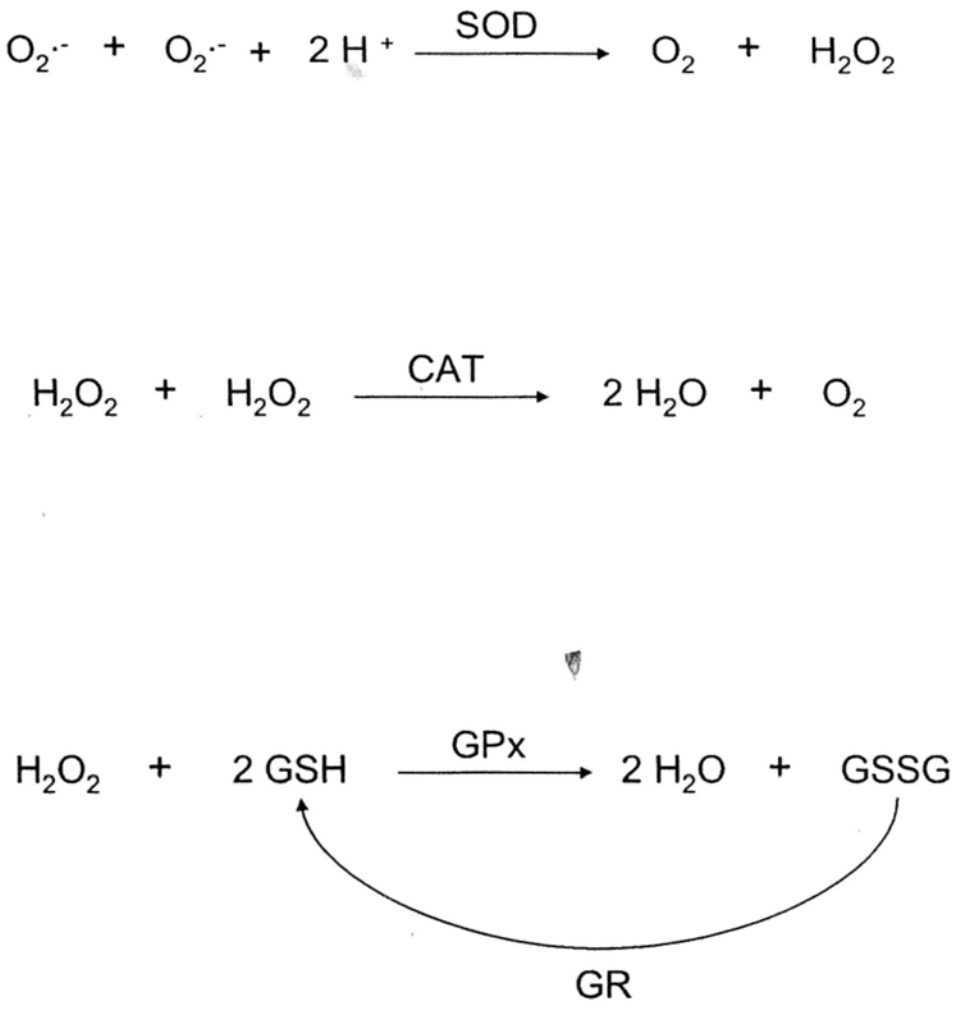


Figure 2 Main enzymatic antioxidant defense system in vivo and their reactions on scavenging free radicals and hydrogen oxide

1.2.1.4.2 Non-enzymatic antioxidants

According to previous publications, non-enzymatic antioxidants can not only provide direct protection against oxidative damages, but more importantly enhance the function of endogenous enzymatic antioxidants, working with them together to scavenge reactive species more efficiently (Balsano and Alisi, 2009). Vitamin C and E are the most renowned antioxidants in this category. However, recent study revealed that, under certain circumstances, they might function as pro-oxidants (Weinberg et al., 2001).

In addition to Vitamins, there are many small molecules which serve as non-enzymatic antioxidants, such as, flavonoids, carotenoids, etc. They can be obtained from our daily diets, belonging to a group called nutraceuticals (Kharb and Singh, 2004; Petchetti et al., 2007).

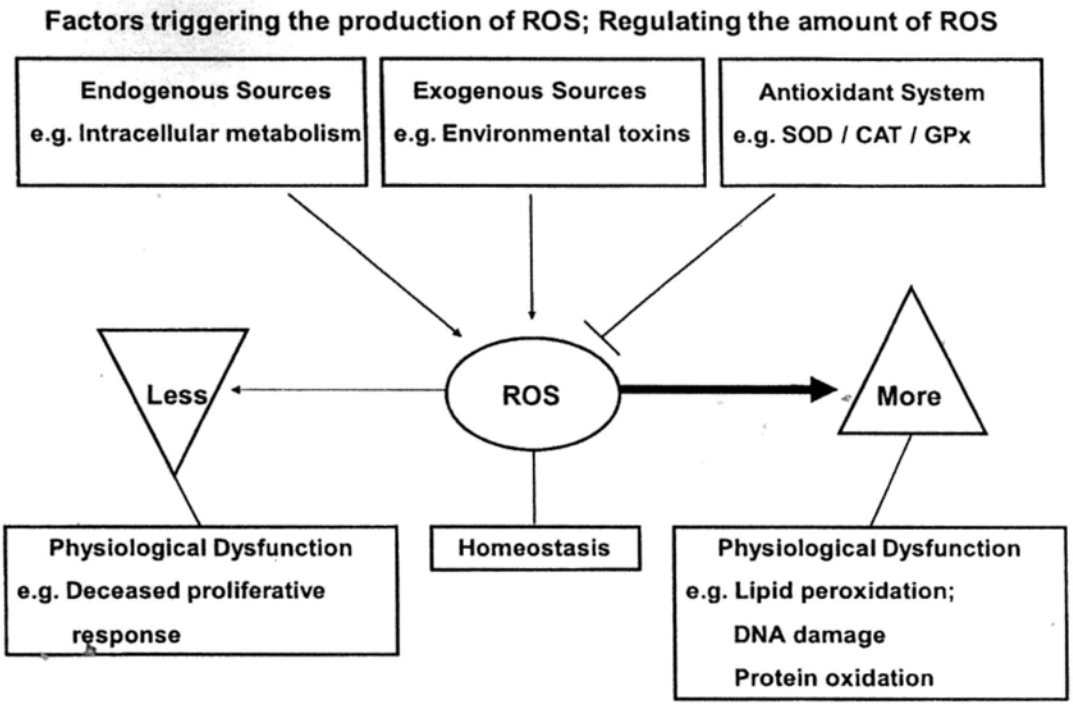


Figure 3 The homeostasis of ROS. Normal intracellular metabolism and external toxins can both result in ROS production. SOD, CAT, GPx form the main enzymatic antioxidant defense system in vivo, scavenging those highly reactive species. The homeostasis condition represents the ideal amount of ROS in vivo. Insufficient ROS might lead to physiological dysfunctions, such as, decreased proliferative response, deficient differentiation. On the other hand, excess amount of ROS would induce oxidations and damages to lipids, DNA, and proteins.

1.2.2 Programmed theories of aging (PTA)

1.2.2.1 General development of PTA

The rate of living theory was first stated by Buffon in 1740s, and then further popularized by Pearl in 1920s. This theory postulated that there was an inverse relation between longevity and metabolic rate, which sounded plausible and actually stayed popular for a while. However, the predictions for which this theory based on was then challenged and disproved by later researches. Experiments conducted in 1980s showed that not only did the metabolic rate vary among species, it did not even keep at a constant value for diverse populations within the same species (Arking et al., 1988). Mitochondrial decline theory now plays dominant role in aging mechanism elucidation in this category.

1.2.2.2 Mitochondrial decline theory of aging (MDTA)

MDTA has for so long been proposed to play a vital role in aging process (Harman, 1972; Beckman and Ames, 1998). Mitochondrial respiratory capacity, showing an inverse correlation with the amount of reactive oxygen species, declines with aging. Cytochrome c oxidase (CcO), the terminal oxidoreductase of mitochondrial electron transport chain (ETC), is consistently reported exhibiting an age-related decline in both invertebrates and vertebrates (Benzi et al., 1992;

Schwarze et al., 1998). Especially, its subunits Ⅲ and Ⅵb are significantly reduced in aging flies (Ren et al., 2010). It has been reported that CcO deficiency would result in reduction of total ETC activity due to increased production of either superoxide anion radicals or hydrogen peroxide in mitochondria. Therefore, there are solid connections between MDTA and FRTA. Theoretically speaking, enhancing antioxidant defense system will not only lead to reduced amount of free radicals, but also ameliorate the function decline of mitochondrial, if it does not totally reverse the reduction.

1.2.3 Other theories of aging and age-related diseases

1.2.3.1 Longevity determined genes

There are many other theories trying to elucidate aging process and age-related diseases. As one of the most complicated biological processes, aging involves factors covering a wide range from genetic to environmental ones. Single gene mutation has been proved to be one of the most useful techniques to understand aging mechanisms at molecular level. Previous studies in *C. elegans*, *Drosophila*, and rats have revealed dozens of genes, whose mutation would lead to extended life span. Those genes were named as longevity determined genes (Murakami and Johnson, 1996; Hekimi et al., 2001). In *Drosophila*, single P-element insertion mutation lines

can be easily generated (Cooley et al., 1988) and the newly inserted locus could be identified by flanking sequence of the inserted transposon (Spradling et al., 1995).

Lin et al (1988) published a paper stating that a P-element insertion line was identified with an extra 35% longer life span, compared to wild type flies. At the same time, they claimed that these *methuselah* (Mth) mutant flies showed higher resistance to various stresses, such as, high temperature, starvation, diet paraquat, etc. (Lin et al., 1998). Later on, it has been revealed that the Mth protein belongs to class B of G protein-coupled receptors (GPCRs), a protein family, with their iconic, large ligand-binding N-terminal extracellular domains, playing key role in intracellular signal transduction (Kikkou et al., 2007; Harmar, 2001). To date, the specific function of Mth is still unknown. However, it has been demonstrated that flies expressing a Mth antagonist peptide live significantly longer (Ja et al., 2007). Furthermore, at least 30% of current drugs act through GPCRs (Wise et al., 2002) and human homologous gene to Mth (APG1) was reported by Kikkou et al. (2006), who indicate that Mth can be a promising candidate for anti-aging research.

Table 1 Selected longevity determined genes recently recognized in fruit flies, for which allelic variation is associated with extension in longevity

Selected Longevity determined genes whose decrease lead to extended life span in *D. melanogaster*	
Gene name	**Brief description**
mth	A P-element insertion at *mth* increase life span by 35% (Lin et al., 1998)
Indy	P-element insertions at *Indy* show extended mean life span (Rogina et al., 2000)
chico	Heterozygous for chico show median life span increase (Clancy et al., 2001)
dTOR	Inhibition of TOR pathway leads to 24-26% life span extension (Kapahi et al., 2004)

1.2.3.2 Ubiquitin proteasomal system (UPS)

Protein misfolding and aggregation are essential factors, contributing significantly to aging process and especially to the formation and development of neurodegenerative diseases, such as, Parkinson's disease (PD), Alzheimer's disease (AD) (Lee et al., 2009). They can be cleared mainly by UPS (Savitt et al. 2006; Thomas and Beal 2007). It is reported that age-related decline of the 26S proteasome activity is an important element, which is evolutionarily conserved in aging process. Thus, maintenance of the 26S proteasome activity with age is vital for promoting longevity. The 26S proteasome is a complex of the 20S core chamber attached with two 19S caps on each end. The 20S proteasome itself cannot degrade multi-ubiquitinated proteins since the pores leading into the catalytic chamber are closed. The opening of the gates is triggered by the 19S attached to the ends of the 20S core chamber (Verma et al., 2001; Thrower et al., 2000).

Rpn11 is one lid component of the multiple subunits making up the 19S, which can be divided into two subcomplexes, i.e., the base and lid (Figure 4). It is reported that knock down of Rpn11 will reduce 26S proteasome activity, leading to increased age-related accumulation of ubiquitinated proteins and shortened life span. On the contrary, overexpressing Rpn11 can reduce age-related accumulation of ubiquitinated proteins, thus extends life span (Tonoki et al., 2009).

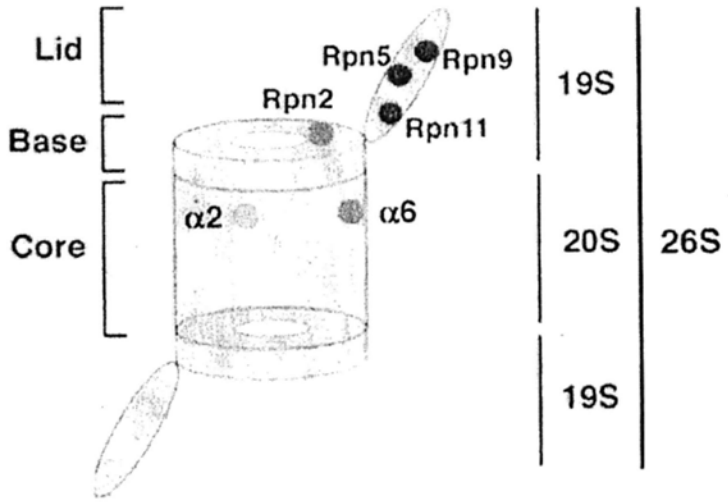


Figure 4 Structure of the 26S proteasome and subcellular localization of Rpn11 (Tonoki et al., 2009)

1.3 Nutraceuticals

The term 'nutraceutical' is actually a combined form of 'nutrition' and 'pharmaceutical'. The generally accepted definition is 'a food or part of a food which provides health benefits, including the prevention and / or treatment of a disease'. Most nutraceuticals are dietary supplements. Their beneficial effects have been demonstrated by previous investigations. Studies both in vitro and in vivo reveal that consumption of nutraceuticals, especially the ones with high antioxidant capacity, has an inverse relation with cardiovascular diseases, various cancers, diabetes, etc. However, their anti-aging activity is yet to be proven. On the basis of FRTA, it is reasonable to postulate that any substance with great antioxidant capacity can be a potential candidate for anti-aging research, which serves as theoretical supports for the hypothesis in this project. The nutraceuticals involved in my study are as follows.

1.3.1 Black tea extracts

Tea, next to water, is the second most popular beverage around the world. Black tea is more widely consumed in western countries while green tea is preferred in the eastern world. Black tea extracts mainly contain epicatechins and theaflavins.

Evidences from clinical trials suggest that consumption of tea has various health

benefits. Leenen et al. (2000) demonstrated that drinking either green tea or black tea would lead a significant increase in plasma antioxidant potential by ferric-reducing antioxidant power (FRAP) assay. Furthermore, it has been reported in different population studies that consumption of green tea or black tea could significantly reduce DNA oxidation and lipid peroxidation (Meng et al., 2001; Rietveld et al., 2003).

As to the anti-aging activity of tea, previous studies conducted in our lab revealed that green tea extracts could extend mean life span of *Drosophila* with the enhancement of flies' endogenous antioxidant defense system (Li et al., 2007; Li et al., 2008). Studies on *C.elegans* also showed similar results, indicating that treatment of EGCG would lead a significant longer lifetime (Abbas et al., 2009; Zhang et al., 2009). In mice, consuming tea polyphenol, starting from 13 month till death, could increase average life span by more than 6% (Kitani et al., 2007). However, reports for the anti-aging property of black tea extracts are lacking.

1.3.2 Apple polyphenols

'One apple a day keeps doctors away'. Apple has been recognized as healthy fruit in many cultures. It contains a large number of phytochemicals, mainly polyphenols, including chlorogenic acid, phloretin, proanthocyanidin B2, epicatechin, catechin and rutin.

Consumption of apple has been inversely associated with the risk of cardiovascular disease, hypercholesterolaemia, and various cancers. The Women's Health Study, involving almost 40,000 women with a 6.9 year follow-up, examined the correlation between flavonoids and cardiovascular disease, finding an inverse correlation between cardiovascular disease and consumption of apples (Sesso et al., 2003). In Iowa, a study of nearly 35,000 women revealed that apple consumption was inversely related to the death caused by coronary heart diseases in postmenopausal women (Arts et al., 2001). Furthermore, several clinical studies have linked apple consumption with a lower risk of cancers, especially lung cancer. It was found that eating apples would reduce the risk of lung cancer, with being more effective in women (Le Marchand et al., 2000; Feskanich et al., 2000).

Experiments on animals showed similar results and revealed some potential mechanisms of the beneficial effects of apple. It was reported that, in cholesterol-fed rats, there was a significant reduction of plasma and liver cholesterol level along with increased amount of high density lipoproteins (HDL) (Aprikian et al., 2001). Another study conducted by Leontowicz et al. (2002) has demonstrated that apples have much better cholesterol lowering effects than pears and peaches, suggesting that, having similar amount of fiber content, apples' superior activity might be due to its larger quantity of phenolic components. Apple has been proved effective in

inhibiting LDL oxidation while the greatest inhibitory effect comes from apple peels (Pearson et al., 1999). In addition, apple can greatly inhibit the growth and proliferation of liver and colon cancer cells (Wolfe et al., 2003; Eberhardt et al., 2000). Moreover, apple juice concentrate has been demonstrated effective in neuroprotection in both genetically compromised and normal aged mice (Rogers et al., 2004; Tchantchou et al., 2004; Tchantchou et al., 2005). However, anti-aging activity of apple and the underlying mechanisms remain elusive.

1.3.3 Soybean isoflavones

Soybeans are considered a great source of complete protein, which contains all the essential amino acids in sufficient amounts for human use (Henkel, 2000). They can serve as a good alternative to animal proteins for vegetarians. Daidzein and genistein are the main isoflavones in soybeans.

The notion that consumption of soy protein could offer health benefits has been popular during the past decades. It has been inversely associated with hypercholesterolaemia, bone loss, and various cancers. According to Food and Drug Administration (FDA), '25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease'. The meta-analysis conducted by Anderson et al. (1995) demonstrates that consumption of soy protein can decrease serum total cholesterol, LDL cholesterol and triglyceride

concentrations. Meanwhile, it is claimed that the decreasing effects are at least partially related to subjects' initial cholesterol concentrations and isoflavones might account for at least 60% of the cholesterol-lowering effects of soy protein (Anderson et al., 1995). More than 50 trials since then, investigating health benefits of isoflavones, have been conducted (Lethaby et al., 2007; Howes et al., 2006). It has been further demonstrated that LDL reduction induced by soy protein without isoflavones is mild, indicating that isoflavones might be the main active compounds, contributing to the cholesterol-lowering effects (Sacks et al., 2006; Crouse et al., 1999). Besides that, evidences from clinical studies reveal that consumption of soy foods, especially isoflavones, leads to higher femoral / lumbar spine bone mineral density in postmenopausal women (Setchell et al., 2003; Peeters et al., 2003; Sarkar and Li, 2003; Magee et al., 2004; Fukui et al., 2000; Somekawa et al., 2001; Mei et al., 2001). It is also reported that in Asian countries where soy foods are more prevalent, the incidence of breast and endometrial cancer is relatively low. Actually plasma genistein in Japanese can reach 4μM while the one can be as low as 40nM in people consuming a typical western diet (Mann et al., 2007; Peeters et al., 2003). Moreover, the case-control studies carried out by Shu et al. (2001) and Wu et al. (2002) have proved that high amount of soy intake was associated with low risk for breast cancer. However, epidemiological findings on its anti-cancer activity are not

as consistent as the ones on its cholesterol-lowering effect.

Though the underlying mechanisms for the efficacy of soybean isoflavones are still not fully understood, studies on cells, isolated arteries and animals provide insightful clues. It is stated that isoflavones are able to activate endothelial nitric oxide synthase, exerting vasodilatory effect (Mann et al., 2007). Moreover, studies on isoflavones' effects on vascular smooth muscle cells (VSMC) reveal that isoflavones can inhibit cell proliferation and DNA synthesis (Pan et al., 2001). Generally speaking, it is believed that actions of isoflavones largely overlap with that of estrogens, especially for its influence on cardiovascular diseases (Cano et al., 2010).

However, few reports exist on demonstrating soybean isoflavones' effects on life span; the conclusions are also controversial. Borra´s et al. (2006) show that antioxidant activity of genistein is mediated via the up-regulation of antioxidant gene expression, such as increased mRNA levels of MnSOD and activation of NFκB, suggesting that supplementation of isoflavones may be beneficial in decreasing oxidative stress, thus contribute to life span extension. However, Altun et al. (2011) recently point out that genistein would decrease the maximum life span of *D. melanogaster*, which is similar to the observation in *C. elegans* by Wu et al. (2002).

1.3.4 Blueberry extracts

Blueberries, containing large amounts of polyphenols, possess greater antioxidant capacity than most other fruits and vegetables (Prior et al., 1998). It has been reported that consumption of compounds in blueberries can retard age-related physiological and functional deficits (Joseph et al., 2005). Krikorian et al. (2010) have finished recently their human trial study evaluating the health benefits of blueberry supplementation, revealing that daily consumption of wild blueberry juice for 12 weeks would improve memory function in older adults with early memory decline. However, larger sample size and more consistent clinical data are lacking to draw a conclusion.

Studies in vitro and on experimental animal models also provide solid and inspiring results. Galli et al. (2006) claimed that blueberry supplemented diet could reverse age-related decline in hippocampal heat shock protein (HSP) neuroprotection in rats. Similarly, blueberries are also suggested effective in enhancing cognitive and motor behavior as well as attenuating cognitive declines in object recognition memory in aged rats (Shukitt-Hale et al., 2006; Goyarzu et al., 2004). Furthermore, age-related deficits in NMDAR-dependent long-term potentiation, a cellular substrate for learning and memory, are also reported to be ameliorated by blueberry enriched diet (Coultrap et al., 2008). As to the anti-aging property of blueberries,

Wilson et al. (2006) has demonstrated that blueberry extract, mainly the fraction enriched in proanthocyanidin compounds, in diet could increase life span and slow aging related declines in *C. elegans*.

1.4 Paraquat, oxidative stress and neurodegenerative diseases

Once widely used an herbicide, paraquat (PQ), is now prohibited in most European countries, and under strict restriction in America. It has the bad reputation as one of the most widely used suicide agents in third world countries. Its potential role in the development of neurodegenerative disorder is of greater concern. Previous reports state that long term exposure to PQ might be an environmental factor inducing neurodegenerative syndrome, especially PD.

PD is a neurodegenerative disorder in central nervous system. The main symptoms include rigidity, tremor, bradykinesia, and cognitive deficiency (Dauer and Przedborski, 2003). The name of the disease is actually after an English doctor J. Parkinson, the first one to publish detailed descriptions of this syndrome in 1817. As the second most common neurodegenerative disease after AD, the incidence of PD is reported from 8 to 18 per hundred thousand person each year (de Lau and Breteler, 2006). There are intensive studies on this age-related neurodegenerative disease during past years. However, the underlying mechanisms and specific biochemical factors inducing the initiation and progression of the disease are still not clear.

The brain is only about 2% of the total body weight, yet consuming around 20% of body oxygen (Moreira et al., 2005). Meanwhile, the brain contains high levels of polyunsaturated fatty acids, which are vulnerable to lipid oxidation. In addition, it is claimed that cells in the brain have relatively low activity of endogenous antioxidant enzymes. Thus, the brain is more susceptible to oxidative stress compared to any other organ (Kedar, 2003).

The occurrence of PD is believed to be related to both genetic and environmental factors. Environmental toxins, such as PQ and rotenone, have been demonstrated to increase the risk of PD in humans (Miller et al., 2009). It is proposed that cellular toxicity of PQ mainly comes from its redox cycle (Figure 5); oxidative stress is still the mainstream theory illustrating the formation of PD (Dinis-Oliveira et al., 2006). Previous work have revealed the positive correlation between amounts of oxidation of lipids, DNA, proteins and risk of neurodegenerative diseases, such as AD, PD, etc (Cardoso et al., 2005).

There are mainly two types of PQ exposure. Intensive challenge of PQ in fruit flies would lead to extremely high mortality rate in a short time, which might be, at least partially, due to its pulmonary toxicity. This assay is commonly used to evaluate fruit flies' resistance to oxidative stress. On the other side, chronic exposure to PQ is reported to induce damage to the basal ganglia and Parkinsonism. Previous work has

also demonstrated that PQ can permeate the blood brain barrier (BBB) into the CNS (McCormack and Di Monte, 2003). In neurons, PQ is reduced by NADPH to produce PQ radicals, superoxide anions, and eventually hydroxyl radical, one of the most destructive reactive species to living cells (Thiruchelvam et al., 2000). What's more, PQ can disturb mitochondrial respiration chain, inducing proteasome dysfunction, causing the accumulation of misfolded proteins (Yang and Tiffany-Castiglioni, 2005).

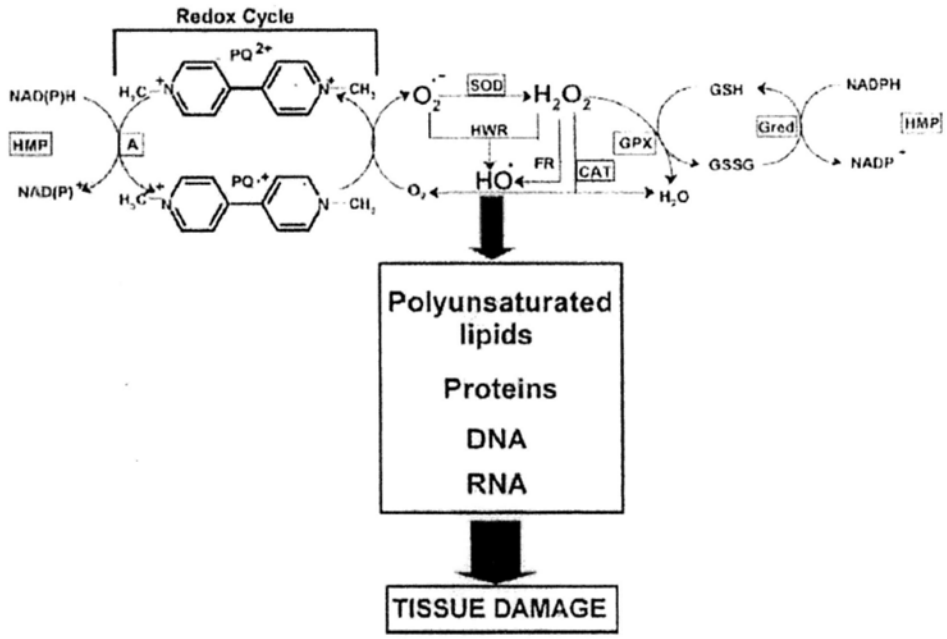


Figure 5 Schematic representation of PQ cellular toxicity. (A, cellular diaphorases; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; Gred, glutathione reductase; PQ^{2+}, paraquat; $PQ^{\cdot+}$, paraquat cation radical; HMP, hexose monophosphate pathway; FR, Fenton reaction; HWR, Haberweiss reaction) (Dinis-Oliveira et al., 2006)

1.5 Laboratory models on aging study

As stated by August Krogh, 'for many biological problems, there is an animal on which it can be most conveniently studied', it is critical to conduct a study on proper models in order to elucidate aging mechanisms more thoroughly. Studies on humans are most straight-forward. However, the duration of human aging is an important limiting factor since researchers themselves also, at the same time, go through the same process. Meanwhile, ethical issues also block many research experiments from human beings. Therefore, scientists turn to laboratory model systems and then try to extrapolate laboratory data to clinical value. The selection of models is diverse and under debate (Gershon and Gershon, 2000; Partridge and Gems, 2007).

1.5.1 Major model systems to study human aging

The mainstream model systems to conduct human aging study include: cells, yeast (*Saccharomyces cerevisiae*), roundworms (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), mice (*Mus musculus*), and rats (*Rattus norvegicus*).

Human cells are one of the major model systems in studying aging mechanisms. Scientists can easily focus on human biology when carrying out experiments on human cells. Nevertheless, in vitro data might not be always consistent with in vivo one. Meanwhile, the most widely employed parameters for cellular models on aging

study are cell proliferation and stress resistance. However, the correlation of those factors with organismal aging is still under serious debate (Cristofalo, 2001 and de Magalhaes, 2004).

Non-mammalian model systems, such as, yeast, roundworms, and flies, share a large number of key biological pathways with humans (Jafari et al., 2006), though their physiology and phenotypes are way from alike with mammals. Meanwhile, aging researches are always based on statistical analysis and comparison at the population level. Non-mammalians models are comparatively easier and cheaper to manipulate in large numbers. On the other hand, aging is so complex a biological process, involving too many factors at the same time. It is reasonable and practical to conduct assays on relatively simpler systems first to observe more direct and immediate response after certain treatment. Actually, many genes and signal pathways modulating aging process have already been identified in yeast (Jazwinski, 2000), worms (Johnson, 2002), and fruit flies (Tower, 2000), which serve as basis for further understanding human aging mechanisms. On the contrary, it is quite difficult to demonstrate the effect of a specific signal pathway in an intricate mammal system.

As to the mammal systems, such as, mice and rats, their physiology and daily activities are more parallel to humans, compared to those non-mammalian models. At the same time, as the mainstream animals employed in laboratory work for

decades, the related experiment protocols are quite mature and stable. However, there is still no solid evidence indicating that those rodents age for the same causes and mechanisms as humans (Gershon and Gershon, 2000). Therefore, if a particular age-related mechanism is investigated, simpler non-mammalian models might be more preferred a choice.

Last but not least, non-human primates are recently regarded as potential alternative for human aging studies (Nadon, 2006). It is claimed that Rehesus monkeys (*Macaca mulatta*) share about 90% of their genome with human beings (Roth et al., 2004). In addition, age-related changes in neurological structure and function of monkeys also share great similarity with those of humans (Small et al., 2004; Smith et al., 2004). Nevertheless, the actual employment of this non-primate model in aging study is still quite rare. First of all, those primates have comparatively long life span, which will be a practical problem for laboratory manipulation. Secondly, the costs to conduct experiments on those animals are relatively high (Nadon, 2006). Another serious issue is the moral concern by animal rights groups.

1.5.2 *Drosophila* model in aging research

1.5.2.1 History of fruit flies as a laboratory model

It is believed that *Drosophila* was first raised in quantity by Charles W. Woodworth when he was working at Harvard between 1900 and 1901. He then suggested to William E. Castle that fruit flies might be used for genetical work. Castle and his colleagues employed *Drosophila* in their work, which actually further inspired Thomas H. Morgan to work with those insects, serving as the basis of his 1933 Nobel Prize.

Drosophila model has been widely used for biological researches, especially in the field of genetics and developmental biology. In light of the study on genetics of longevity in fruit flies, specific genes regulating life span have been revealed during the past decades, which involved in stress response, antioxidant system, insulin signaling pathway and TOR pathway. It is reported that SOD or CAT mutant flies (partially knock out either SOD or CAT genes) will lead much shorter life spans along with a greater sensitivity to oxidative stress (Mackay and Bewley, 1989; Philips et al., 1989). On the contrary, transgenic flies with additional copies of CAT and SOD show median life span increase ranging from 6% to 33%; overexpression of SOD increases mean life span up to 40% (Orr and Sohal, 1994; Orr and Sohal, 2003; Sun and Tower, 1999).

Studies on the relation between diet supplements and life span of fruit flies have been continuously producing inspiring results. Experiments conducted by Bonilla et al (2002 and 2006) have demonstrated that melatonin in diet can significantly increase lifetime and the resistance to PQ challenge in *Drosophila*. Similarly, resveratrol has been proved effective in life span extension in fruit flies by Bauer et al. (2004) and Wood et al. (2004). In our laboratory, anti-aging activities of green tea and broccoli extracts in fly diet have been well studied (Li et al., 2007; 2008).

1.5.2.2 The advantage of *Drosophila* model in aging research

Serving as an efficient model in aging research for decades, fruit flies (*Drosophila melanogaster*) possess unique advantages over other animal models. Fruit flies and humans share many conserved physiological pathways, such as, superoxide metabolism, insulin-like signaling, etc., many of which have been proposed as vital elements for aging regulation (Jafari, 2006). Moreover, more than 70% of known disease-causing genes in humans are conserved in fruit flies and 50% of fly protein sequences have mammalian homologs (Reiter et al., 2001; Minois, 2006). In addition, the technique of genetic manipulation in *Drosophila* is now quite mature. There are a wide variety of transgenic flies available, which simplify the exploration for the targets, or influencing pathways of a specific compound in vivo

(Jafari, 2010). Meanwhile, fly strains with longer life span are reported to have no reduction in metabolic rate (Rose et al., 2002). Furthermore, *Drosophila* have complex nervous system yet relatively weak BBB, which make it suitable a model system for screening and evaluation of effects of drugs and functional compounds on neurodegenerative diseases (Bilen and Bonini, 2005; Sang and Jackson, 2005). In addition, fruit flies are comparatively easier and cheaper to maintain in large numbers, which is essential for cohort study. The short life cycle, tiny body size, high fecundity, and known sequence of full genome make it an ideal model for aging research at population level (Rose et al., 1992). On the other hand, the effects of diet supplements in fruit flies have been investigated, providing promising results in the last 20 years, which not only construct practical bench methods to do related analysis, but also package powerful statistical protocols to systematically estimate and assess the effects of certain supplement compound (Nusbaum et al., 1996). By and large, *D. melanogaster* model is more than simple and valid to be employed in the study of universal aging mechanisms.

Chapter 2

Anti-aging activity of black tea theaflavins (BTE) in *Drosophila melanogaster*

2.1 Introduction

Interest in relationship between diet and aging is growing. Research has shown that moderately reduced nutrient intake or dietary calorie restriction extends lifespan in rodents (McCay et al., 1935), fruit flies (Partridge et al., 2005), nematode worms (Lee et al., 2006), and yeast (Lin et al., 2002). The proposed underlying mechanisms associated with dietary restriction on lifespan mainly include its effect on growth retardation, reduction in body fat, decrease in metabolic rate, and attenuation of oxidative stress (Masoro, 2009). Some earlier researches have also suggested that not only total energy intake but also the composition of nutrients affect the lifespan and aging of an organism (Piper et al., 2008).

Dietary antioxidants have become popular in prevention of aging (Willis et al., 2009). Oxygen is essential to aerobic organisms because it is a final electron acceptor. However, oxygen in electron transport chain can continuously generate the by-products, reactive oxygen species (ROS), which is believed to cause aging of an organism (Gutteridge et al., 2007). To remove these ROS in cells, aerobic organisms possess an antioxidant defense system which consists of a series of enzymes, namely

superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Cutler, 1991). In addition, dietary antioxidants including ascorbic acid, vitamin A, vitamin C, α-tocopherol and plant flavonoids are also able to scavenge ROS in cells (Ames et al., 1993). Both endogenous antioxidant enzymes and exogenous dietary antioxidants build a defense base to terminate the propagation of the free radicals reactions, limit the formation of new free radicals and slow down the aging process.

Green tea is one source of excellent dietary antioxidants because it contains four epicatechin derivatives namely (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC). Green tea extract have been previously demonstrated to prolong the lifespan in fruit flies (Li et al., 2007). Black tea is also rich in antioxidants. In addition to EGCG, EGC, ECG and EC, black tea contains a mixture of four theaflavin derivatives including theaflavin-1 (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3'-gallate (TF_2B), and theaflavin-3,3'-digallate (TF_3).

2.2 Objectives

The present study was to investigate if black tea extract (BTE) could prolong the lifespan and have capacity of scavenging the free radicals in *Drosophila melanogaster*. The objective was to study the interaction between dietary BTE and gene expression of endogenous antioxidant enzymes namely SOD and CAT in

Drosophila melanogaster.

2.3 Materials and methods

2.3.1 Fly strains

Fly strains used in this study included Oregon-R-C (OR), SOD^{n108}/TM3 (SOD^{n108}), and OE/SM5 x Cat^{n1}/TM3 (Cat^{n1}) (Bloomington Drosophila Stock Center, Department of Biology, Indiana University, Bloomington, IN, USA). OR is a wild type fly which was used in all experiments unless specified otherwise. SOD^{n108} is a mutant with one pair of single SOD gene on 3L chromosome being knocked out while Cat^{n1} is a mutant with CAT gene on chromosome 3L being knocked out by a point mutation.

2.3.2 Diet

A basal diet was prepared according to the standard formulation described previously (Li et al., 2007; Roberts et al., 1998). In brief, 1000 ml diet contained 105g cornmeal, 21g yeast, 105g glucose, and 13g agar. Ethyl-4-hydroxybenzoate (0.4%) was added into diet to prevent mold growth. BTE was added into the basal diet at 5 and 10 mg/ml, respectively. For the fat-induced mortality experiments, the fatty acids derived from lard were added into the basal diet at 10% on a weight basis. The mixture was cooked and poured into each vial (5 ml each). For rearing the

stocks, 15 ml of the basal diet was poured and set into a vial. For the experimental flies, 5 ml of the basal or experimental diets was prepared per vial. BTE was obtained from Professor You-Ying Tu, Tea Research Institute of Zhejiang University, Hangzhou, China, while the lard fatty acids were prepared according to the method previously described (Li et al., 2008).

2.3.3 Effect of BTE on longevity of OR flies fed the basal diet

Male flies (2-day-old) developed from eggs were divided into 3 groups with 200 flies in each group rearing in 10 vials (20 flies per vial). The first group was maintained on the basal diet, while the rest two groups were fed one of the two diets containing 5 or 10 mg BTE/ml. The dead flies were counted every 2-3 days and the remaining alive flies were then transferred to a new vial containing the same diet. The feeding lasted 76 days. The two sets of experiments described above were similarly repeated and the fruit flies were killed at various time points to quantify the expression of SOD, CAT and MTH.

2.3.4 Paraquat treatment

Paraquat (1,1'–dimethyl-4,4'–bi-pyridinium dichloride; PQ^{2+}) (Sigma, St. Louis, MO, USA) is able to generate superoxide anion radicals (Michaelis et al., 1933). To examine the resistance of flies against superoxide-induced stress, both OR flies (n=400 in 20 vials) and SOD^{n108} mutant flies (n=400 in 20 vials) were maintained on

their corresponding control diet and experimental diet containing 10 mg BTE /ml, and incubated at 25℃. At day 25, the fruit flies in two groups were first starved for 2 hours, and then transferred into new vials containing a filter paper saturated with 1 ml of 20mM paraquat in a 6% glucose solution. Every 4-6 hours, dead flies were counted until all flies died.

2.3.5 Hydrogen peroxide (H_2O_2) treatment

H_2O_2 is able to generate a hydroxyl radical in the presence of some metal ions. Therefore, H_2O_2 was also used to examine the resistance of flies against OH-induced oxidative stress. OR flies (n=400) and Cat^{n1} mutant flies (n=400) were maintained on their corresponding control diet or experimental diet containing 10 mg BTE /ml and incubated at 25℃. Similarly, the fruit flies in the two groups were first starved for 2 hours, and then were transferred into new vials containing a filter paper saturated with 1 ml of 30% H_2O_2 in a 6% glucose solution at day 25. Every 4-6 hours, dead flies were counted until all of the flies died.

2.3.6 Effect of BTE on longevity of OR flies fed a high-fat diet

Similarly, 2-day-old male flies were divided into 3 groups with 200 flies in each group rearing in 10 vials (20 flies per vial). The first group was maintained on the basal diet containing no lard fatty acids (CTL), while the two experimental groups were fed diets containing 10% lard fatty acid only [CTL(Lard)] or with addition of 10 mg BTE/ml [BTE(lard)]. The dead flies were counted every 2-3 days and the remaining alive flies were then transferred to a new vial containing the same diet.

The feeding lasted 32 days. The two sets of experiments described above were similarly repeated and the fruit flies were killed at day 0, 5 and 10 to quantify the expression of SOD, CAT and MTH.

2.3.7 Measurement of lipid hydroperoxides (LPO)

A LPO assay kit (Cayman Chemical, Michigan, USA) was used to quantify the lipid oxidation in fruit flies. In brief, hydroperoxides could react with ferrous ions to produce ferric ions, which was detected using thiocyanate ion as the chromogen. The fruit flies (200) were homogenized in 2 ml of HPLC grade water and centrifuged at a speed of 1,500 g for 5 minutes at 0℃. The supernatant (800 µl) was aliquoted into a tube in triplicates followed by being deproteinized and extracted using a mixture of methanol and chloroform (1:2, vol/vol) saturated with nitrogen gas. Afterward, the sample was centrifuged at 1,500g for 5 min at 0℃, and the chloroform layer was collected into a test tube on ice. To initiate the reaction, 500µl of chloroform extract together with 450µl of methanol / chloroform solvent (1:2, vol/vol) was mixed with chromogen, which contained 50µl FTS Reagent 1 (4.5mM ferrous sulfate in 0.2M hydrochloric acid) and 50µl FTS Reagent 2 (3% methanolic solution of ammonium thiocyanate). After incubation at room temperature for 5 min, absorbance of each sample was measured in a spectrophotometer at 500 nm in quartz cuvette.

2.3.8 SOD activity

An assay kit (Cayman Chemical, Michigan, USA) was used to quantify the SOD activity in fruit flies. The principle is that a tetrazolium salt can detect superoxide anion radicals generated by xanthine oxidase and hypoxanthine while SOD is able to remove the superoxide anion. In general, one unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The fruit flies (n=100 in 5 vials) were homogenized in 1 ml of cold 20mM HEPES buffer (pH 7.2, with 1mM EGTA, 210mM mannitol and 70mM sucrose) followed by centrifugation at a speed of 1,500 g for 5 minutes at 4℃. The supernatant was transferred into a new tube on ice and then subjected to centrifugation at 10,000 g for 15 minutes at 4℃. The supernatant contained the cytosolic copper zinc containing SOD (CuZnSOD or SOD1), and the pellet contained mitochondrial manganese containing SOD (MnSOD or SOD2). The supernatant was removed into a new tube and the mitochondrial pellet was suspended in 0.5 ml cold HEPES buffer. The sample (10 µl) in triplicates was used for each test. The diluted radical detector containing tetrazolium salt (200 µl) was added onto 96 well plates together with 10 µl sample. The reaction was initiated by adding 20 µl of diluted xanthine oxidase followed by shaking the plate for 20 minutes at room temperature. After incubation, the absorbance was recorded at 450

nm in a micro-plate reader.

2.3.9 CAT activity

CAT was measured using a catalase assay kit (Sigma, St. Louis, MO, USA). The principle is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase present in the sample. In brief, flies (n = 100) were homogenized in 1mL enzyme dilution buffer followed by centrifugation at a speed of 1,500g for 5 min at 4°C. The supernatant was moved into a new tube and diluted 15 times by 1×assay buffer (5mM potassium phosphate buffer, pH 7.0) in triplicates. The resultant sample (10μL) was diluted again with 65μL of 1×assay buffer. Then, 25μL of 200mM hydrogen peroxide solution was added to initiate the reaction. At exactly 1 minute, 900μL of stop solution (15mM sodium azide) was added. The reaction mixture (10μL) was mixed with 1mL color reagent containing 0.25mM 4-aminoantipyrine, 2mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, and freshly added peroxidase (0.8–1.2U/mg). After incubation at room temperature for 15 minutes, absorbance of each sample was measured in a spectrometer at 520 nm.

2.3.10 Real-Time PCR

Total RNA was extracted using the commercial extraction agent TRIzol (Invitrogen, Carslbad, CA, USA). Fruit flies (n=15) were homogenized in 800μL of

TRIzol solution, and then centrifuged at 12,000g at 4°C for 10 min and the supernatant was transferred to another a new tube containing 160μL chloroform. The mixture were then subjected to centrifugation at 12,000g at 4°C for 15 min. The upper layer was mixed with 400μL isopropanol. After 10 min of incubation at room temperature, the samples were centrifuged at 12,000g at 4°C for 10 min, and the pellet was saved and washed in 1ml of 75% ethanol followed by re-centrifugation. Finally, 25μL DEPC water was employed to resuspend the RNA pellet. The concentration and purity of RNA obtained were determined by measuring their absorbance at 260nm and 280nm. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to construct cDNA. RNA (2μg) was used for each reaction together with $MgCl_2$, 10X RT buffer, dNTP, random hexamers, RNase inhibitor, and MultiScribe Transcriptase. The final volume was adjusted to 10μL. cDNA was synthesized in the thermocycler GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) and stored at -20 °C.

Real-time PCR amplification was carried out on a Fast Real-time PCR System 7500 (Applied Biosystems, Foster City, CA, USA). Four target genes included: CuZnSOD (SOD1, NCBI Reference Sequence NM_057387.3), MnSOD (SOD2, NCBI Reference Sequence NM_057577.2), Catalase (CAT, NCBI Reference Sequence NM_080483.2), and methuselah (MTH, NCBI Reference Sequence

NM_079147.2). The expressions of target genes were normalized with that of rp49 (NCBI Reference Sequence NM_079843.2), a housekeeping gene used as the internal control. Gene expressions were calculated based on the comparative Threshold cycle (C_T) value. Levels of gene expressions in all groups were shown as a ratio of the day 0 control group.

2.3.11 Western Blot analysis

Total proteins were extracted and subjected to western blot analysis. In brief, 50 flies were homogenized in a 1.5mL tube containing 500μl homogenizing buffer (20mM Tris-HCl, 2mM $MgCl_2$, 0.2M sucrose and protease inhibitor cocktail (Roche, Mannheim, Germany)). The extracts were centrifuged at 13,000 g for 5 min at 4°C and the supernatant were collected. Protein concentration was determined using a protein concentration assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). After adding 6×loading dye and homogenizing buffer to adjust the volume, the protein were boiled at 95 °C for 5 min, and then stored at -80°C. For the measurement of catalase and β-actin, 20μg total protein were size-fractionated by 7% SDS-PAGE at 130V for 70 minutes; on the other hand, the same amount of total protein were loaded to measure CuZnSOD and MnSOD, which would be size-fractionated by 15% SDS-PAGE at 130V for 180 minutes. The proteins were then transferred to a Hybond-P PVDF membrane (Millipore, Billerica, MA, USA).

The membrane was incubated for one hour in blocking solution (5% non-fat milk) at room temperature and then in the same solution containing diluted anti-catalase / anti-actin / anti-CuZnSOD / anti-MnSOD antibodies respectively at 4°C overnight. The membrane was then washed in 1×TBST and was then incubated for one hour at 4°C in diluted horseradish peroxidase-linked goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., California, USA) or anti-mouse IgG (Santa Cruz Biotechnology, Inc., California, USA). The washes were repeated before the membranes were developed with ECL enhanced chemiluminescence agent (Santa Cruz Biotechnology, Inc., California, USA) and subjected to autoradiography for one second to five minutes on SuperRX medical X-ray film (Fuji, Tokyo, Japan). Densitometry was quantified using the computer software Quantity one (Bio-Rad, CA, USA).

2.3.12 Statistics

Data were expressed as mean ± standard deviation. The Kaplan-Meier test was employed to compare the difference between the survival curves using SPSS 15.0 (Statistical Package for the Social Sciences software, SPSS Inc, Chicago, USA). The significance of difference between means was assessed using T-test and one way ANOVA. Differences were considered significant when $p<0.05$.

2.4 Results

2.4.1 Composition of BTE

HPLC analysis found that BTE used in the present study was a mixture of epicatechins and theaflavins (Figure 1). Results revealed that BTE contained 11% TF_1, 12% TF_2A, 13% TF_2B, 24% TF_3, 12% EC, 12% EGC, 10% EGCG, and 6% ECG.

2.4.2 Effect of BTE on longevity of OR flies fed the basal diet

BTE at both 5 and 10 mg/ml showed lifespan extension effect on OR wild type male flies. The maximum life span increased more than 4% in BTE groups compared with that of the control. Meanwhile, 50% survival time was elevated from 50 days to 55 and 56 days when 5 and 10 mg BTE/ml was added into diet, respectively. The mean lifespan for the control and the two BTE groups were 51, 55, and 56 days respectively (Figure 2, Table1) The Kaplan-Meier test demonstrated that both 5 and 10 mg BTE/mL could significantly extend the mean lifespan of fruit flies ($P<0.01$) while there was no difference between the two experimental groups.

The present study also investigated effect of 10 mg BTE/ml diet on activity of SOD1, SOD2 and CAT in OR wild type male flies at day 0, 25 and 45 (Figure 3). It was found that both SOD1 and SOD2 activity decreased with aging. BTE groups had greater activity of SOD1 and SOD2, although significant difference was always seen between the control and BTE-fed group. CAT activity also decreased with aging. BTE group had greater CAT activity than the control at day 25. However, no difference was seen at day 0 and 45. The total body LPO level increased with aging. Supplementation of 10 mg BTE/ml diet could lower the LPO formation significantly at day 45 ($p<0.05$).

Gene expression of SOD1, SOD2, CAT and MTH in general was characterized by an increase and then a decrease with age in both the control and BTE-fed group (Figure 4). The expression level of SOD1 was greater in BTE-fed group compared with that in the control at day 35 and 55 while gene expression of CAT in BTE-fed group was greater than that in the control at day 15 and 25. Otherwise, no significant differences in gene expression of SOD1, SOD2, CAT and MTH were seen between the control and BTE-fed group.

Western blot analyses found that the changes in protein abundance of SOD1, SOD2 and CAT were consistent with their patterns of gene expression (Figure 5). Similarly, the protein mass of SOD1, SOD2 and CAT increased and then decreased with age in both the control and BTE-fed group. No differences in protein mass were seen between the control and BTE-group except for CAT, which was greater in BTE-fed group than that in the control at day 15 and 25.

2.4.3 Effect of BTE on Paraquat and H_2O_2 Resistance in OR, *Cat*^{n1} and *SOD*^{n108} flies

Results from paraquat challenge test showed that supplementation of 10 mg BTE per ml diet only prolonged the survival time of OR wild type flies ($p<0.05$) but it did not affect that in *SOD*^{n108} mutant significantly. To be specific, OR wild type fly had a maximum survival time increased from 70 hr in the control to 74 hr in BTE-fed group with 50% survival time being prolonged by 18% ($P<0.05$). In contrast, no significant difference in the resistance was observed between the control and the BTE-fed group. Similar results were seen for the H_2O_2 challenge test. The maximum survival time, 50% survival time and mean survival time were all prolonged in the BTE-fed OR wild type but not in BTE-fed *Cat*^{n1} mutant fly strain (Figure 6).

2.4.4 Effect of BTE on longevity of OR flies fed a high-fat diet

Addition of 10% fatty acid derived from lard could induce the oxidative stress and significantly shorten the maximum lifespan to 15 days. This fat-induced high mortality could be partially reversed by the administration of 10 mg/ml BTE along with basal diet. In this assay, the maximum lifespan was prolonged from 15 days in the control group to 28 days in BTE-fed group, increasing by 87%. Addition of fatty acids into the diet increased the LPO values and BTE supplementation could partially reduce the value, although no statistical difference was seen (Figure 7).

Western blot analyses did not find any changes in protein abundance of SOD1, SOD2 and CAT between the control and BTE-fed OR flies (Figure 8). SOD1 gene expression demonstrated an increase pattern during the period of study. At day 5, the control (lard) had a significant decrease in SOD gene expression while BTE increased the SOD gene expression to a level similar to the control (no lard). In contrast, SOD2 gene expression decreased during the period of the study. At day 10, addition of lard fatty acid down-regulated and BTE had no effect on the SOD2 gene expression. Both CAT and MTH gene expressions were down-regulated significantly with addition of lard fatty acids into diet while BTE supplementation could up-regulate these genes to the levels of the control (no lard) (Figure 9).

SOD1 and SOD2 had lower activity at day 10 compared with that at day 0 and 5 in all three groups. However, no difference could be observed among the three groups. CAT activity decreased at day 5 and 10 compared with that at day 0. At day 10, addition of lard fatty acid decreased CAT activity while addition of BTE could recover it completely (Figure 10).

Table 1 Lifespan of OR wild type flies fed the control diet and the two experimental diet containing 5 and 10 mg BTE /ml.

	Maximum Lifespan of last fly (Day)	50% Survival (Day)	Mean Lifespan (Day)
Control	74	50	51 ± 2^{a}
5 mg BTE	77	55	55 ± 2^{b}
10 mg BTE	77	56	56 ± 2^{bc}

[a,b] Means with different letters differ significantly at $p<0.05$.

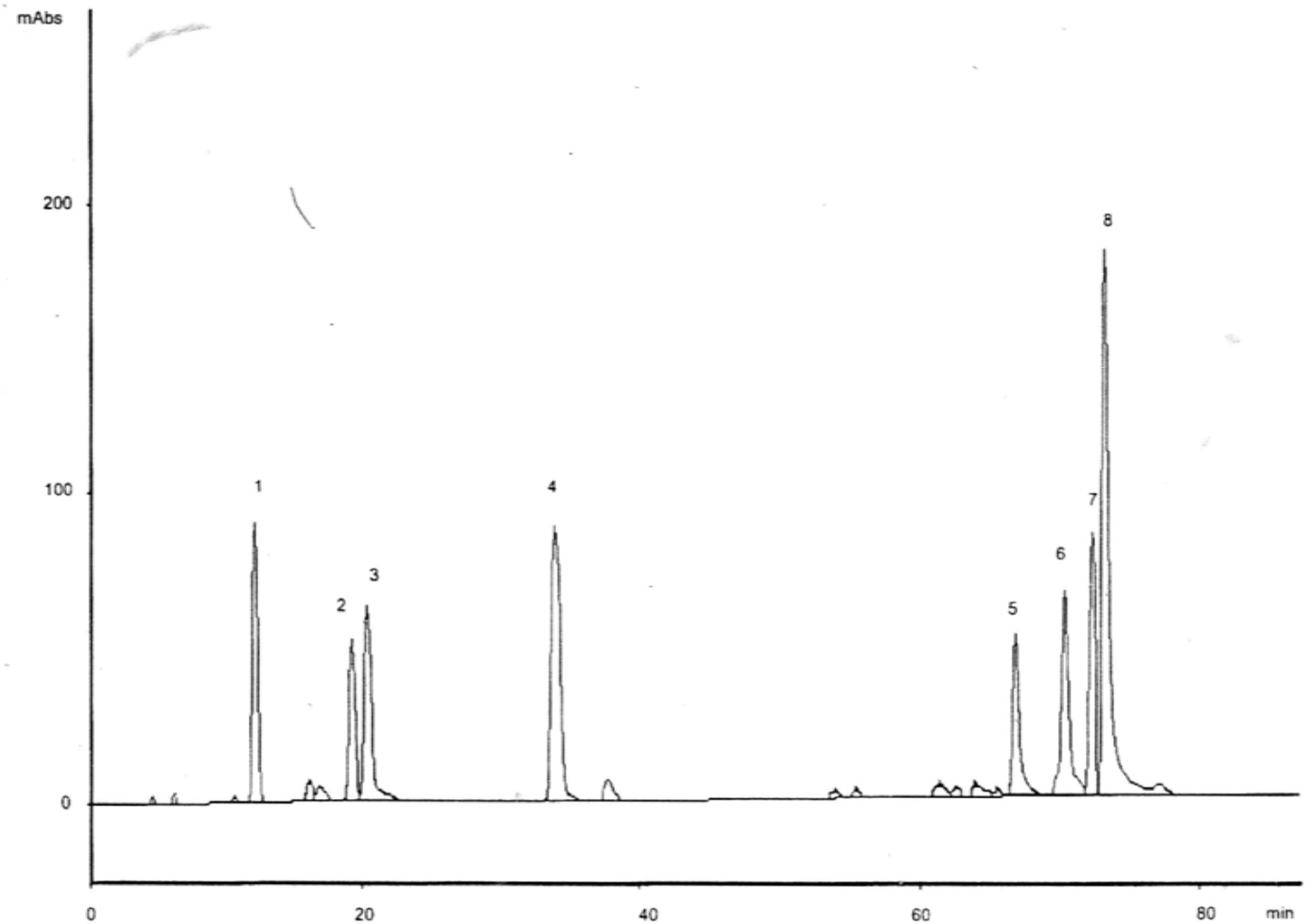


Figure 1 HPLC chromatogram of black tea extract (BTE). Peak identification: 1, epigallocatechin (EGC); 2, epicatechin (EC); 3, epigallocatechin gallate (EGCG); 4, epicatechin gallate (ECG); 5, theaflavin-1 (TF1); 6, theaflavin-3-gallate (TF_2A); 7, theaflavin-3'-gallate (TF_2B); 8, theaflavin-3,3'-digallate (TF_3)

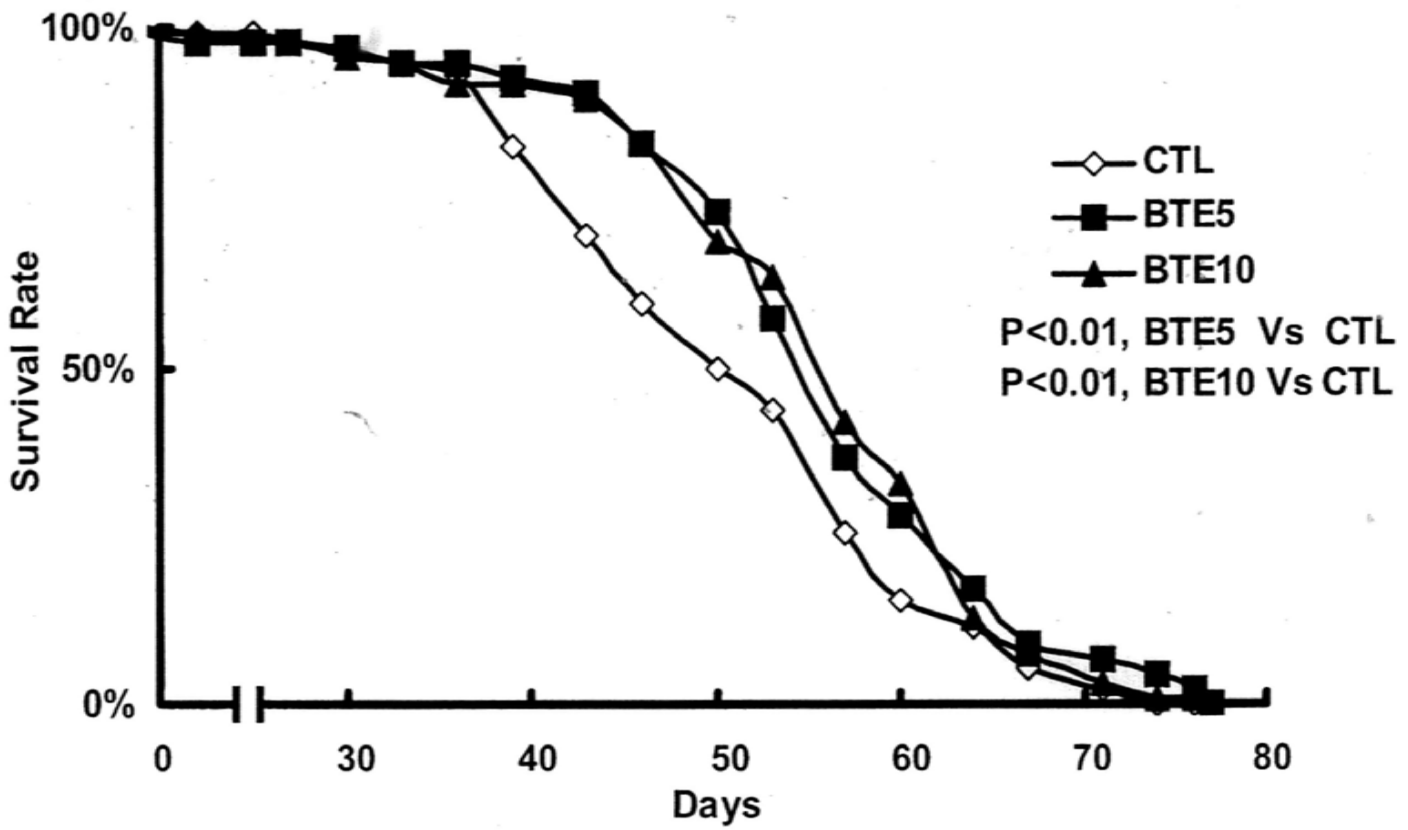


Figure 2 Lifespan curve of wild type flies (OR) fed diets containing 0 mg/ml (control), 5 mg and 10 mg black tea extract (BTE5 and BTE10) per milliliter diet. Data were expressed as the maximum lifespan of last fly, 50% survival time and mean lifespan (n=200 flies) for each group (Table 1). The Kaplan-Meier test found both BTE5 and BTE10 could significantly extend the mean lifespan of fruit flies ($P<0.01$).

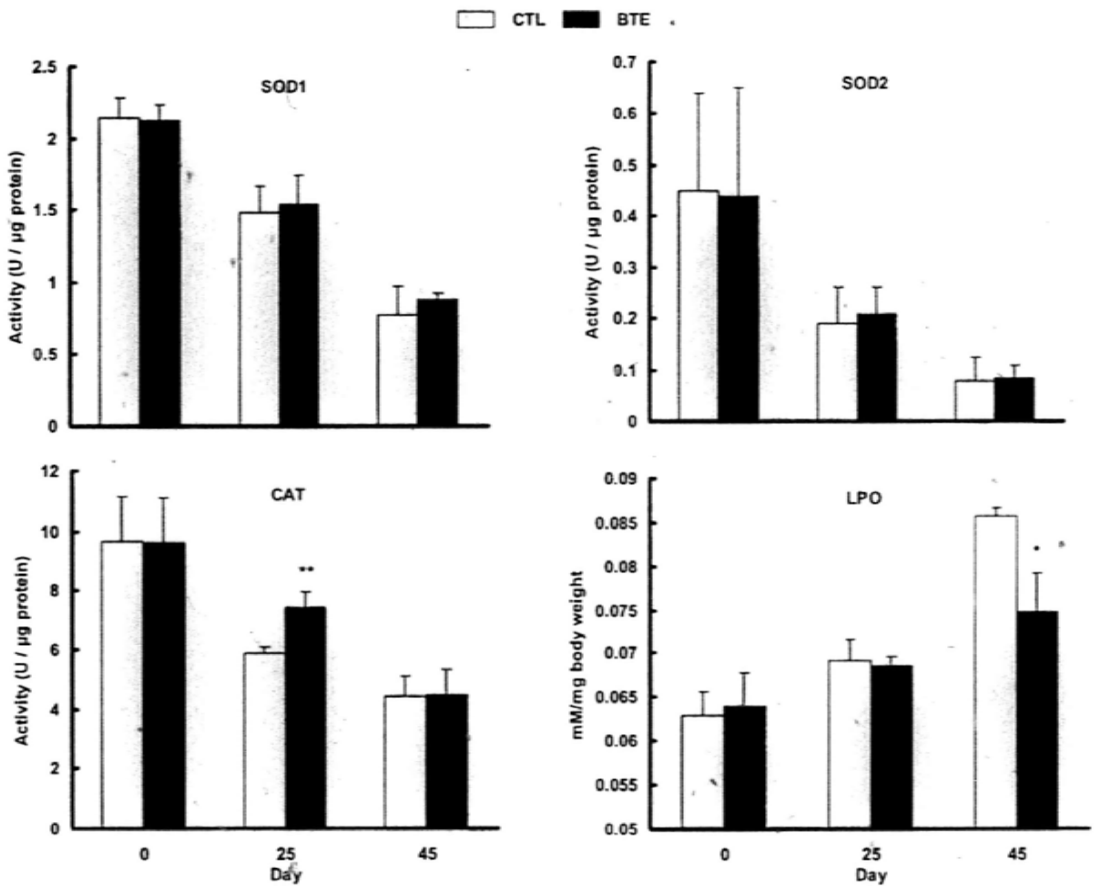


Figure 3 Effect of black tea extract (BTE) supplementation (10 mg/ml diet) on the whole body lipid hydroperoxide (LPO) level, and enzymatic activity of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2) and catalase (CAT) compared with the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 25 and 45 days. Data are expressed as mean + S.D. * $P<0.05$; ** $P<0.01$ compared with the control value.

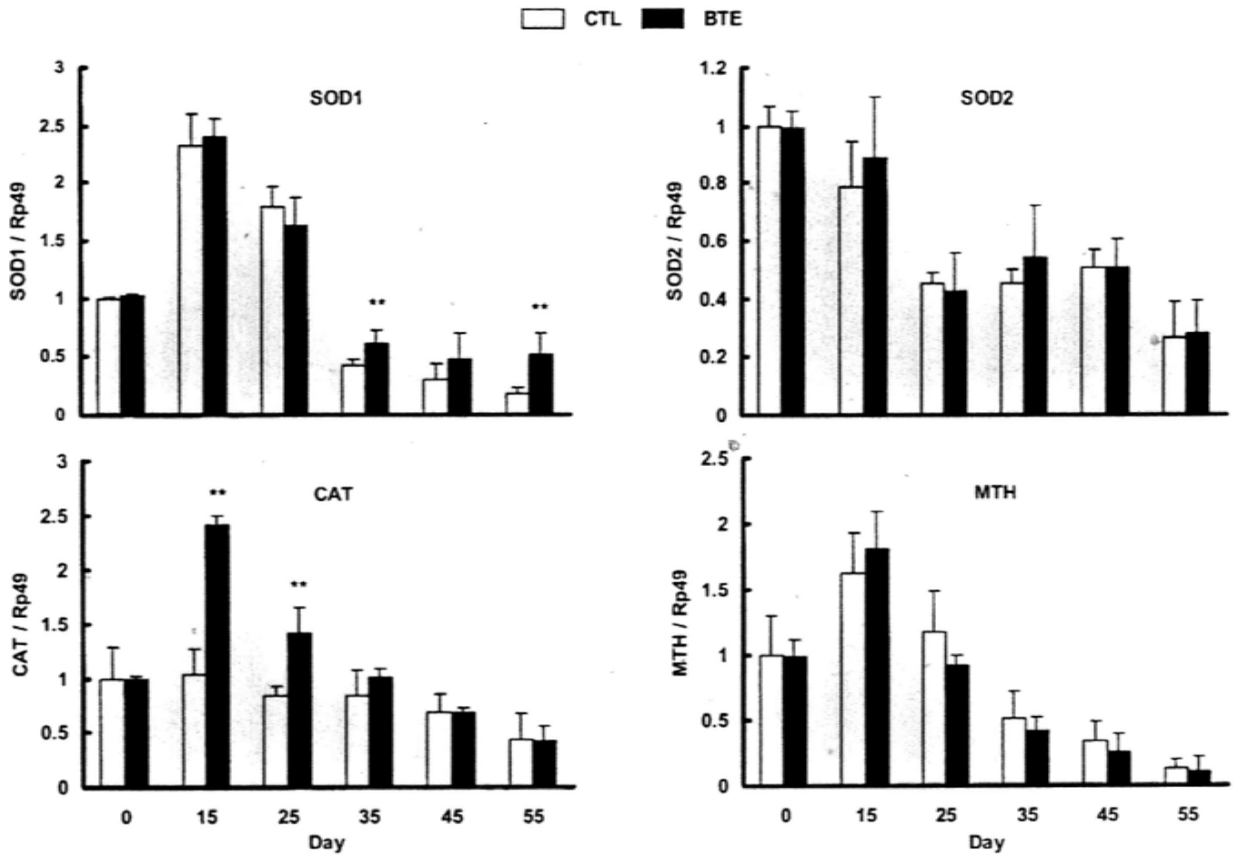


Figure 4 Effect of black tea extract (BTE) supplementation (10 mg/ml diet) on mRNA of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2), catalase (CAT) and Methuselah (MTH) compared with the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 15, 25, 35, 45 and 55 days. Data are expressed as mean $\pm$ S.D. * $P<0.05$; ** $P<0.01$ compared with the control value.

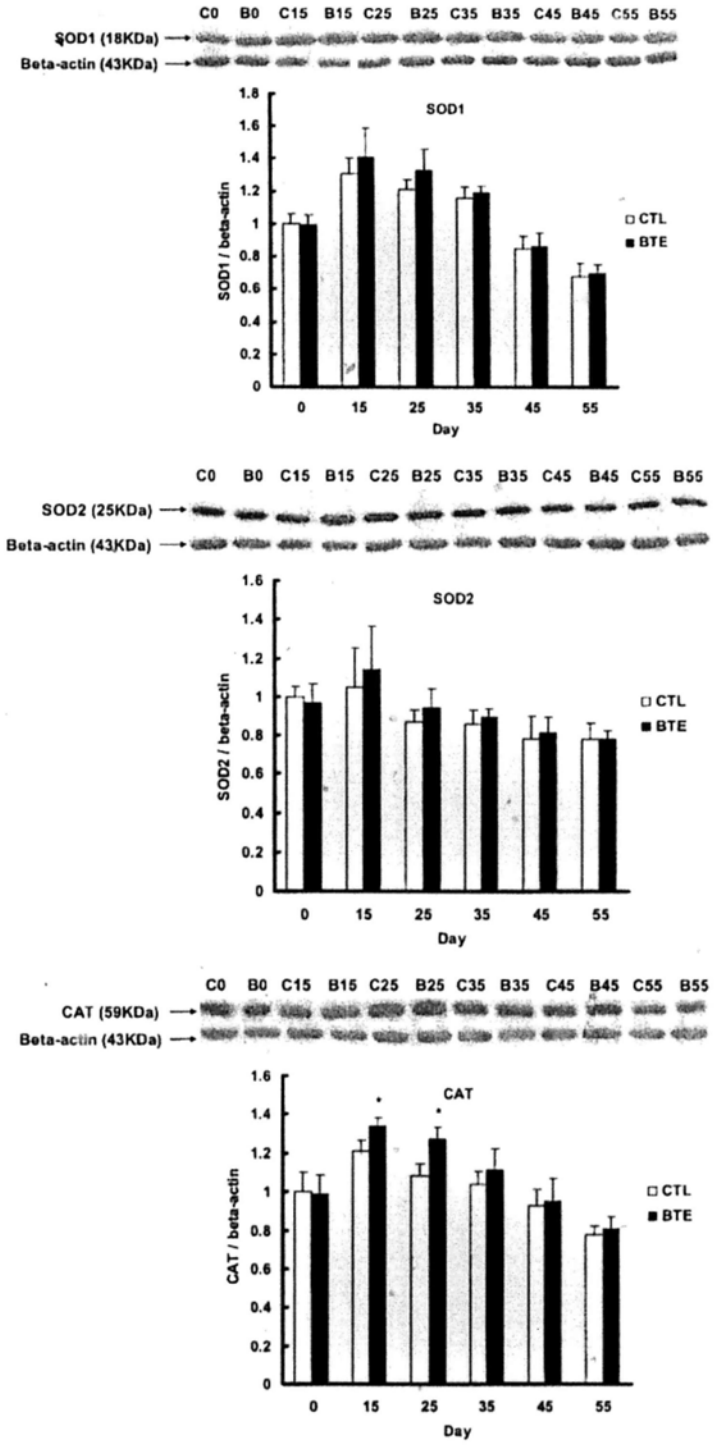


Figure 5 Effect of black tea extract (BTE) supplementation (10 mg/ml diet) on the relative immunoreactive mass of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2) and catalase (CAT) compared with the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 15, 25, 35, 45 and 55 days. Data are expressed as mean $\pm$ S.D. * $P<0.05$ compared with the control value.

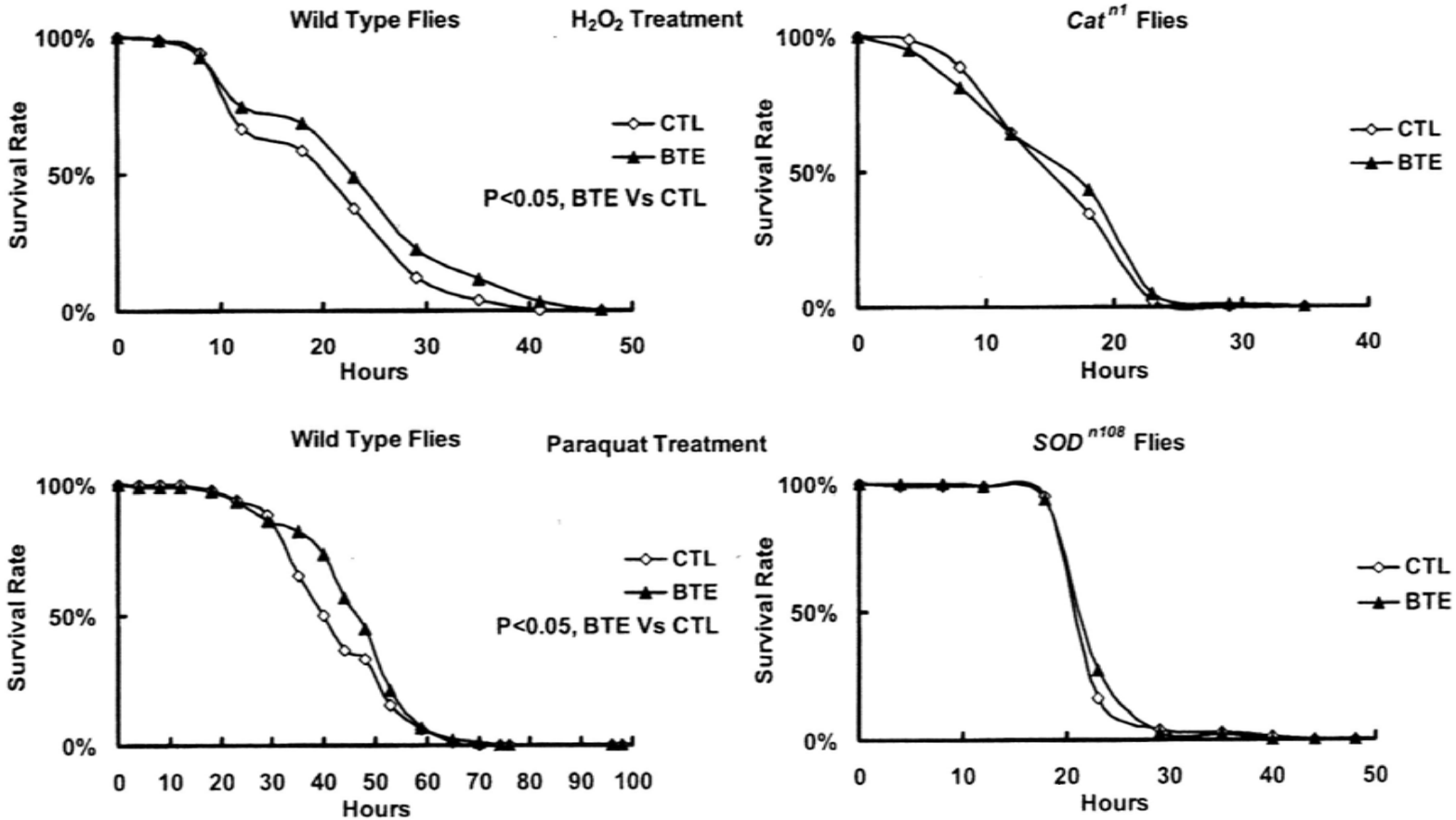


Figure 6 Effect of paraquat treatment or hydrogen peroxide treatment on the survival time the mutant flies (SOD^{n108}) or mutant flies (Cat^{n1}) fed the diets containing 0 mg/ml (CTL) or 10 mg black tea extract (BTE) /ml compared with that of the wild type (OR) flies. The Kaplan-Meier test found both that BTE-fed OR group survived better than its corresponding OR control ($P<0.05$) while the survival rate of BTE-fed SOD^{n108} and Cat^{n1} groups was not different from their corresponding control groups.

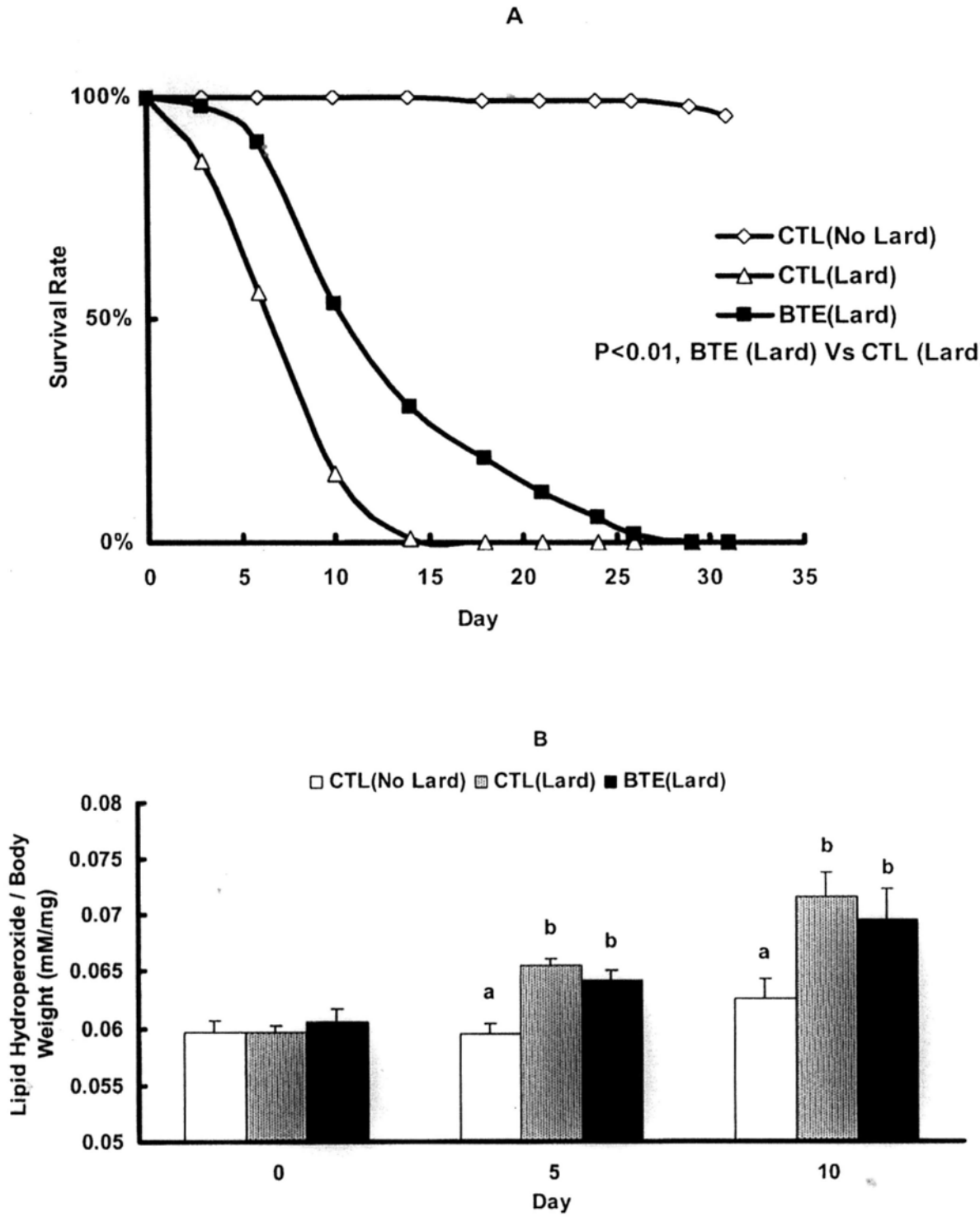


Figure 7 (A) Lifespan of OR fruit flies fed either a diet containing no lard fatty acid [CTL(no lard)] or a diet containing 10% lard fatty acid [CTL(lard)] with addition of 10 mg black tea extract [BTE(lard) at 25°C. The Kaplan-Meier test found both that BTE(lard) group survived better than the CTL(lard) ($P<0.01$). (B) Effect of BTE on the whole body lipid hydroperoxide (LPO) level in CTL(no lard), CTL(lard) and BTE(lard) groups. [a,b]Means at the same time point differ significantly at $P<0.05$.

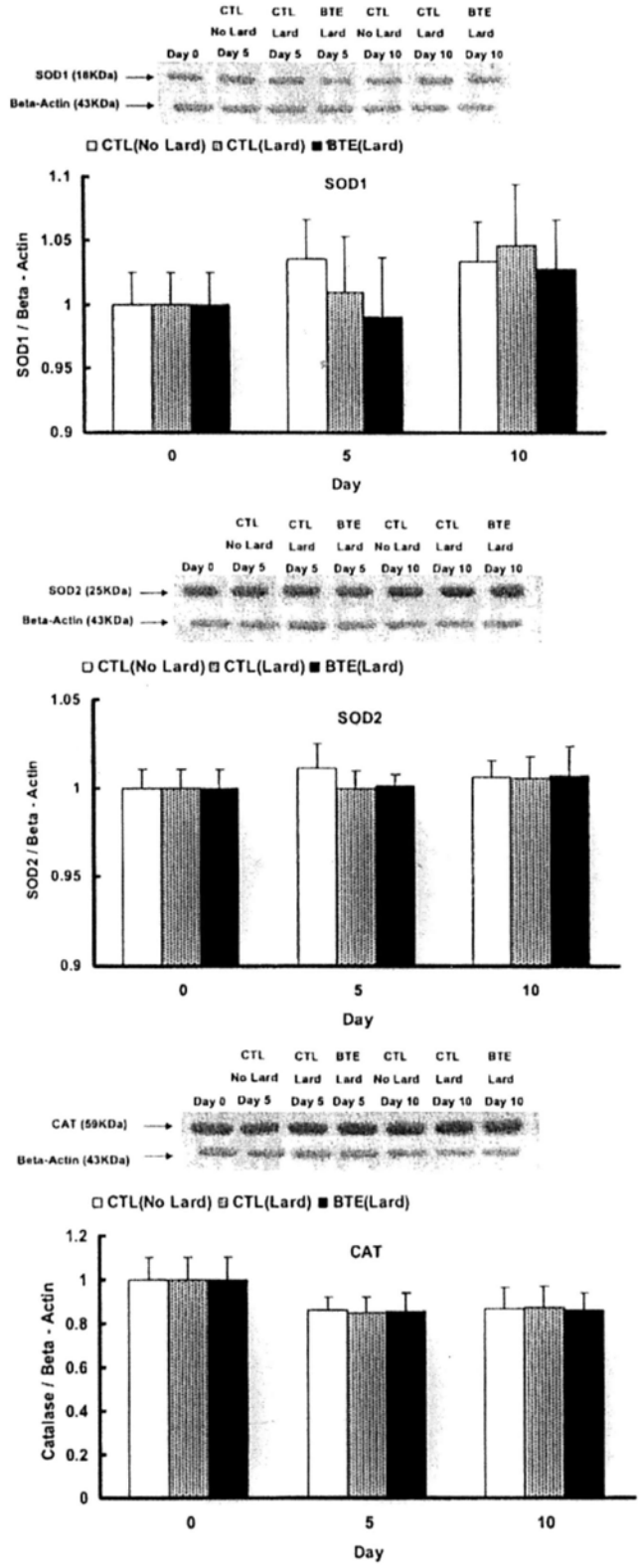


Figure 8 The relative immunoreactive mass of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2) and catalase (CAT) in OR fruit flies fed either a diet containing no lard fatty acid [CTL(no lard)] or a diet containing 10% lard fatty acid [CTL(lard)] with addition of 10 mg black tea extract [BTE(lard) at 25°C.

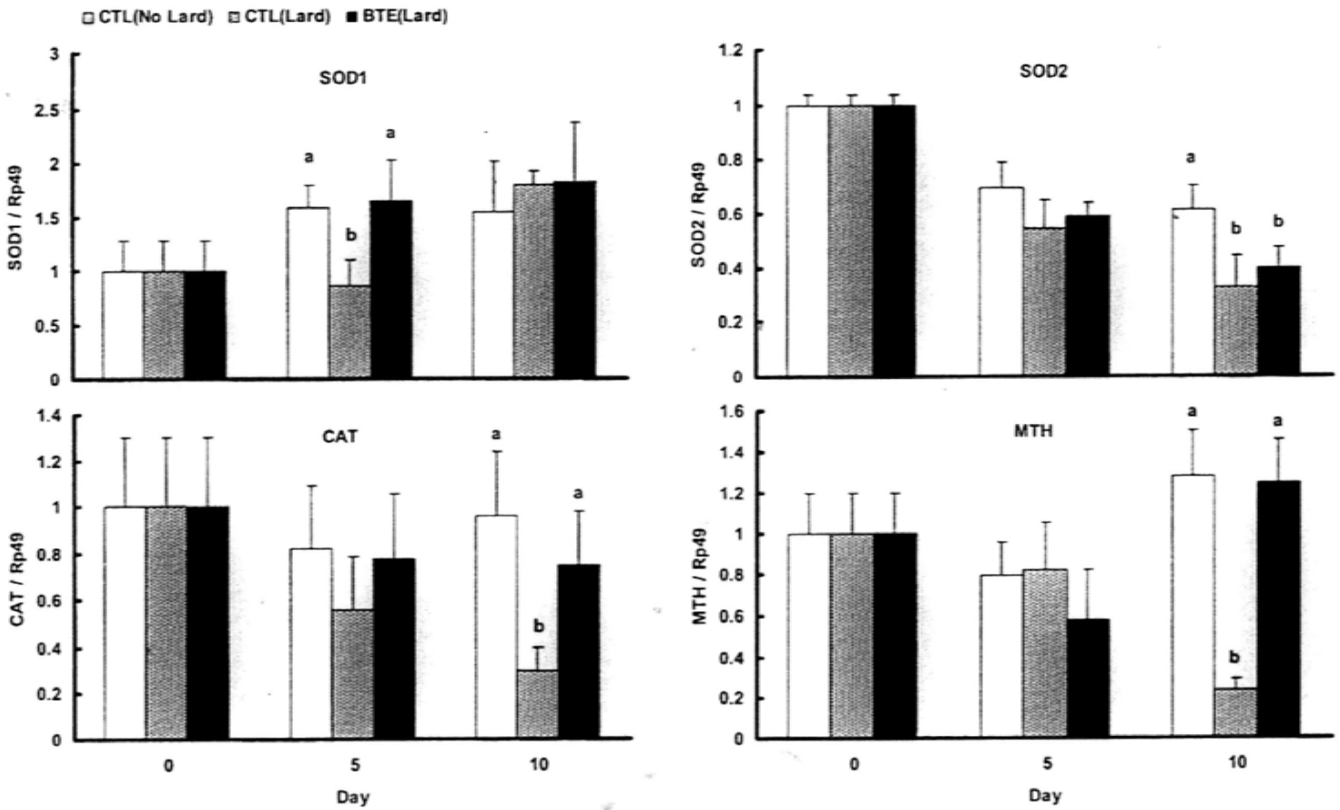


Figure 9 mRNA of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2) and catalase (CAT) in OR fruit flies fed either a diet containing no lard fatty acid [CTL(no lard)] or a diet containing 10% lard fatty acid [CTL(lard)] with addition of 10 mg black tea extract [BTE(lard) at 25°C. [a,b]Means at the same time point differ significantly at $P<0.05$.

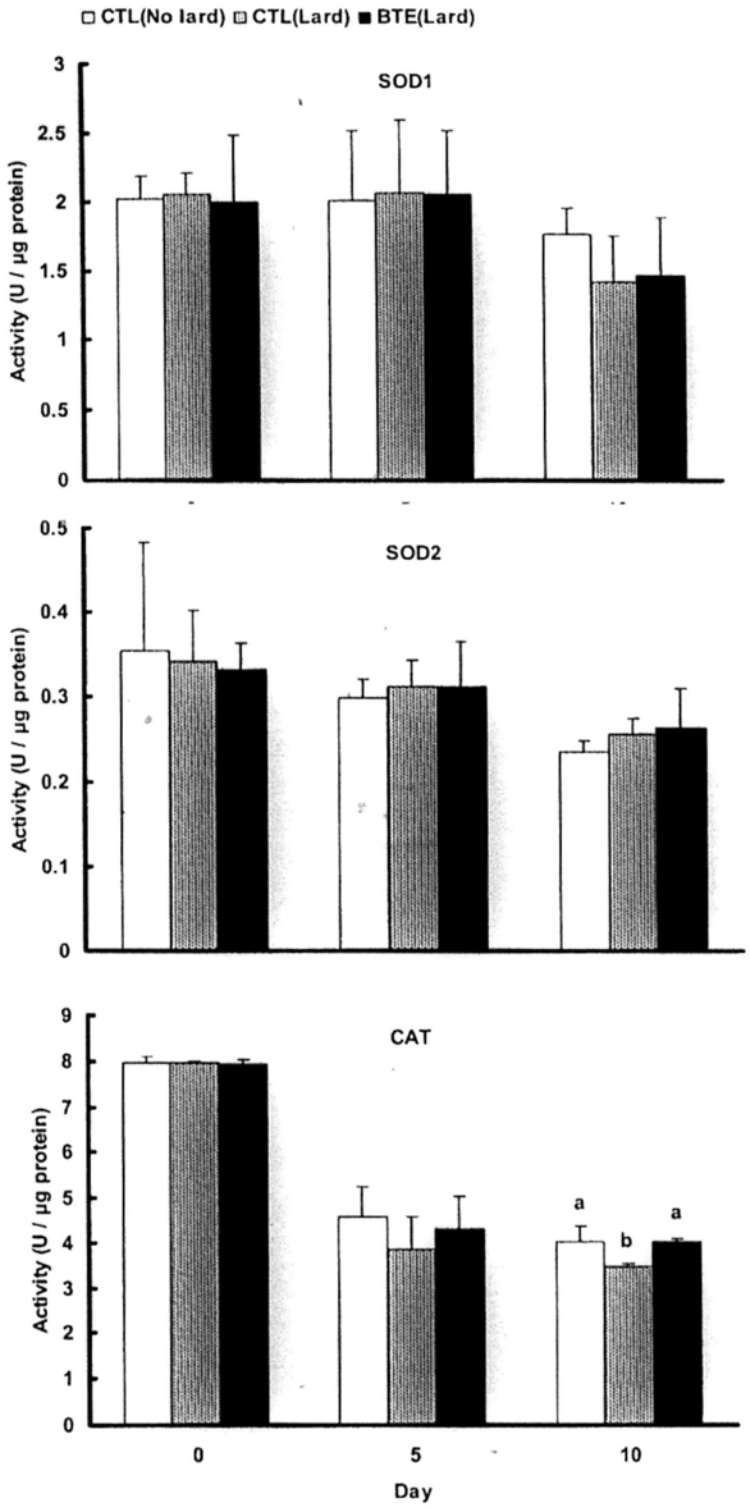


Figure 10 Enzymatic activity of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2) and catalase (CAT) in OR fruit flies fed either a diet containing no lard fatty acid [CTL(no lard)] or a diet containing 10% lard fatty acid [CTL(lard)] with addition of 10 mg black tea extract [BTE(lard) at 25°C. ^{a,b}Means at the same time point differ significantly at $P<0.05$.

2.5 Discussion

The present study is the first time to investigate the effect of black tea in a form of BTE on lifespan of *Drosophila melanogaster*. Results clearly demonstrated that BTE could prolong the mean lifespan and 50% survival time of fruit flies. This observation was in agreement with the previous studies showing that green tea extracts could extend the lifespan in fruit flies (Li et al., 2007; Li et al., 2008) and with the study of Cui et al.(Cui et al., 1999), who found that finding *Drosophila's* lifespan increased by 33% when 7 mg/ml of green tea catechins was added into the diet. In *C. elegans* given EGCG, life span could be significantly extended by 10-170% (Abbas et al., 2009; Zhang et al., 2009). In male mice given a water solution containing 80 mg/l of tea polyphenols at the age of 13 month until death, it was found that mice had an increase in an average lifespan by 6.4% (Kitani et al., 2007). Although there is no direct evidence showing drinking tea increases lifespan in humans, it has been reported that daily consumption of green tea in sufficient amounts could prolong life by avoiding pre-mature death, particularly death caused by cancer (Nakachi et al., 2003).

The underlying mechanisms by which BTE extends the mean lifespan of fruit flies remain poorly understood. One of the possible mechanisms is probably related to the free radical scavenging activity of BTE. It is generally believed that the free radical species can cause deterioration of an organism while the antioxidant can delay the process of aging (Gutteridge et al., 2000). HPLC analysis showed that BTE used in the present study contained 60% theaflavins and 40% epicatechins. It is known that both theaflavins in black tea and epicatechins in green tea are equally effective antioxidants (Leung et al., 2001). In fact, the present study demonstrated

that BTE group had a decrease in total body LPO level compared with the control fruit flies, although a significant difference was not always observed (Figures 3 and 7). In HPF-1 cells, theaflavins exhibit generally a greater antioxidant activity than EGCG (Yang et al., 2008). In this regard, the genetically manipulated long lived strain of *Drosophila melanogaster* has been shown to produce a lower LPO level with a greater activity of SOD and CAT activities in every time points throughout the whole life compared with the short lived strain, suggesting that the oxidative stress is at least one of the factors leading to aging (Arking et al., 2000).

The second mechanism by which BTE extends the lifespan of fruit flies may be mediated by up-regulation of endogenous antioxidants enzymes of SOD1 and CAT in both transcriptional and translation levels. In general, expression of these enzymes was characterized by an increase up to age 15 days followed by a decrease thereafter (Figures 4 and 5), suggesting the ability of scavenging the free radical in fruit flies decreases with aging. In general, BTE supplementation could up-regulate SOD1 and CAT at some time points while at other times up-regulation was not statistically significant compared with the control fruit flies. In this regard, the following two explanations are offered. First, both western blot and RT-PCR analyses are semi-quantitative and are not sensitive enough to detect the difference in gene expression of these enzymes associated with BTE supplementation. Second, three vials of fruit flies per time point might not generate enough power to conduct the statistical analyses. There has been no report up to date regarding the effect of BTE on lifespan of fruit flies, it can only make comparison with the data published on

green tea epicatechins in literature. It has been previously demonstrated that green tea epicatechins could up-regulate expression of both SOD and CAT in flies maintained on a diet containing 10 mg/ml green tea epicatechins (Li et al., 2007; Li et al., 2008). Sohal et al. (Sohal et al., 1995) found that flies had increased SOD activity by 26% and CAT activity by 73% in response to 34% increase in lifespan. In one previous report from this laboratory (Li et al., 2007), it was found that supplementation of green tea epicatechins into diet increased the mean lifespan by 16% in response to 32% activity enhancement in SOD1, 40% in SOD2 and 19% in CAT. However, Orr and Sohal (1993) showed that over-expression of SOD1 alone did not increase the lifespan of the flies and the increase in the oxidative resistance induced by the paraquat challenge was insignificant. In another study, over-expression of CAT gene alone did not increase the longevity of the flies (Mockett et al., 2003). It is believed that longer lifespan of the flies is associated with up-regulation of gene expression of not only SOD but also CAT.

Additional evidence supported that lifespan prolonging effect of BTE in the fruit flies was associated with up-regulation of genes for SOD and CAT. Paraquat and H_2O_2 challenge test demonstrated BTE prolonged the survival time only in OR wild type flies but it did not affect that of SOD^{n108} or Cat^{n1} mutants, in which gene of either SOD or CAT was knocked out (Figure 6). Results implied that BTE prolonged the mean lifespan of fruit flies at least mediated in part by interaction of BTE with genes of SOD and CAT. In this regard, BTE has been shown to significantly elevate the activities and gene of SOD in various models (Khan et al., 2006; Das et al., 2002).

Similarly, green tea epicatechins have been also shown to up-regulate genes SOD and CAT (Chow et al., 2002; Das et al., 2002; Mori et al., 2003; Yamamoto et al., 2003; Ying et al., 2004).

It was noteworthy that gene expression of SOD1, SOD2 and CAT decreased after day 15 (Figure 4), suggesting the oxidative defense system becomes weaker in aged fruit flies compared with that of the young. In this regard, overexpression of these genes in *S. cereviseae* and *D. melanogaster* has been shown to reduce oxidative damage and extend lifespan (Landis and Tower, 2005). Similarly, a gradual decrease in gene expression of MTH was also observed after day 15 in aged fruit flies (Figure 4). MTH gene in *D. melanogaster* has been a major target of interest in the biology of aging (Lin et al., 1998; Paaby et al., 2008). MTH encodes the G-protein coupled receptor and its mutants have extended longevity in *D. melanogaster*. However, we did not find any significant effect of BTE on expression of MTH.

In conclusion, gene expression of SOD1, SOD2, CAT and MTH decreased with aging. The present study showed that BTE supplementation could increase the mean lifespan and survival time of *Drosophila melanogaster* under various oxidative with reduction of the LPO level. BTE prolonged lifespan in fruit flies was associated partially with up-regulation of the expression of endogenous SOD1 and CAT but unlikely with that of SOD2 and MTH.

Chapter 3
Dietary soybean isoflavones (SIF) delay aging in fruit flies

3.1 Introduction

Extending life span through dietary manipulation has been one of the most inspiring topics in anti-aging research community. Based on free radical theory of aging (Harman, 1956), life span can be extended by scavenging free radicals more efficiently, resulting in fewer oxidative damages to cells. This can be achieved by either strengthing endogenous antioxidant system, or directly eliminating free radicals by exogenous reinforcement, or the combination of the two (Calabrese and Maines, 2006).

Drosophila melanogaster has been regarded as one of the most efficient models to investigate the genetic determinants of aging and to evaluate the bio-activity of compounds in vivo. Firstly, fruit flies and humans have many conserved physiological and biological pathways (Jafari, 2010). Secondly, there are abundant competent genetic resources and practical tools available in *Drosophila* research community (Minois, 2006). Furthermore, systematic protocols have been established and modified on screening the anti-aging candidate compounds in *Drosophila* during the past years. Previous studies conducted by our group demonstrated the anti-aging activity of green tea, broccoli, and black tea extracts in fruit flies (Li et al., 2007; Peng et al., 2009).

The main components in soybean isoflavones (SIF) are daidzein, genistein, and their glycoside derivatives. Besides its cholesterol-lowering effect (Lethaby et al., 2007; Anderson et al., 1995), SIF has also been regarded as a competent candidate for anti-cancer nutraceuticals (Mann et al., 2007; Wu et al., 2002). In addition, its antioxidant capacity, especially the ability to attenuate DNA oxidative damage and enhance endogenous antioxidant enzymes, has been confirmed by previous studies both in vitro and in vivo (Barbosa et al., 2010; Lien et al., 2009; Kwak et al., 2007; Borrás et al., 2006; Liu et al., 2005; Yen et al., 2003) However, no study to date has addressed the issue if SIF possesses the anti-aging activity in fruit fly.

3.2 Objectives

The present study was to investigate (a) the anti-aging activity of SIF; (b) the potential interaction between dietary SIF and expression levels of genes encoding endogenous antioxidant enzymes, including SOD, CAT, and also the longevity determined gene, MTH in fruit flies.

3.3 Materials and methods

3.3.1 Fly strains

Fly strain employed in this study was called Oregon-R-C (OR), which had been obtained from Bloomington Drosophila Stock Center, Department of Biology, Indiana University, Bloomington, IN, USA.

3.3.2 Diet

The basal diet was prepared according to the standard formula suggested by Li et al. (2007) as described previously in Chapter 2 (2.3.2).

3.3.3 Effect of SIF on life span of OR flies fed the basal diet

Newly eclosed male flies (2-day-old) were divided into 3 groups with 200 flies in each group rearing in 10 vials (20 flies per vial). The first group was maintained on the basal diet, while the rest two groups were fed one of the two diets containing 5 or 10 mg SIF/ml. The dead flies were counted every 2-3 days and the remaining alive flies were then transferred to a new vial containing the same diet. The feeding lasted 77 days. The survival assays described above were similarly repeated and the fruit flies were killed at various time points to quantify the expression of SOD, CAT and MTH.

3.3.4 Effect of SIF on life span of OR flies fed the high-fat diet

Similarly, newly eclosed male flies (2-day-old) were divided into 3 groups with 200 flies in each group rearing in 10 vials (20 flies per vial). The first group was maintained on the basal diet containing no lard fatty acids (CTL), while the two experimental groups were fed diets containing 10% lard fatty acid only [CTL(Lard)] or with addition of 10 mg SIF/ml [SIF(lard)]. The dead flies were counted every 2-3 days and the remaining alive flies were then transferred to a new vial containing the same diet. The feeding lasted 33 days.

3.3.5 Body weight tracking and Gustatory assay

To exclude the possibility that lifespan extension in survival assay might be induced by dietary restriction, body weights were tracked and gustatory assay was carried out (Peng et al., 2010). The body weight of fruit flies in the control and SIF groups was measured at day 0, 15, 25, 35, 45, 55. As to gustatory assay, 60 newly eclosed male flies were collected (20 flies per vial) and reared on a standard diet for 5 days and then starved for 24 h on Kimwipes paper soaked with distilled water. Afterward, flies were maintained on the basal or SIF supplemented diets containing 0.2% sulforhodamine B sodium salt (Acid-Red) for 2 hours. The degree of fly abdomen redness was blind-scored using a grading scale ranging from grade 0 (colorless abdomen) to grade 5 (fully red abdomen). Food intake was compared on the basis of the difference in the degree of abdomen redness between the control and SIF-fed group (Figure 3).

3.3.6 Measurement of lipid hydroperoxides (LPO)

LPO assay was conducted as previously described in Chapter 2 (2.3.7) by using a LPO assay kit (Cayman Chemical, Michigan, USA).

3.3.7 SOD activity

SOD activity assay was conducted as previously described in Chapter 2 (2.3.8) by using a SOD activity assay kit (Cayman Chemical, Michigan, USA).

3.3.8 CAT activity

CAT activity was measured as described previously in Chapter 2 (2.3.9) using a catalase assay kit (Sigma, St. Louis, MO, USA).

3.3.9 Real-Time PCR

For real-time PCR assay details and primers information, please refer to Chapter 2 (2.3.10).

3.3.10 Western Blot analysis

Protein abundance detection and related anti-bodies information were mentioned in Chapter 2 (2.3.11).

3.3.11 Statistics

Data were expressed as mean $\pm$ standard deviation. The Kaplan-Meier test was employed to compare the difference between the survival curves using SPSS 15.0 (Statistical Package for the Social Sciences software, SPSS Inc, Chicago, USA). The significance of difference between means was assessed using T-test and one way ANOVA. Differences were considered significant when $p<0.05$.

3.4 Results

3.4.1 HPLC profile of SIF

HPLC profile provided by Shaanxi Sciphar Biotechnology Co., Ltd. showed that

SIF used in the present study mainly included daidzein, geinistein, and their glycoside forms, i.e., Daidzin, Genistin. This SIF extract product provided 60% total isoflavones.

3.4.2 Effect of SIF on life span of fruit flies

Both 5 and 10 mg/mL SIF could extend life span of OR wild type male flies to certain degrees. The maximum life span increased by around 10% in 10mg/mL SIF group compared with that of the control. Meanwhile, 50% survival time was increased from 46 days to 49 and 50 days when 5 or 10 mg SIF/ml was added into daily diet, respectively. The mean lifespan for the control and the two SIF groups were 48, 49, and 53 days respectively (Figure 2; Table 1) The Kaplan-Meier test demonstrated that 10 mg SIF/mL could significantly extend the mean lifespan of fruit flies ($P<0.05$) while there was no difference between the two dose groups. Meanwhile, body weight measurement and gustatory assay did not reveal any significant difference between control and SIF-fed group (Figure 3).

Furthermore, addition of either 5% or 10% fatty acids could induce severe oxidative stress and significantly shorten life span of fruit flies. SIF supplementation could not reverse the fat-induced mortality in fruit flies (Figure 4).

3.4.3 Effect of SIF on endogenous antioxidant enzyme activity of fruit flies

Enzyme activities of SOD1, SOD2, and CAT in OR male flies were investigated in the present study (Figure 5). The result showed that the activity of these

antioxidant enzymes decreased with aging. SIF could help attenuate the decrease in SOD2. LPO increased with aging while it decreased with supplementation of 10mg/mL SIF at day 25. Nevertheless, similar to the results in enzyme activity study, no significant difference was observed in LPO level between the control and SIF groups (Figure 6).

3.4.4 Effect of SIF on mRNA expression level of fruit flies

Real-time PCR data revealed that gene expression level of SOD1, SOD2, CAT and MTH was, in general, decreasing with aging in both the control and SIF groups (Figure 7). The expression level of SOD2 was greater in SIF group compared with that in the control at day 35 and 45 while gene expression of CAT in SIF group was greater than that in the control at day 35. Other than that, no further significant difference in gene expression was observed between the control and SIF groups.

3.4.5 Effect of SIF on antioxidant protein mass of fruit flies

Western blot analyses found that protein abundance of SOD1 was greater in SIF group than that in the control at day 55 while protein mass of SOD2 was greater in SIF than that in the control at day 35 (Figure 8). As to CAT, no significant difference was found between the SIF and control group at the selected time points.

Table 1 Lifespan of OR wild type flies fed the control diet and the two experimental diet containing 5 and 10 mg SIF /ml.

	Maximum Lifespan of last fly (Day)	50% Survival (Day)	Mean Lifespan (Day)
Control	70	46	48 ± 2^{a}
5 mg SIF	72	49	49 ± 2^{ab}
10 mg SIF	77	50	53 ± 2^{b}

[a,b] Means with different letters differ significantly at $p<0.01$.

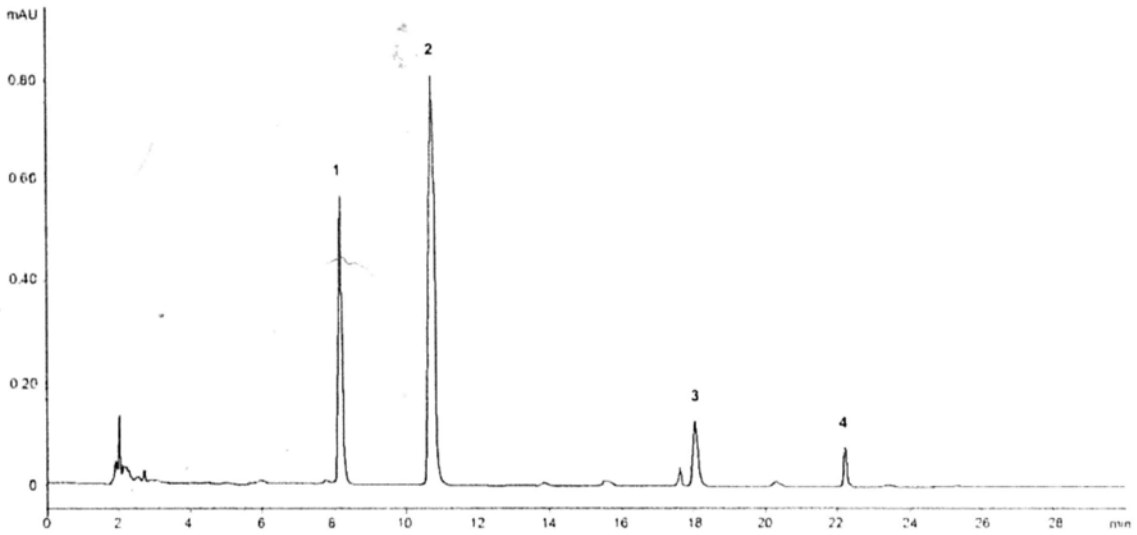


Figure 1 HPLC chromatogram of soybean isoflavones (SIF). Peak identification: 1, Daidzin; 2, Genistin; 3, Daidzein; 4, Genistein

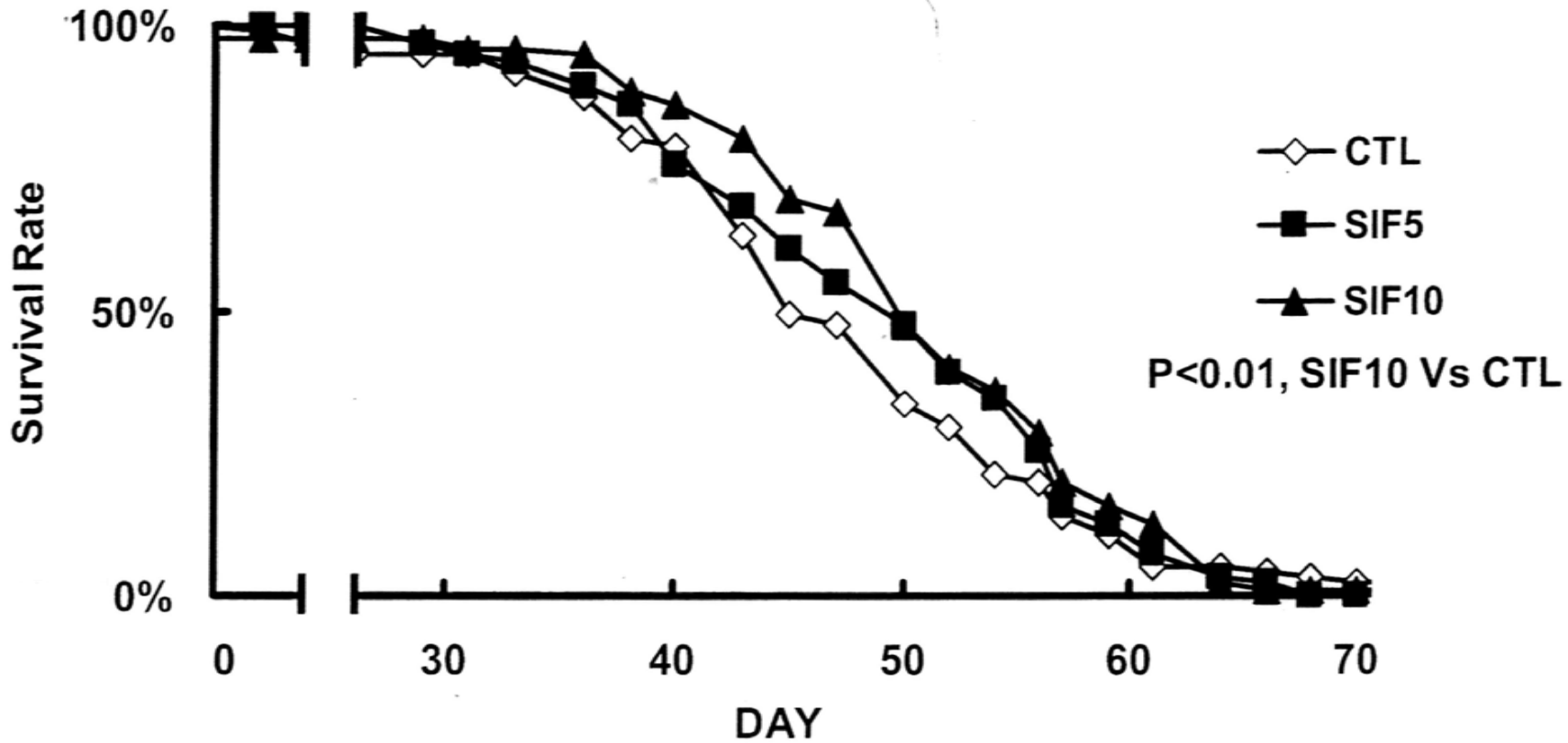


Figure 2 Lifespan curve of wild type flies (OR) fed diets containing 0 mg/ml (CTL), 5 mg and 10 mg soybean isoflavones (SIF5 and SIF10) per milliliter diet. Data were expressed as the maximum lifespan of last fly, 50% survival time and mean lifespan (n=200 flies) for each group (Table 1). The Kaplan-Meier test found that SIF10 could significantly extend the mean lifespan of fruit flies ($P<0.01$).

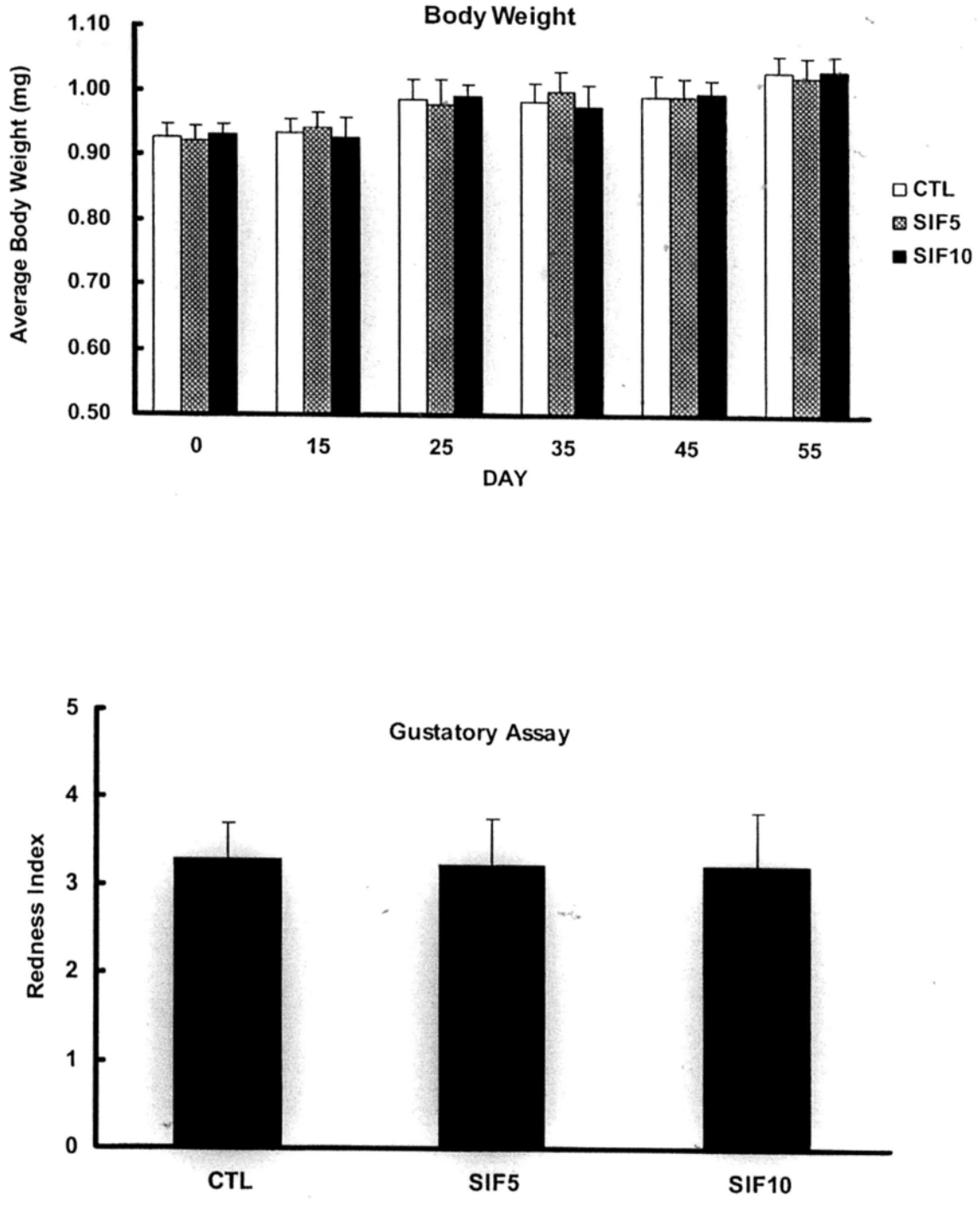


Figure 3 Body weights were measured at day 0, 15, 25, 35, 45, and 55 for flies in control (CTL), SIF 5mg/mL (SIF5), and SIF 10mg/mL (SIF10) groups; Gustatory Assay compared the food intake on the basis of the differences in the degree of abdomen redness among CTL, SIF5, and SIF10 groups. Data are expressed as mean ± SD.

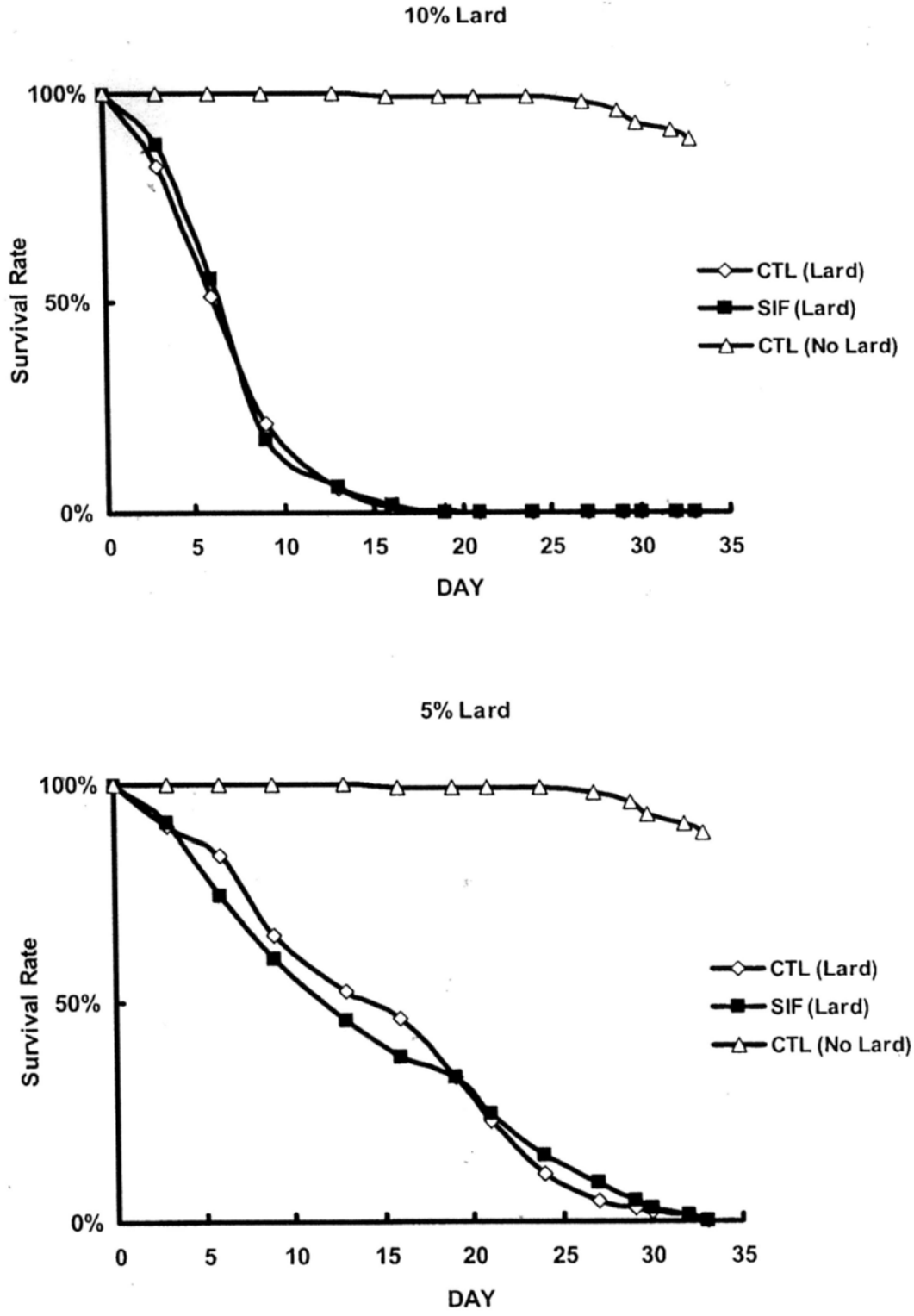


Figure 4 Lifespan of OR fruit flies fed either a diet containing no lard fatty acid [CTL(no lard)] or a diet containing 10% / 5% lard fatty acid [CTL(lard)] with addition of 10 mg/mL SIF [SIF(lard) at 25°C.

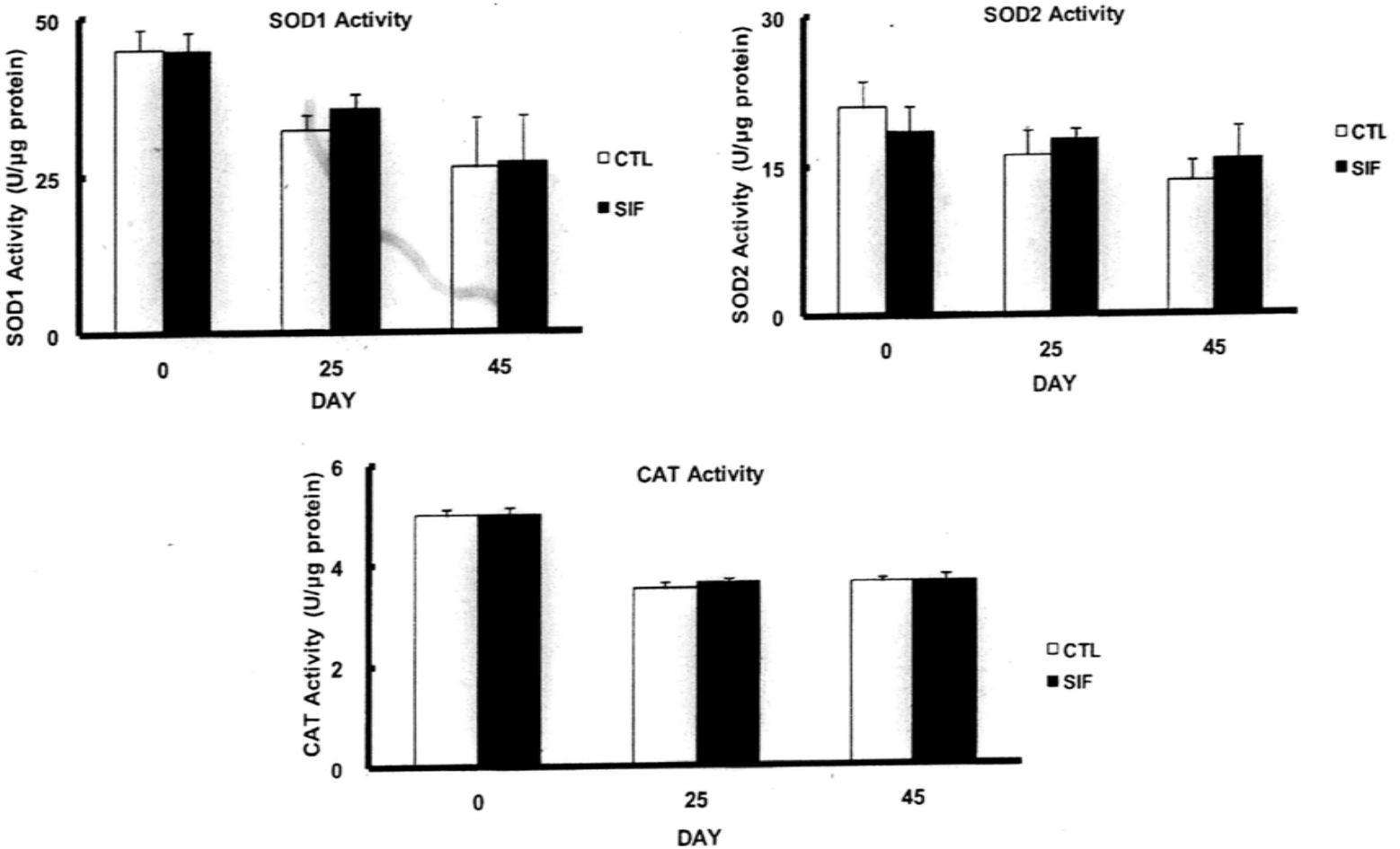


Figure 5 Effect of 10mg/mL SIF on enzymatic activity of SOD1, SOD2 and CAT compared with the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 25 and 45 days. Data are expressed as mean ± S.D.

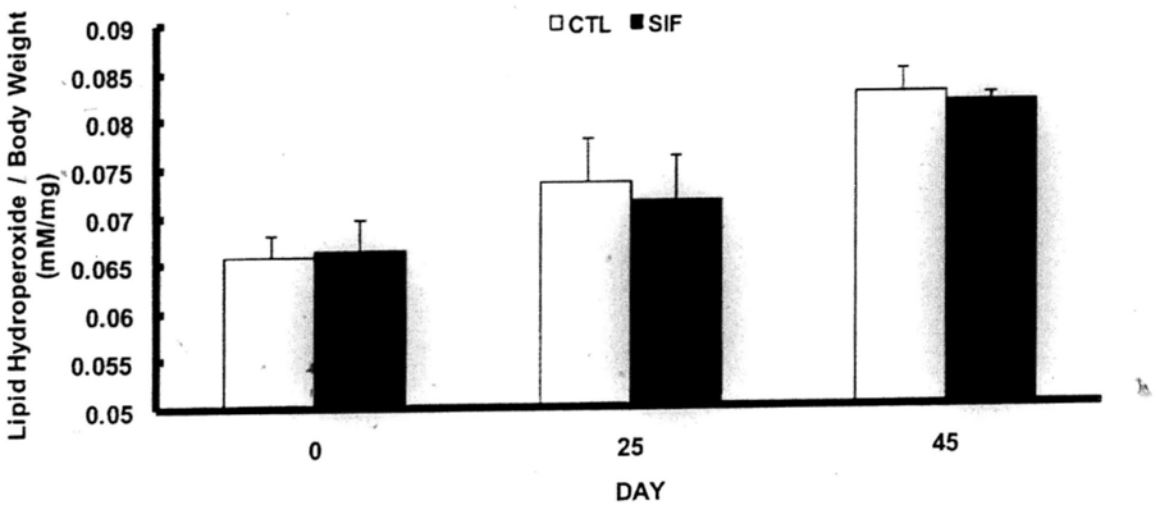


Figure 6 Effect of SIF on the whole body lipid hydroperoxide (LPO) level in control (CTL) and SIF groups. The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 25 and 45 days. Data are expressed as mean ± S.D.

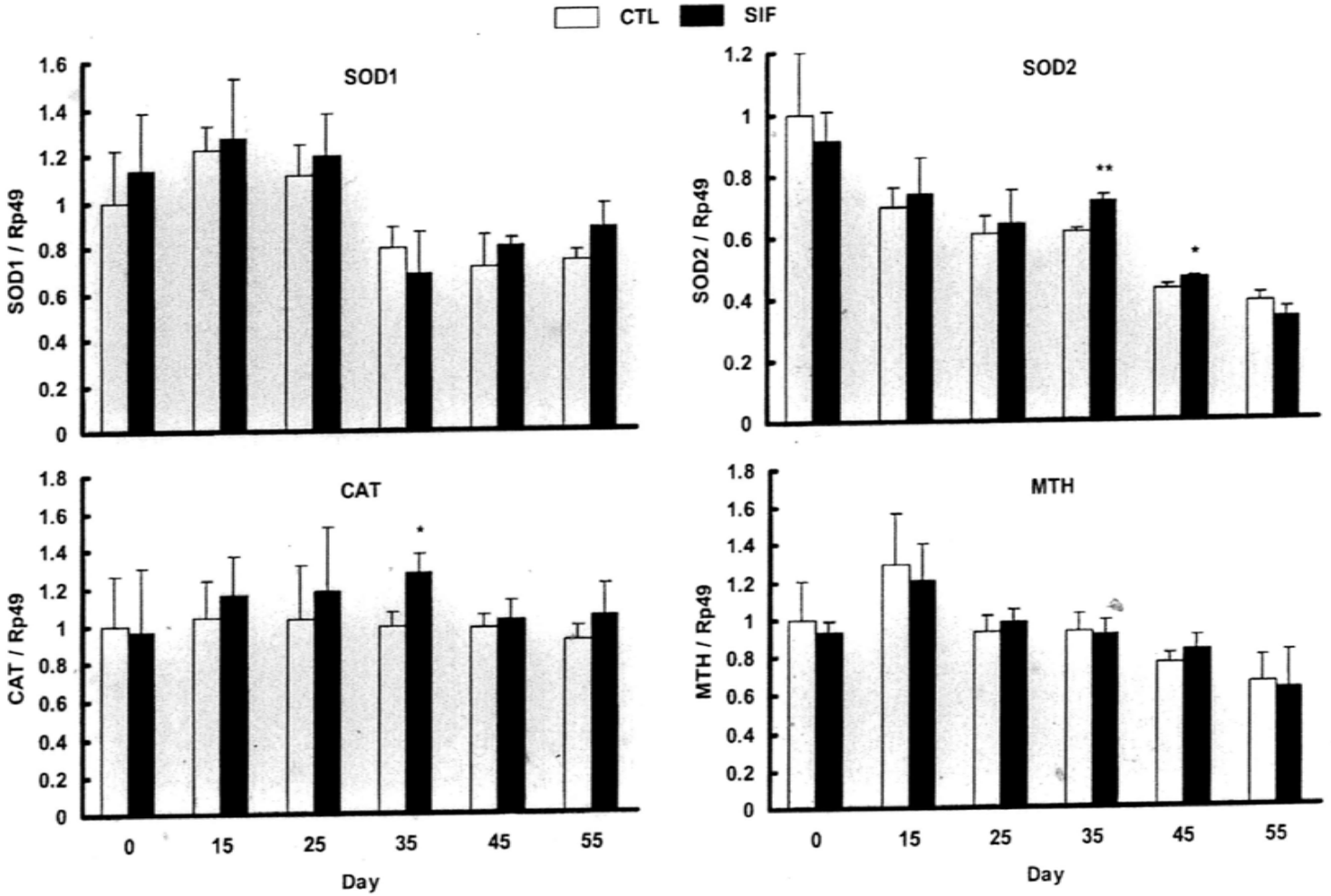


Figure 7 Effect of SIF supplementation (10 mg/ml) on mRNA of SOD1, SOD2, CAT and MTH compared with the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 15, 25, 35, 45 and 55 days. Data are expressed as mean ± S.D. * $P<0.05$; ** $P<0.01$ compared with the control value.

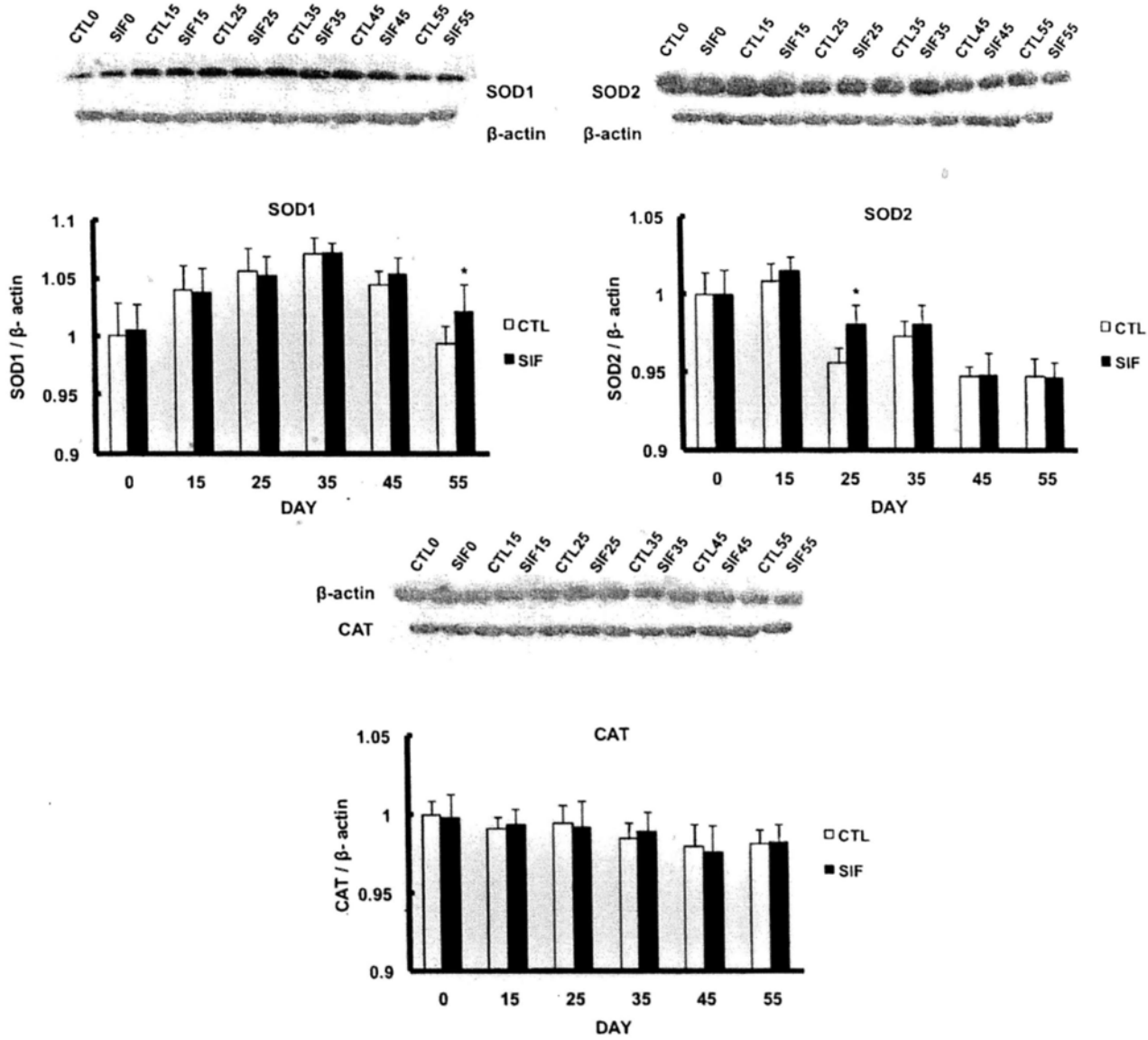


Figure 8 Effect of black SIF supplementation (10 mg/ml diet) on the relative immunoreactive mass of SOD1, SOD2, and CAT compared with the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 15, 25, 35, 45 and 55 days. Data are expressed as mean ± S.D. * $P<0.05$ compared with the control value.

3.5 Discussion

The present study is the first report to reveal the anti-aging activity of SIF in *Drosophila melanogaster*. Results demonstrated that SIF in diet could extend the mean life span of fruit flies by more than 10% compared with the control group. However, SIF had no significant effect on the maximum life time. Data were in consistent with those in a previous study showing that SIF could significantly attenuate the oxidative damage and improve the parameters related to aging and Alzheimer's disease in mice (Hsieh et al., 2009), In another study conducted by Baeza et al. (2010), it was found that the administration of SIF and green tea could improve the immune and redox state in ovariectomized mice. However, the study on the anti-aging property of SIF in humans is still lacking.

The underlying mechanisms for SIF's anti-aging activity remain elusive. One potential explanation is that SIF could directly help scavenge free radicals in vivo. HPLC analysis revealed that SIF mainly contain aglycones genistein, daidzein, and their glycosides, genistin, daidzin (Figure 1). Previous studies showed that SIF possessed the antioxidant activity, attenuating oxidative damage both in vitro and in vivo (Sierens et al., 2001; Kawakami et al., 2004; Mahn et al., 2005). Furthermore, it has been demonstrated that oral administration of soy isoflavones would significantly reduce the lipid peroxidation and DNA damage in human runners (Di Giocamo et al., 2009). The present result showed that supplementation of SIF in diet exhibited a decreasing tendency in LPO level compared with the control fruit fly,

although no statistical significance was seen at the selected time points (Figures 6).

Another potential mechanism by which SIF extend life span of fruit flies might be due to its interaction with endogenous antioxidant defense system. This study demonstrated that SIF administration could significantly up-regulate gene expression of SOD2 and CAT at selected time points. This was in agreement with the study of Borrás et al., who demonstrated that the antioxidant activity of genistein was mediated via the up-regulation of antioxidant gene expression, such as increased mRNA levels of MnSOD and activation of NFκB (Borrás et al., 2006). In this regard, it has been shown that over-expression of SOD and CAT in *S. cereviseae* and *Drosophila melanogaster* would lead to reduced oxidative damages and extended life span (Landis and Tower, 2005). The present study found that supplementation of SIF has no effect on the gene expression level of MTH and the high fat-induced mortality (Figure 4; Figure 7), indicating that life prolonging activity of SIF in fruit flies was associated with its effect on endogenous antioxidants SOD and CAT, and most unlikely related to the longevity determined gene. In fact, SIF only prolonged the mean lifespan and had no effect on the maximum lifespan.

Data gathered from western blot confirmed that endogenous antioxidants were indeed strengthened by the supplement of SIF. In general, SOD1 and SOD2 showed decreasing trends with peaks at different life time points. For SOD1, the protein

mass increased in SIF group compared with the control at late stage of life span, with the appearance of significant difference at day 55. As to SOD2, the most obvious difference appeared at the middle stage of life span. However, no significant difference in CAT protein mass was observed. Two reasons are provided in response to the data variation between western blot and real time PCR. First of all, as a semi-quantitative analysis conducted on SDS-PAGE, western blot is relatively less sensitive. Thus, minor differences in protein mass might not be effectively detected. Secondly, antibodies employed in the study are not all specific to the species of *Drosophila melanogaster*, which would lead to the lack of strict consistency in protein mass and mRNA expression level.

Effect of SIF supplementation on antioxidant enzyme activities was minor. Total activity of antioxidants, which refers to a certain enzyme activity per unit protein times the relative protein mass, is directly responsible for the ROS scavenging capacity of the enzyme. In this study, with no difference observed in unit enzyme activity, SIF still could enhance the antioxidant defense system by elevating protein abundance of SOD1 and SOD2. In addition, this might help explain why SIF could extend mean life span of fruit flies but it was not able to rescue the elevated mortality rate caused by a high-fat diet (Figure 4).

In conclusion, the present study demonstrated that SIF in diet could

significantly increase mean life span of fruit flies with up-regulation of endogenous antioxidant defense system, such as, SOD1, SOD2, CAT, on both mRNA and protein level at selected time points. However, its influence on either longevity determined gene MTH, or antioxidant enzyme activity in fruit flies was trivial.

Chapter 4

Apple polyphenols (AP) extend mean lifespan of *Drosophila melanogaster*

4.1 Introduction

Organisms become aged partially due to accumulation of free radical damage in their cells over time. Reactive oxygen species (ROS), namely hydroxyl radical, superoxide anion and hydrogen peroxide, are produced as natural byproducts of the normal cellular metabolism of oxygen. The oxidative damage initiated by ROS is believed to be a major contributor to the functional decline associated with characteristic of aging. In this regard, organisms have two antioxidant systems to scavenge these ROS and minimize their associated adverse effect. First, they endogenously produce a group of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), which serves as a first line of defense against ROS. Second, exogenous antioxidants like vitamin E and C may build a secondary defense base to terminate the propagation of ROS reactions and slow down the aging process (Willis et al., 2009).

Various predictive biomarkers have been suggested for physiological aging and age-linked diseases. First, it is widely accepted that mitochondrial respiratory capacity declines during the aging process. Cytochrome c oxidase (CcO), the

terminal component of the mitochondrial electron transport chain (ETC), has been reported to show an age-related decline with its subunits III and VIb being significantly reduced in the aging flies (Ren et al., 2010). It has been also reported that CcO deficiency would lead to reduction of ETC activity as a whole due to increased production of either superoxide anion radicals or hydrogen peroxide in mitochondria. (Sohal et al., 2008). Second, Rpn11 has been recognized as a suppressor of progressive neurodegeneration. Knocking down of Rpn11 can lead to the accumulation of ubiquitinated proteins, shorten life span, and reduce 26S proteasome activity (Tonoki et al., 2009). Third, *Methuselah* (MTH) gene has been shown to be one of the longevity determined genes. It has been reported that MTH mutant flies can live 35% longer than wild type flies and exhibit higher resistance to oxidative stress (Lin et al., 1998), even though the underlying mechanism remains poorly understood.

Fruit flies (*Drosophila melanogaster*) have been employed as a model in aging research because they share many conserved biological pathways and more than 70% of known diseases-causing genes in humans are conserved (Minois et al., 2006). In addition, the benefits of using fruit flies as an aging model are associated with their short lifespan, tiny body size, easy maintenance and known sequence of the full *Drosophila* genome. Dietary modification is thought of being able to prolong

lifespan and slow the age-related diseases. On the one hand, calorie restriction has been shown to extend lifespan in various animal models (McCay et al., 1935; Lin et al., 2002; Partridge et al., 2005; Lee et al., 2006); on the other hand, supplement of different nutraceuticals into daily diet has been also recognized as a potential way to prolong lifespan (Piper and Bartke, 2008). Apple is also excellent source of polyphenol antioxidants and has been regarded as a healthy fruit in many cultures.

4.2 Objectives

The present study was therefore to investigate (i) the lifespan-prolonging activity of apple polyphenols (AP); and (ii) the interaction between dietary AP and gene expressions of endogenous antioxidant enzymes; CcO subunits Ⅲ and Ⅵb, Rpn11, and MTH in fruit flies.

4.3 Materials and methods

4.3.1 Fly strains

Fly strains used in this study were Oregon-R-C (OR), SOD^{n108}/TM3 (SOD^{n108}), and OE-/SM5 x Cat^{n1}/TM3 (Cat^{n1}) (Bloomington Drosophila Stock Center, Department of Biology, Indiana University, Bloomington, IN, USA). OR is a wild type fly which was used in all experiments unless specified otherwise. SOD^{n108} is a mutant with one pair of single SOD gene on 3L chromosome being knocked out while Cat^{n1} is

a mutant with CAT gene on chromosome 3L being knocked out by a point mutation.

4.3.2 Diet

A control diet was prepared according to the standard formulation suggested by Li et al. (2007) as described previously in Chapter 2 (2.3.2).

4.3.3 Isolation of AP and HPLC analysis

AP was isolated from the pomace of Red Fuji as we previously described (Lam et al., 2008). In brief, AP was extracted into ethanol and then concentrated followed by purification through adsorption on an AB-8 macroporous resin column. The yield of AP was about 0.4 kg AP/100 kg apple pomace. Individual polyphenol in AP was separated on a Luna C18 column (100A, 250×4.6mm ID) and quantified on a Shimadzu LC-10AT HPLC system equipped with a UV detector at 280 nm. The flow rate was set at 1 ml/ min, while the gradient mobile phase consisted of 2% acetic acid (solvent A) and 0.4% acetic acid with 80% acetonitrile (solvent B). The ratio of A to B was programmed from 10:1 to 2:8 in 80 minutes and then back to 10:1 in 3 minutes, and then was held for another 22 minutes (Figure 1). The peaks were identified according to the retention times of standards. AP contained chlorogenic acid most (16.4%) followed by phloretin (6.2%), proanthocyanidin B2 4.3%, epicatechin (2.6%), catechin (1.2%), rutin (0.2%); and proanthocyanidins (dimmer, trimer and tetramer,

69.0%).

4.3.4 Effect of AP on longevity of OR flies fed the basal diet

Newly eclosed male flies were divided into 3 groups (n=200 flies each), and housed in 10 vials (20 flies per vial). The first group was maintained on the basal diet, while the two AP groups were fed one of the two diets containing 2 or 10 mg AP/ml, respectively. Dead flies were counted every 2-3 days and the remaining alive flies were transferred to a new vial containing the same diet. The feeding lasted 74 days (Figure 2A). The same experiments described above were similarly repeated and the fruit flies were killed at selected time points in order to quantify the expression of SOD, CAT and MTH.

4.3.5 Gustatory assay

To exclude the possibility that lifespan extension in survival assay might be induced by dietary restriction, gustatory assay was carried out (Bahadorani et al., 2008). Details of this assay was described in Chapter 3 (3.3.5).

4.3.6 Intensive paraquat challenge experiment

Paraquat chemically named 1,1'–dimethyl-4,4'–bi-pyridinium dichloride (Sigma, St. Louis, MO, USA) is able to generate superoxide anion radicals (Michaelis et al.,

1933). To examine the resistance of flies against superoxide-induced stress, both OR flies (n=400 in 20 vials) and SOD^{n108} mutant flies (n=400 in 20 vials) were maintained on their corresponding control diet and experimental diet containing 10 mg AP /ml, and incubated at 25°C. At day 25, the fruit flies in two groups were first starved for 2 hours, and then transferred into new vials containing a filter paper saturated with 1 ml of 20mM paraquat in a 6% glucose solution. Every 4-6 hours, dead flies were counted until all flies died.

4.3.7 Hydrogen peroxide (H_2O_2) challenge test

H_2O_2 is able to generate hydroxyl radicals and therefore, it was used to examine the resistance of flies against OH-induced oxidative stress. OR flies (n=400) and Cat^{n1} mutant flies (n=400) were maintained on their corresponding control diet or experimental diet containing 10 mg AP /ml and incubated at 25°C. Similarly, the fruit flies in the two groups were first starved for 2 hours, and then were transferred into new vials containing a filter paper saturated with 1 ml of 30% H_2O_2 in a 6% glucose solution at day 25. Every 4-6 hours, dead flies were counted until all flies died.

4.3.8 Chronic paraquat challenge

A long term exposure to paraquat has been recognized as a potential risk factor to get neurodegenerative diseases such as Parkinson's disease. To examine the

resistance of flies against paraquat-induced mortality and locomotor deficiency, 900 newly eclosed male OR flies were randomly divided into three groups, namely blank control group (BCTL), control group (CTL), and the experiment diet containing 10 mg AP/ml (AP10). Every 3 days, flies, after 2-hour starvation, were transferred into vials containing a filter paper had saturated with 1ml of 20 mM paraquat in a 6% glucose solution. After 24 hours, flies were moved to new vials containing only water-saturated filter paper for 2 hours before they were transferred back into vials containing respective diets. The experiment lasted 42 days until all the fruit flies in AP10 group died. Another set of the experiment described above was similarly repeated and the fruit flies were killed at day 0, 10, 20, and 30 to quantify the expression of SOD, CAT, MTH, Cco subunits, ubiquitinated proteins and Rpn11.

4.3.9 Climbing assay

Locomotor function of fruit flies was assessed using a climbing assay as previously reported with slight modifications (Lee et al., 2009). In brief, 10 male flies were placed in a plastic vial, given 20 seconds to climb up. At the end of each trial, the number of flies that climbed up to a vertical distance of 15 cm or above was recorded. Each trial was performed three times. Flies were tested at selected time points during the chronic paraquat challenge survival assay.

4.3.10 SOD Activity

SOD activity assay was conducted as previously described in Chapter 2 (2.3.8) by using a SOD activity assay kit (Cayman Chemical, Michigan, USA).

4.3.11 CAT activity

CAT activity was measured as described previously in Chapter 2 (2.3.9) using a CAT assay kit (Sigma, St. Louis, MO, USA).

4.3.12 Real-time PCR

Gene expression of SOD1, SOD2, CAT, MTH and Rpn11 was measured as previously described (Peng et al., 2009). Total RNA was extracted using the commercial extraction agent TRIzol (Invitrogen, Carslbad, CA, USA). Fruit flies (n=15) were homogenized in 800μL of TRIzol solution, and then centrifuged at 12,000g at 4°C for 10 min and the supernatant was transferred to another new tube containing 160μL chloroform. The mixture was then subjected to centrifugation at 12,000g at 4°C for 15 min. The upper layer was mixed with 400μL isopropanol. After 10 min of incubation at room temperature, the samples were centrifuged at 12,000g at 4°C for 10 min, and the pellet was saved and washed in 1ml of 75% ethanol followed by re-centrifugation. Finally, 25μL DEPC water was employed to resuspend the RNA pellet. The concentration and purity of RNA obtained were

determined by measuring their absorbance at 260nm and 280nm. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to construct cDNA. RNA (2μg) was used for each reaction together with MgCl2, 10X RT buffer, dNTP, random hexamers, RNase inhibitor, and MultiScribe Transcriptase. The final volume was adjusted to 10μL. cDNA was synthesized in the thermocycler GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) and stored at -20 °C.

Real-time PCR amplification was carried out on a Fast Real-time PCR System 7500 (Applied Biosystems, Foster City, CA, USA). Five target genes included: SOD1 (NCBI Reference Sequence NM_057387.3), SOD2 (NCBI Reference Sequence NM_057577.2), CAT (NCBI Reference Sequence NM_080483.2), MTH (NCBI Reference Sequence NM_079147.2), and Rpn11 (NCBI Reference Sequence NM_135061.2). The expressions of target genes were normalized with that of rp49 (NCBI Reference Sequence NM_079843.2), a housekeeping gene used as the internal control. Gene expressions were calculated based on the comparative Threshold cycle (CT) value. Levels of gene expressions in all groups were shown as a ratio of the day 0 control group.

4.3.13 Western blot analysis

Total proteins or mitochondria protein were extracted and subjected to western

blot analysis. For total proteins, 50 flies were homogenized in a 1.5mL tube containing 500μl homogenizing buffer (20mM Tris-HCl, 2mM MgCl2, 0.2M sucrose and protease inhibitor cocktail (Roche, Mannheim, Germany). The extracts were centrifuged at 13,000 g for 5 min at 4°C and the supernatant were collected; To isolate the mitochondria, approximately 50 flies per sample (4 samples/group) were homogenized in mitochondrial isolation medium (MIM; 250 mM sucrose, 10 mM Tris pH 7.4, 0.15mM MgCl2). The samples were centrifuged twice at 1,000 × g for 5 min to remove debris. The supernatant was centrifuged at 13,000 × g to obtain a mitochondria-enriched pellet which was washed with 1 ml of MIM. This pellet was re-suspended in 50 μl MIM and the membranes were disrupted by two freeze thaw cycles. Protein concentration was determined using a protein concentration assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). After adding 6×loading dye and homogenizing buffer to adjust the volume, the protein were boiled at 95°C for 5 min, and then stored at -80°C. Mitochondria protein were employed to western blot analysis specifically for Cco subunit Ⅲ, Ⅵb, and porin.

For the measurement of CAT and β-actin, 20μg total protein were size-fractionated on 7% SDS-PAGE gel at 130V for 70 minutes, while the same amount of total protein were loaded to measure SOD1 and SOD2 on 15% SDS-PAGE gel at 130V for 180 minutes. To measure Cco subunit Ⅲ, Ⅵb, and porin,

10μg mitochondria protein was size-fractionated on 10% SDS-PAGE gel at 110V for 100 minutes. The proteins were then transferred to a Hybond-P PVDF membrane (Millipore, Billerica, MA, USA). The membranes with total proteins were incubated for one hour in blocking solution (5% non-fat milk) at room temperature and then in the same solution containing diluted anti-catalase / anti-actin / anti-CuZnSOD / anti-MnSOD/ anti-Ub antibodies respectively at 4°C overnight. For immunodetection of mitochondria proteins, membranes were incubated with the anti- CcO subunits Ⅲ, Ⅵb, and porin at 37 °C for 1 h. The membrane was washed in 1×TBST and was then incubated for one hour at 4°C in diluted horseradish peroxidase-linked goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., California, USA) or anti-mouse IgG (Santa Cruz Biotechnology, Inc., California, USA). The washes were repeated before the membranes were developed with ECL enhanced chemiluminescence agent (Santa Cruz Biotechnology, Inc., California, USA) and subjected to autoradiography for one second to five minutes on SuperRX medical X-ray film (Fuji, Tokyo, Japan). Densitometry was quantified using the computer software Quantity one (Bio-Rad, CA, USA).

4.3.14 Statistics

Data were expressed as mean±standard deviation. The Kaplan-Meier test was employed to compare the difference between the survival curves using SPSS 15.0

(Statistical Package for the Social Sciences software, SPSS Inc, Chicago, USA). The significance of difference between means was assessed using T-test and one way ANOVA. Differences were considered significant when $p<0.05$.

4.4 Results

4.4.1 Effect of AP on longevity of OR flies fed the basal diet and AP diets

AP10 group had the longest lifespan among the three groups of OR wild type male flies (Figure 2A). The maximum lifespan increased about 3% in AP10 group, while 50% survival time increased from 50 days to 55 days compared with that in the control flies. To be specific, the mean lifespan for the control and the two AP groups were 50, 52, and 55 days, respectively (Figure 2A; Table 1). However, a significant difference in the mean lifespan was only found between AP10 and the control ($P<0.01$). Meanwhile, gustatory assay did not reveal any significant difference between control and AP-fed group (Figure 2B).

SOD1 gene was significantly up-regulated in AP10 group compared with that in the control at day 35, 45, and 55, while gene expressions of SOD2 and CAT in AP10 group were greater than those in the control at day 45 and 55 (Figure 3). In contrast, MTH gene was significantly down-regulated in AP10 group than that in the control since day 25 (Figure 3).

Western blot data showed that, at day 15 and 55, AP10 group had protein mass of SOD1 greater than that in the control; while at day 15, significant difference in CAT protein mass was observed between AP10 group and the control. Otherwise, no significant differences in protein mass were seen between AP10 and the control

(Figure 4). The present study also investigated effect of AP on the activity of SOD1, SOD2 and CAT in OR wild type male flies at day 0, 25 and 45. In general, SOD1, SOD2 and CAT activity decreased with aging. Compared with the control group, AP10 could not prevent the decreasing trend in SOD1, SOD2 and CAT activity at all the time points except at day 45 when AP10 showed a greater CAT activity than the control (Figure 5).

4.4.2 Effect of AP on paraquat and H_2O_2-challenged OR, *Cat*^{n1} and *SOD*^{n108} flies

Results from intensive paraquat challenge test showed that AP10 OR group had a longer survival time than the corresponding control ($P<0.05$). To be specific, OR wild type flies had a maximum survival time increased from 70 hr in the control to 98 hr in AP10 group with mean survival time being prolonged by 14% ($P<0.05$). However, no difference was in *SOD*^{n108} mutant flies fed the control and AP 10 diet. Similar results were obtained in the H_2O_2 challenge assay. Supplementation of AP prolonged the maximum survival time, 50% survival time and mean survival time only in OR wild type but not in *Cat*^{n1} mutant fly strain (Figure 6).

4.4.3 Effect of AP on chronic paraquat challenge in OR flies

Long term exposure to 20mM paraquat could induce high mortality rate in fruit flies, shortening their maximum lifespan to 31 days and reducing their climbing ability by more than 60% (Figure 7). Supplementation of AP in diet could partially reverse the paraquat-induced mortality and decline in climbing ability. Results demonstrated that the maximum lifespan was 31 days in the paraquat-control group, while it was 39 days in the paraquat-AP10 group (Figure 7A). At the same time, the

climbing ability was less than 40% in the paraquat-control groups, while it was partially recovered to more than 60% in the paraquat-AP10 flies at day 30 (Figure 7B).

Western blot data did not found any significant difference in protein abundance of SOD1, SOD2, CAT, ubiquitinated protein, Cco subunit III and VIb among the blank control, paraquat-control and paraquat-AP10 groups (Figure 8). mRNA analysis revealed that AP could only up-regulate the gene expression of CAT and Rpn11 at day 30 with no effect on SOD1, SOD2, ubiquitinated protein, Cco subunit III and VIb (Figure 9). Regarding the enzyme activity, SOD1 did not change over time and no difference could be seen among the three groups. In contrast, CAT had a decreasing trend over time and similarly, no difference was found among the three groups. At day 10 thereafter, SOD2 activity showed a decreasing trend over time in the paraquat-control flies and supplementation of AP could partially restore the SOD2 activity (Figure 10).

Table 1 Lifespan of OR wild type flies fed the control diet and the two experimental diet containing 2 and 10 mg AP /ml.

	Maximum Lifespan of last fly (Day)	50% Survival (Day)	Mean Lifespan (Day)
Control	72	50	50 ± 2^{a}
2 mg AP	74	53	52 ± 2^{a}
10 mg AP	74	55	55 ± 2^{b}

[a,b] Means with different letters differ significantly at $p<0.01$.

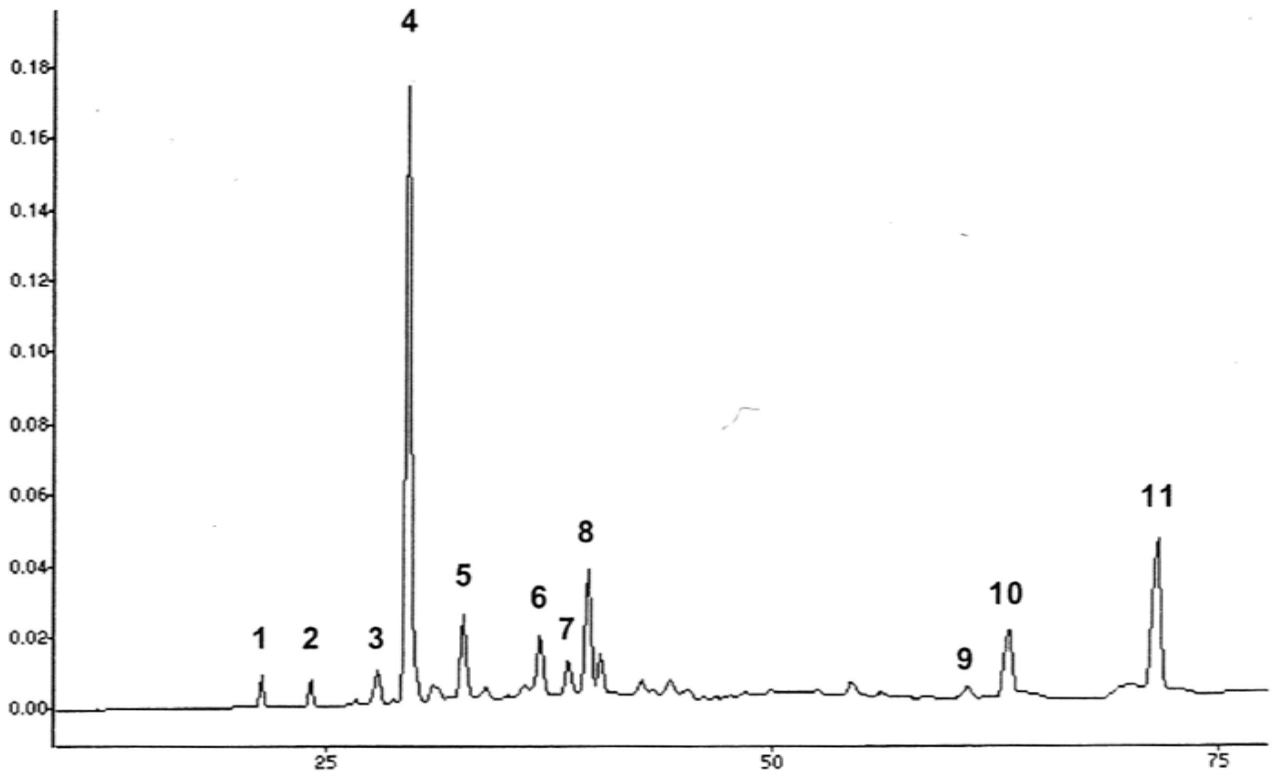


Figure 1 HPLC chromatogram of apple polyphenols (AP). Identification of peaks: 3, catechin; 4, chlorogenic acid; 5, proanthocyanidin B2, 6, epicatechin; 9, rutin; 11, phloridzin; 1,2,7,8,10=unknown.

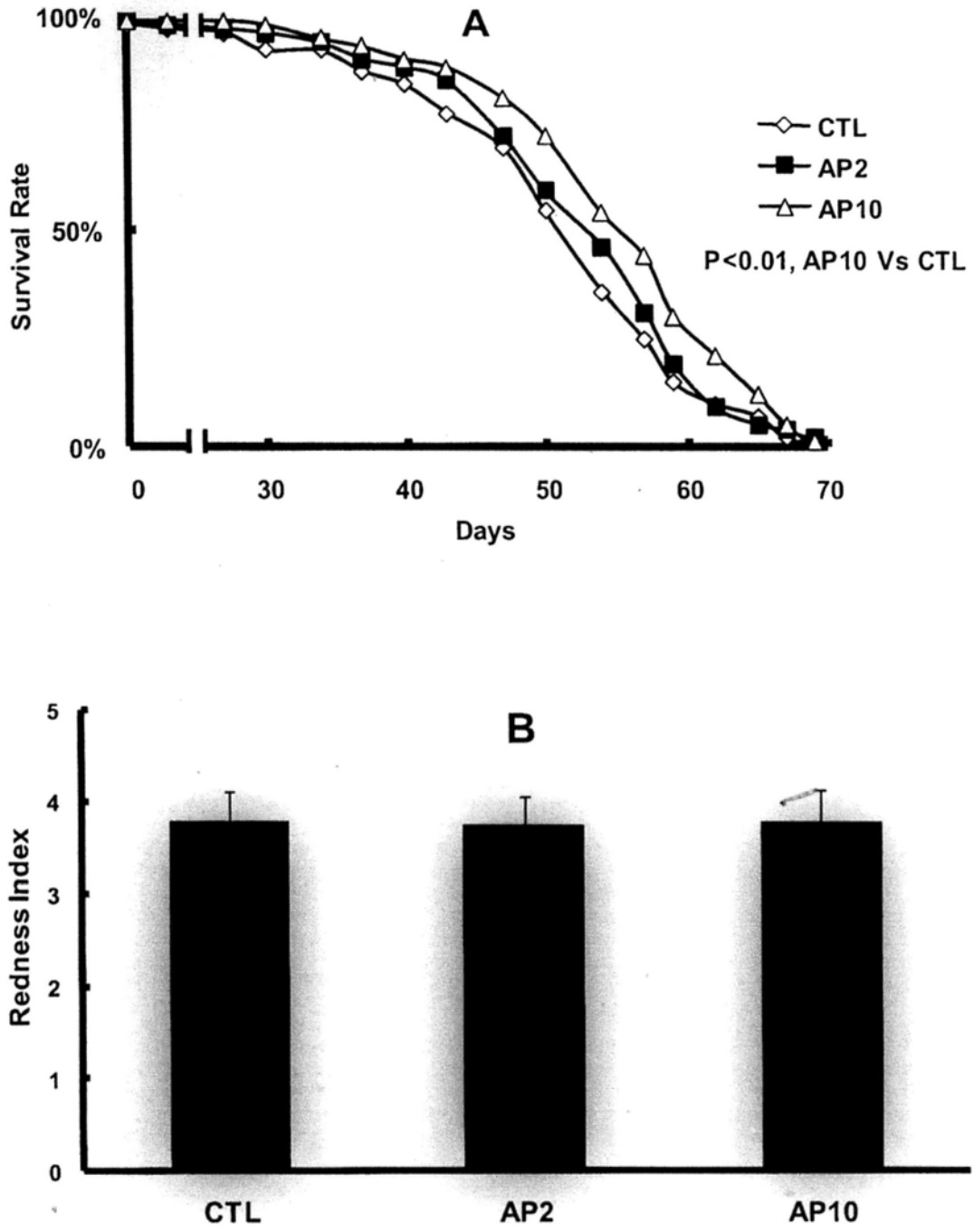


Figure 2. **(A)** Lifespan curve of wild type flies (OR) fed diets containing 0 mg/ml (control, CTL), 2 mg (AP2) and 10 mg AP (AP10) per milliliter diet. Data were expressed as the maximum lifespan of last fly, 50% survival time and mean lifespan (n=200 flies) for each group (Table 1). The Kaplan-Meier test found AP10 could significantly extend the mean lifespan of fruit flies (P<0.01). **(B)** Gustatory Assay compared the food intake on the basis of the differences in the degree of abdomen redness among CTL, AP2 and AP10 groups. Data are expressed as mean ± SD.

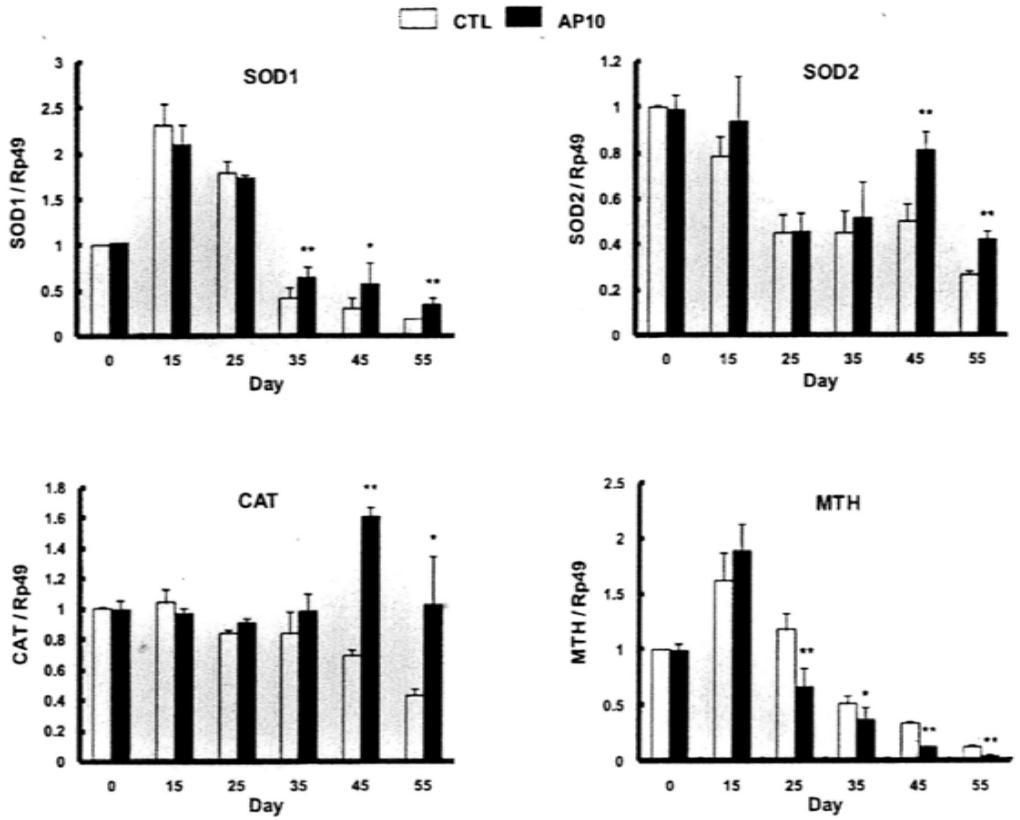


Figure 3 Effect of apple polyphenols (AP) supplementation (10 mg/ml diet) on mRNA of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2), catalase (CAT) and Methuselah (MTH) compared with those in the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 15, 25, 35, 45 and 55 days. Data are expressed as mean ± S.D. * $P<0.05$; ** $P<0.01$ compared with the control value.

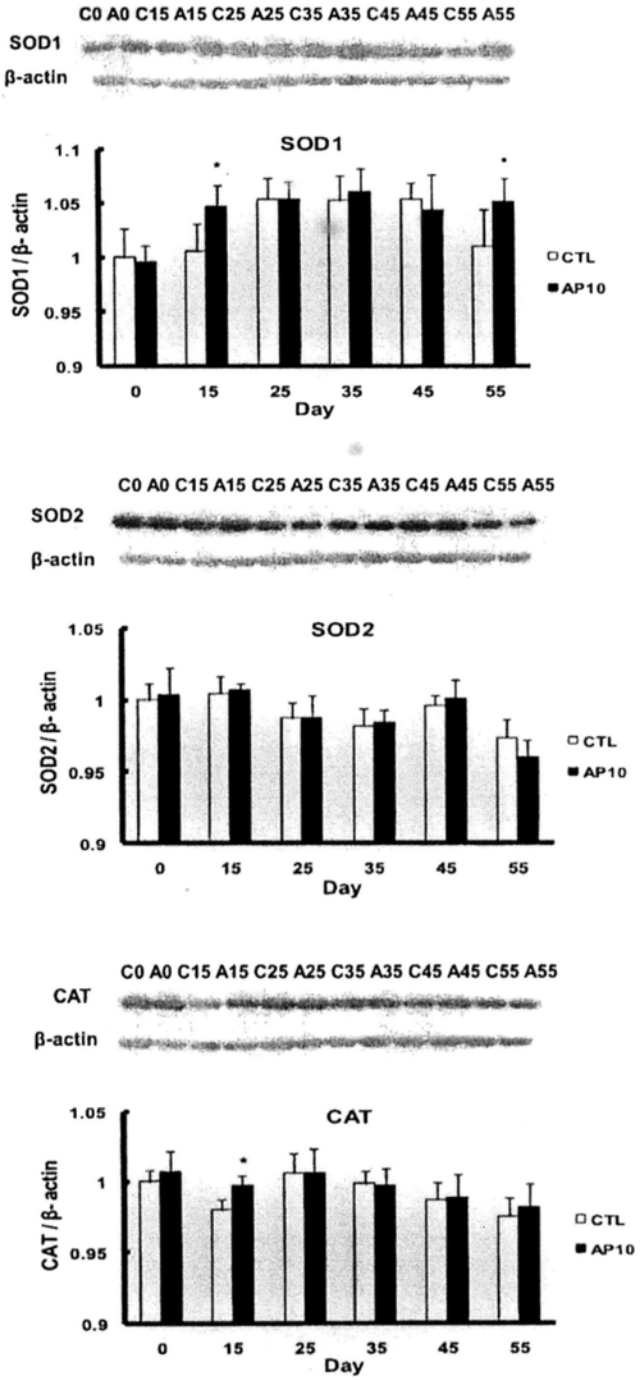


Figure 4 Effect of AP supplementation (10 mg/ml diet) on the relative protein mass of SOD1, SOD2 and CAT compared with those in the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 15, 25, 35, 45 and 55 days. C0-C55 represented the protein bands for the control at day 0-55 days while A0-55 represented the protein bands for AP10 at day 0-55. Data are expressed as mean ±SD. * $P<0.05$ compared with the control value.

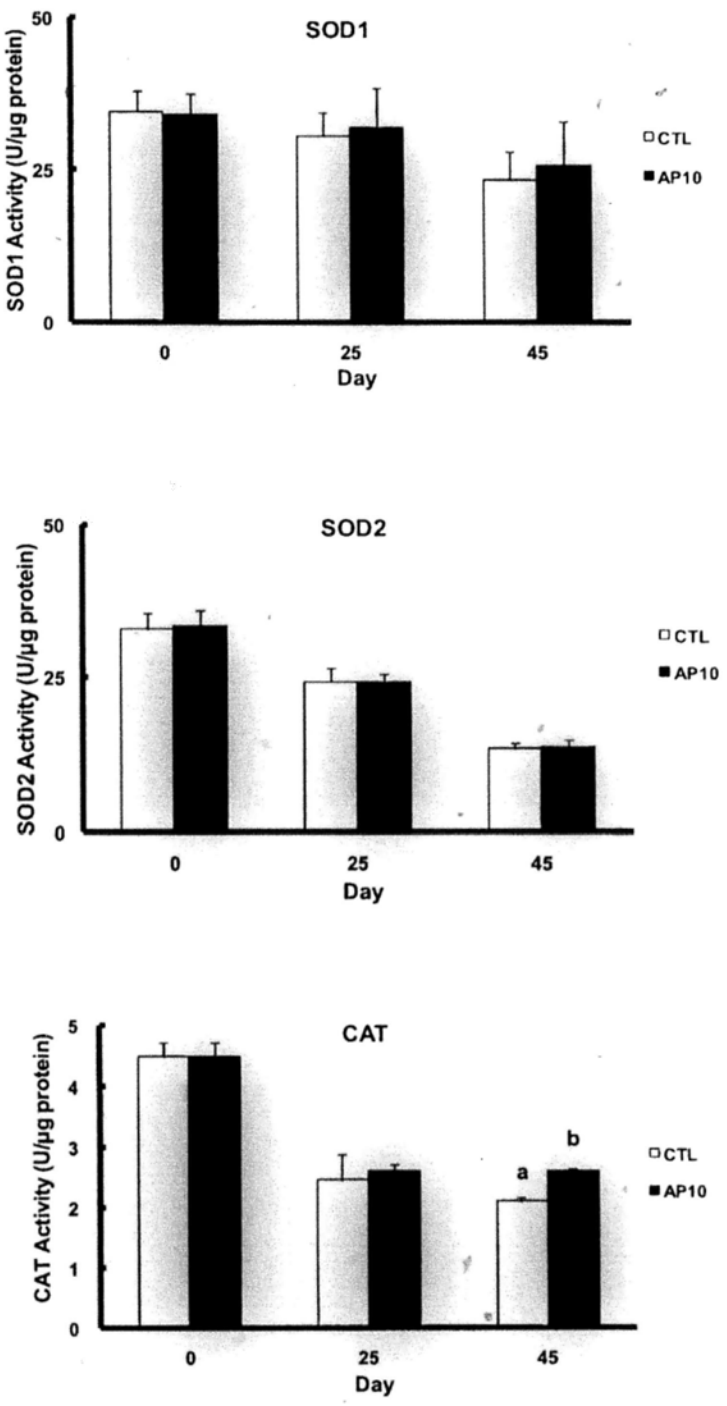


Figure 5 Effect of AP supplementation (10 mg/ml diet) on enzymatic activity of SOD1, SOD2 and CAT compared with those in the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 25 and 45 days. Data are expressed as mean ± SD. [a,b] Means with different letters differ significantly at $p<0.05$.

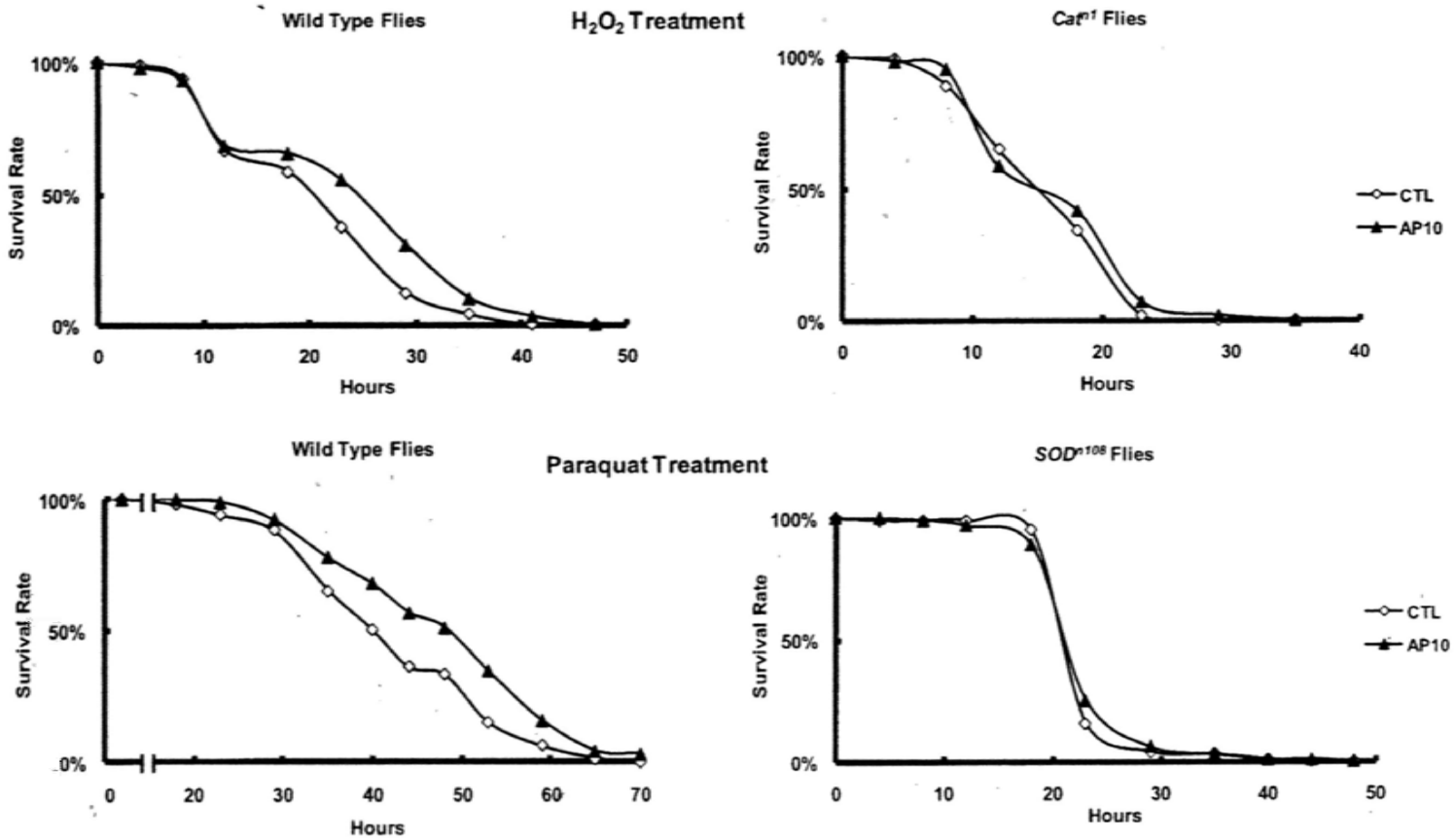


Figure 6 Effect of paraquat treatment or hydrogen peroxide treatment on the survival time of the mutant flies (SOD^{n108}) or mutant flies (Cat^{n1}) fed the diets containing 0 mg/ml (CTL) or 10 mg AP /ml compared with that of the wild type (OR) flies. The Kaplan-Meier test found both that AP-fed OR group survived better than its corresponding OR control ($P<0.05$) while the survival rate of AP-fed SOD^{n108} and Cat^{n1} groups was not different from their corresponding control groups.

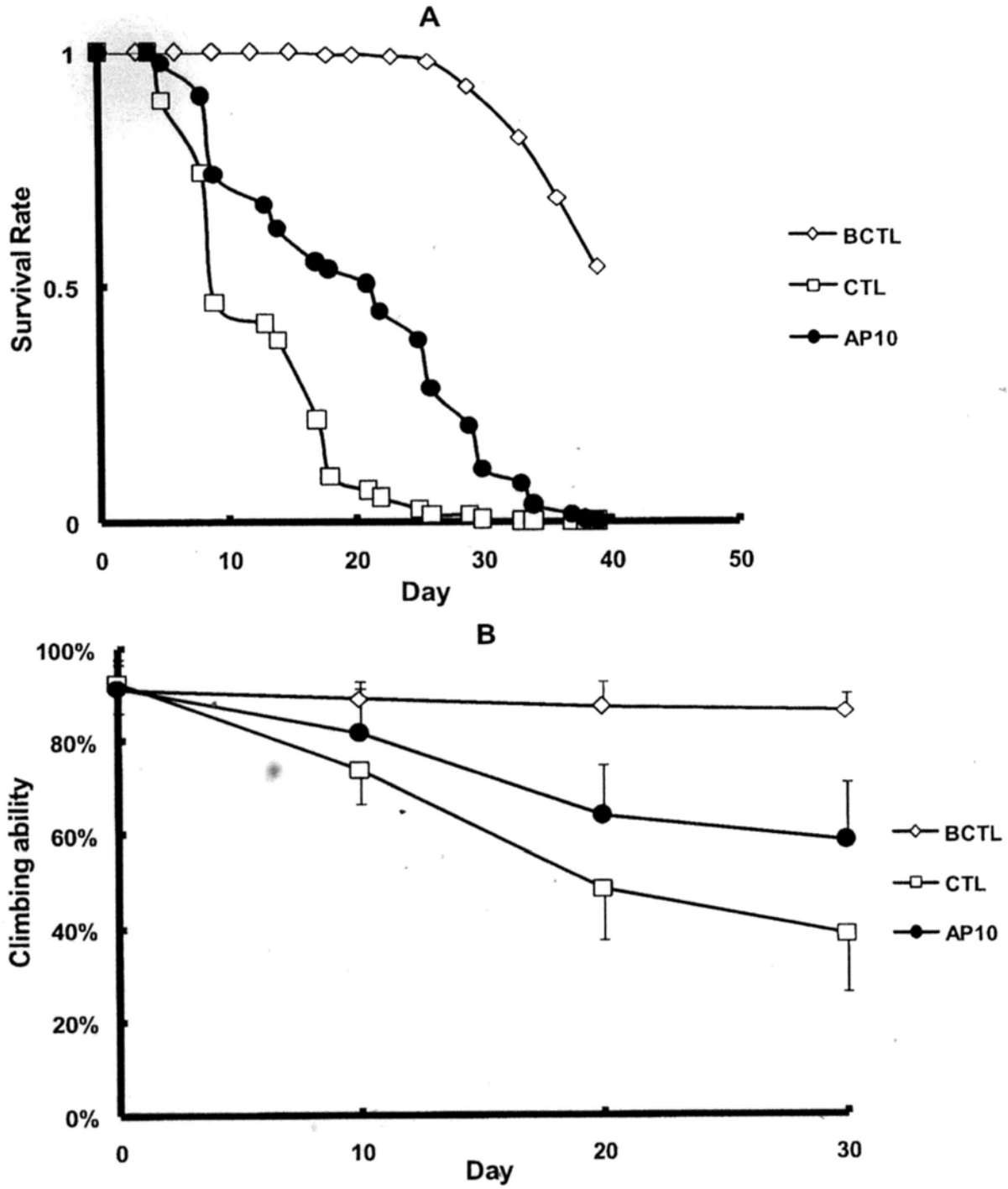
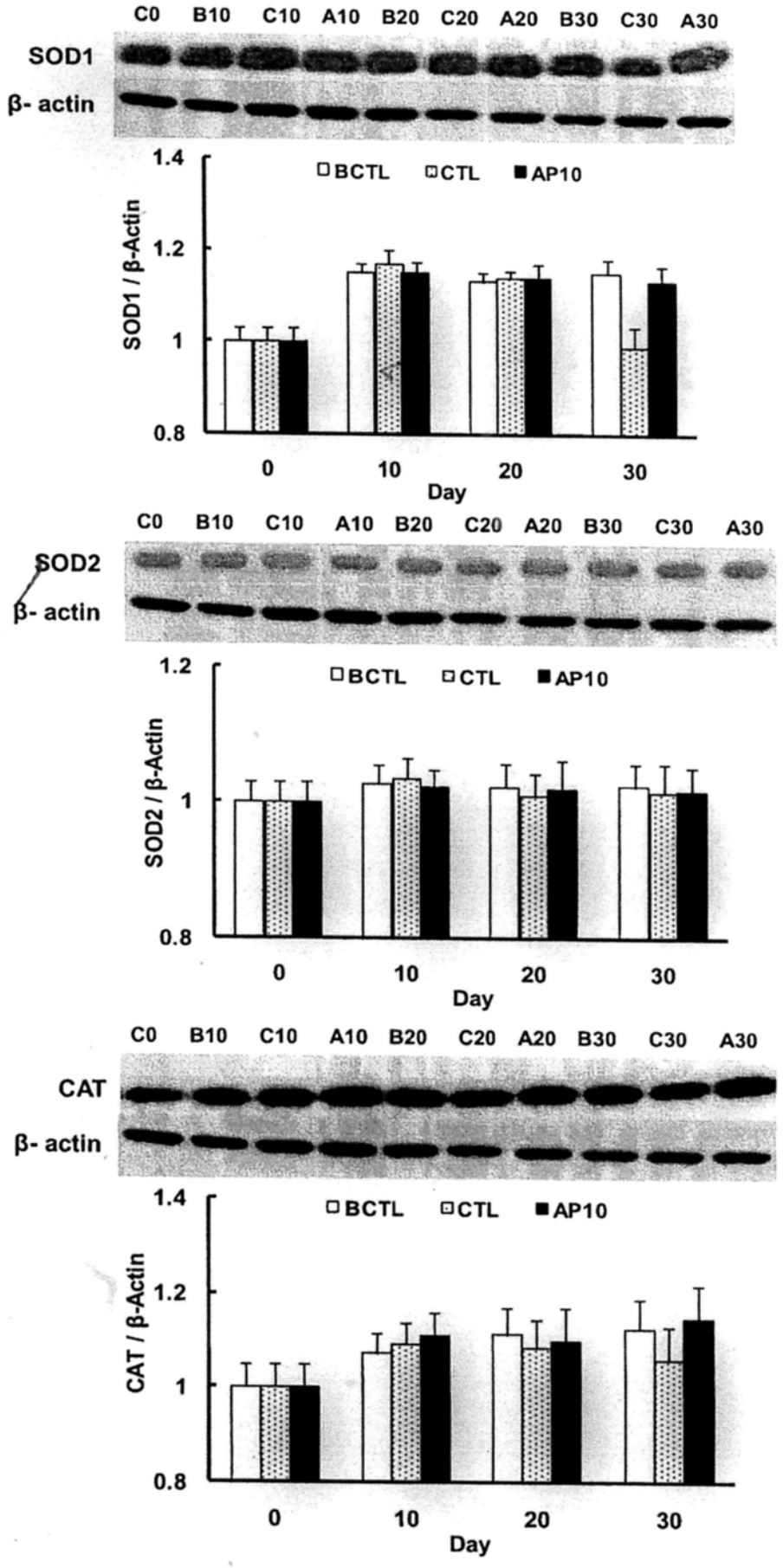


Figure 7 (A) Lifespan of OR fruit flies fed either a basal diet without exposure to paraquat (BCTL) or a basal diet with the paraquat chronic exposure (CTL) with addition of 10 mg / ml AP (AP10) at 25°C. The Kaplan-Meier test found AP10 group survived better than the CTL ($P<0.01$). **(B)** Effect of AP on the climbing ability in BCTL, CTL, and AP10 groups. AP10 group could significantly alleviate locomotor deficiency in CTL group.


C0 B10 C10 A10 B20 C20 A20 B30 C30 A30
SOD1
β- actin
BCTL CTL AP10
SOD1 / β-Actin
1.4
1.2
1
0.8
0 10 20 30
Day
C0 B10 C10 A10 B20 C20 A20 B30 C30 A30
SOD2
β- actin
BCTL CTL AP10
SOD2 / β-Actin
1.2
1
0.8
0 10 20 30
Day
C0 B10 C10 A10 B20 C20 A20 B30 C30 A30
CAT
β- actin
BCTL CTL AP10
CAT / β-Actin
1.4
1.2
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Day

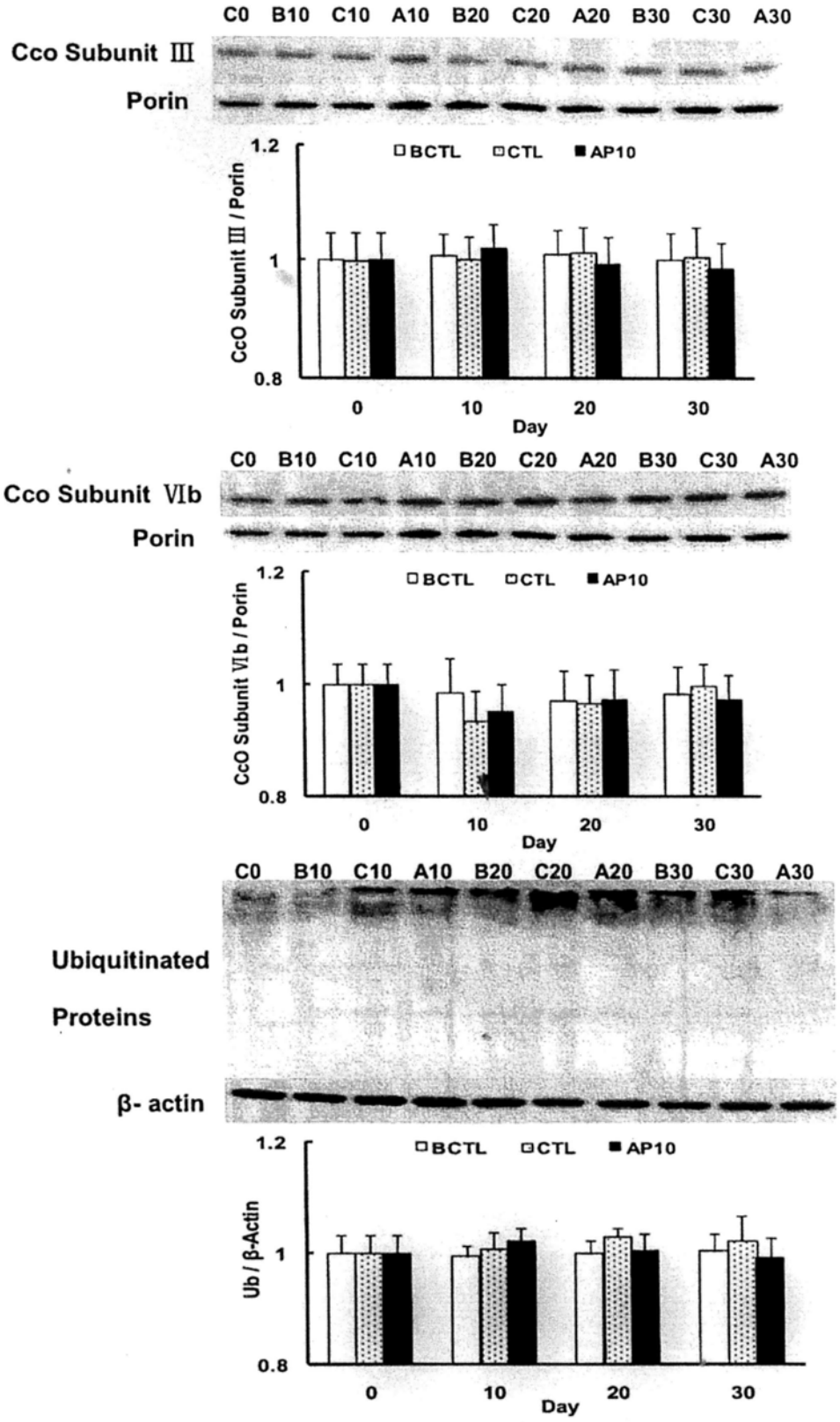


Figure 8 The relative immunoreactive mass of SOD1, SOD2, CAT, CcO Subunit Ⅲ, CcO Subunit Ⅵb, and Ubiquitinated Proteins in OR fruit flies fed either a basal diet without going through the paraquat challenge cycle (BCTL; B10-B30) or a basal diet with the paraquat chronic exposure (CTL; C0-C30) with addition of 10mg / mL AP (AP10; A10-A30).

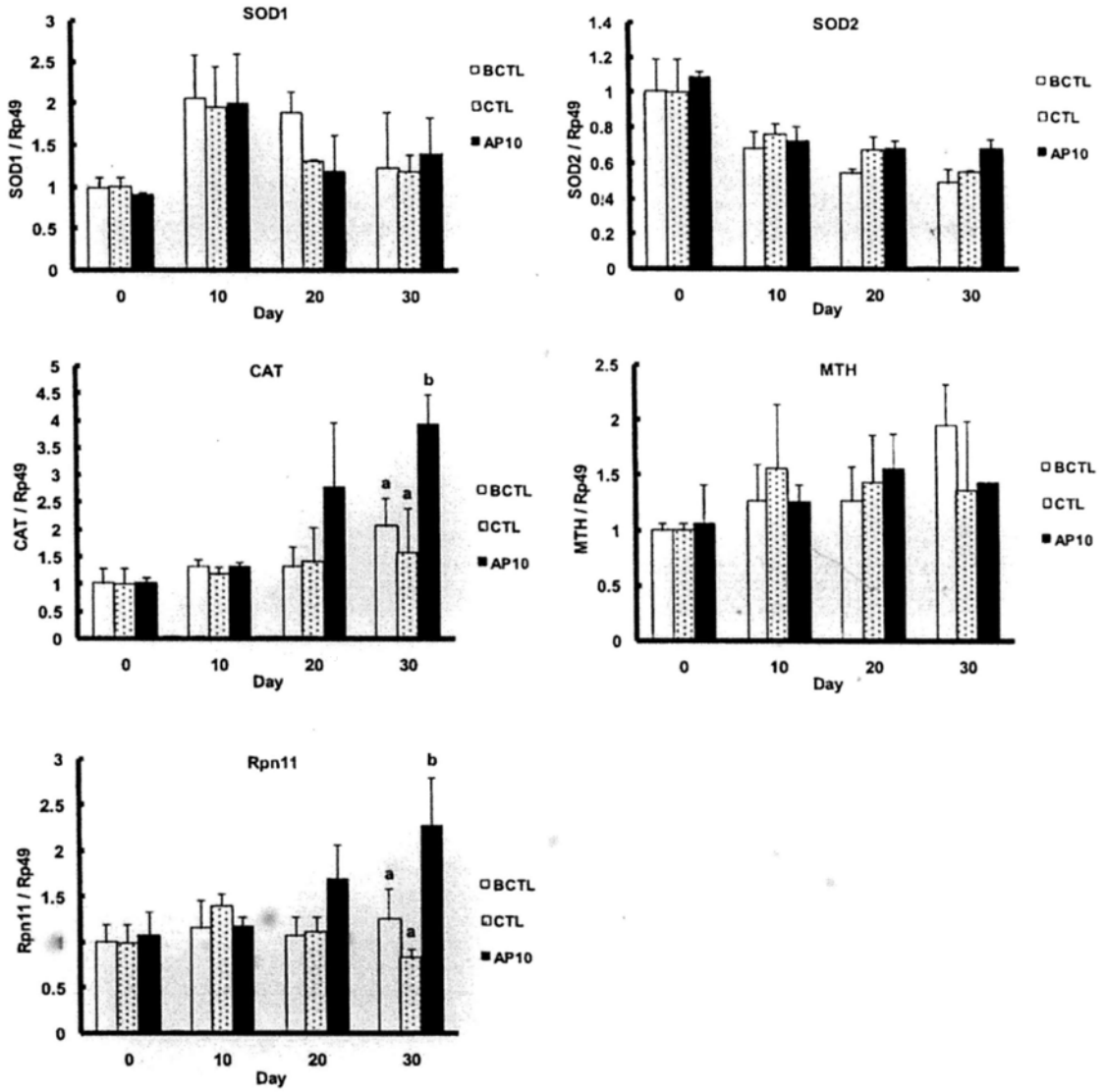


Figure 9 mRNA of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2), catalase (CAT) methuselah (MTH) and Rpn11 in OR fruit flies fed either a basal diet without going through paraquat exposure (BCTL) or a basal diet containing with paraquat challenge cycle (CTL) with addition of 10 mg / mL AP (AP10) at 25°C. [a,b]Means at the same time point differ significantly at $P<0.05$

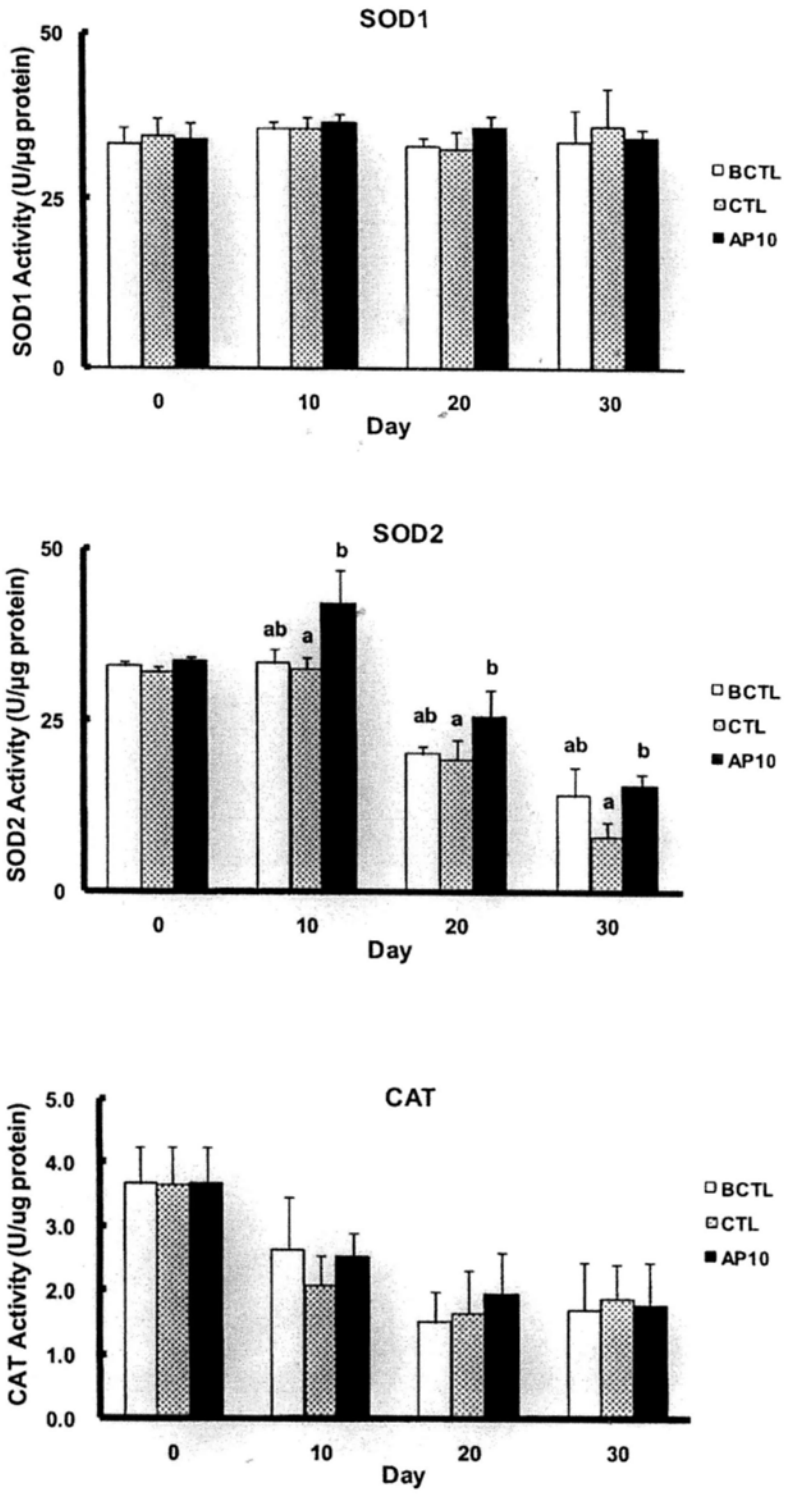


Figure 10 Enzymatic activity of SOD1, SOD2 and CAT in OR fruit flies fed either a basal diet without going through paraquat exposure (BCTL) or a basal diet containing with paraquat challenge cycle (CTL) with addition of 10 mg / mL AP (AP10) at 25°C. [a,b]Means at the same time point differ significantly at $P<0.05$

4.5 DISCUSSION

The present study was the first report to show AP was effective against aging in fruit flies. AP supplementation at 10mg/ml prolonged the mean lifespan by 10% compared with the control (55 versus 50 days, Figure 2). However, AP had little or no significant effect on the maximum lifespan of fruit flies (Table 1). This is in agreement with the study of Sunagawa et al. (Sunagawa et al., 2010), who found that apple procyanidins could extend the mean lifespan of *C. elegans* by 8-12%. No similar lifespan extension study has been conducted in either animals or humans. However, apple juice concentrate, administered ad libitum in drinking water, can compensate for the increased reactive oxygen species and decline in cognitive performance in mice deprived of folate and vitamin E (Rogers et al., 2004). In humans, it has been demonstrated that plasma ROS generation, within 30 minutes after apple juice consumption, could be effectively suppressed in plasma and this radical scavenging effect was maintained for around 2 hours post-consumption. (Ko et al., 2005). Results in a women's health study found that women ingesting apples had a 13–22% decrease in cardiovascular disease risk (Sesso et al., 2003).

The present study found that supplementation of AP was associated with greater mRNA of SOD1, SOD2 and CAT with lesser MTH mRNA only in the aged but not in the young fruit flies, implying the anti-aging activity of AP was mediated at least in part by up-regulation of endogenous antioxidant enzymes while down-regulation of longevity MTH gene (Figure 3). However, up-regulation of mRNA of SOD1, SOD2 and CAT was not accompanied by greater protein masses or increased activity of these antioxidant enzymes (Figure 4; Figure 5). Perhaps, this discrepancy is due to the insensitivity of western blot assay compared with the RT-PCR technique. The

involvement of SOD and CAT associated with AP's anti-aging activity can be further reaffirmed in both paraquat and H_2O_2 challenge tests, which demonstrated that AP could only prolong the survival time in OR wild type flies but it did not affect that of SOD^{n108} or Cat^{n1} mutants, in which gene of either SOD or CAT was knocked out (Figure 6), supporting that AP prolonged the mean lifespan of fruit flies at least mediated in part by interaction of AP with genes of SOD and CAT. Results from intensive paraquat / H_2O_2 challenge assay is in agreement with the previous report of Jimenez-Del-Rio et al. (2010), who found AP in diet could significantly reduce the mortality rate of OR wild type flies. In this regard, SOD activity in the hippocampus of the aged rats fed apple-rich diet could be maintained at the level of the young animals (Viggiano et al., 2006).

Supplementation of AP in diet could partially reverse the chronic paraquat exposure-induced mortality and decline in climbing ability (Figure 7), demonstrating superoxide anion could accelerate while dietary antioxidants could delay the aging process. It is suggested that chronic paraquat exposure may be one of factors contributing to neurodegenerative disorder such as Parkinsonian syndrome (Dinis-Oliveira et al., 2006). Data from survival and locomotor activity in this assay implied that AP could help ameliorate the neurodegenerative disorder.

Administration of AP significantly down-regulated gene expression of MTH in the wild type fruit flies (Figure 3). However, the effect was not evident in chronic paraquat challenge test. It was reported that MTH mutant flies could lead a 35% longer lifespan and acquire higher resistance to oxidative stress, compared with the wild type peers (Lin et al., 1998). With chronic paraquat exposure, gene expression of Rpn11 was down-regulated since day 10. Rpn11 is a suppressor of progressive neurodegeneration and knocking out of Rpn11 could lead to formation of

ubiquitinated proteins and shorten lifespan. This result together with data on MTH suggests an additional mechanism by which AP prolonged the mean lifespan of fruit flies exists in addition to its regulating effect on genes of SOD and CAT.

In conclusion, the present study demonstrated that AP in diet could prolong the mean lifespan, attenuate paraquat-induced mortality rate and partially reverse the decline of locomotor deficiency in fruit flies. The anti-aging activity and anti-neurodegenerative disorder were at least partially mediated by its interaction with genes MTH, Rpn11, SOD and CAT. It was unlikely that the lifespan-prolonging activity of AP in the fruit flies was associated with any changes in food intake, as the gustatory assay found no difference in average body weight and stomach redness index between the control and AP fruit flies.

Chapter 5

Blueberry extracts (BBE) contribute to lifespan extension in *Drosophila melanogaster*

5.1 Introduction

Aging is a complex biological process involving both genetic and environmental factors. During the past decades, various theories have been developed to illustrate the underlying mechanisms of senescence. It is believed that accumulation of oxidative damages caused by free radicals, or rather, reactive oxygen species (ROS) in vivo serve as a major contributor to organisms' functional decline, and eventually aging. In order to scavenge excess amount of reactive radicals, antioxidant systems are indispensable, which mainly include endogenous antioxidant system and exogenous antioxidant source. Firstly, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) serve as primary endogenous antioxidants to defense ROS in vivo. Secondly, exogenous source of antioxidants, such as, vitamin C and E, polyphenols, can help to eliminate free radicals by terminating the propagation of ROS reaction (Willis et al., 2009; Matés et al., 1999).

In addition, mitochondria and longevity-determined genes also play vital roles in aging process. Thus, certain molecules in those categories can serve as biomarkers for aging and age-related diseases. Mitochondrial respiratory capacity declines with aging, revealing an inverse correlation with the amount of ROS in vivo. As the

terminal oxidoreductase of mitochondrial electron transport chain (ETC), Cytochrome c oxidase (CcO) shows an age-related decline in both invertebrates and vertebrates (Schwarze et al., 1998). CcO deficiency would lead to the reduction of total ETC activity, resulting in increased amount of either superoxide anion radicals or hydrogen peroxide in mitochondria (Sohal et al., 2008). Furthermore, expression of Rpn11 has been demonstrated to be inversely correlated with progressive neurodegeneration. Knocking down of Rpn11 would result in accumulated ubiquitinated proteins, reduced 26S proteasome activity, and shortened lifespan (Tonoki et al., 2009). Moreover, one of the longevity-determined genes namely *methuselah* (MTH) is of great interest in *Drosophila* research community ever since the publication in Science by Lin et al. (1998), who reported that MTH mutant flies could live 35% longer than their wild type peers as well as exhibit stronger resistance to exogenous oxidative stress. However, the specific function of MTH and its potential connection with antioxidant defense system remain mysterious.

Fruit flies have been demonstrated as one of the most efficient in vivo model to study aging and age-related diseases during the past decades (Jafari, 2010). Human beings actually share huge amount of conserved biological pathways and diseases-causing genes with those tiny insects (Reiter et al., 2001). Furthermore, fruit flies are relatively easy to maintain in a large quantity due to their tiny body size and short lifespan. The full genome of *Drosophila* has already been sequenced and there are mature and practical genetic tools available in *Drosophila* research community (Bauer et al., 2004). Previous studies have revealed that dietary modification, including calorie restriction and compounds supplementation, can extend lifespan and ameliorate certain age-related diseases (McCay et al., 1935; Lin et al., 2002; Partridge et al., 2005; Lee et al., 2006; Piper and Bartke, 2008). Blueberry is

excellent source of antioxidants. Previous publications have shown that consumption of blueberries has various health benefits. However, no publication to date has demonstrated its anti-aging activity in *Drosophila melanogaster*.

5.2 Objectives

The present study was to investigate (a) anti-aging activity of blueberry extracts (BBE); (b) interaction between supplementation of diets with BBE and gene expressions of endogenous antioxidant enzymes; CcO subunits III and VIb, Rpn11, and MTH in *Drosophila melanogaster*.

5.3 Materials and methods

5.3.1 Fly strains

Fly strains used in this study were Oregon-R-C (OR), SODn108/TM3 (SODn108), and OE-/SM5 x Catn1/TM3 (Catn1) (Bloomington Drosophila Stock Center, Department of Biology, Indiana University, Bloomington, IN, USA). OR is a wild type fly strain which was employed in all experiments unless specified otherwise. SOD^{n108} is a mutant with one pair of single SOD gene on 3L chromosome being knocked out while Cat^{n1} is a mutant with CAT gene on chromosome 3L being knocked out by a point mutation.

5.3.2 Diet

A control diet was prepared according to the standard formulation suggested by Li et al. (2007) as described previously in Chapter 2 (2.3.2).

5.3.3 Effect of BBE on longevity of OR flies fed the basal diet

Newly eclosed male flies were divided into 3 groups (n=200 flies each), and housed in 10 vials (20 flies per vial). The first group was maintained on the basal diet, while the two BBE groups were fed one of the two diets containing 2 or 5 mg BBE/ml, respectively. Dead flies were counted every 2-3 days and the remaining alive flies were transferred to a new vial containing the same diet. The feeding lasted 76 days (Figure 2A). The same experiments described above were similarly repeated and the fruit flies were killed at selected time points (day 0, 15, 25, 35, 45, 55) in order to quantify the expression of SOD, CAT and MTH, Rpn11.

5.3.4 Gustatory assay

To exclude the possibility that lifespan extension in survival assay might be induced by dietary restriction, gustatory assay was carried out (Bahadorani et al., 2008). Details of this assay were described in Chapter 3 (3.3.5).

5.3.5 Intensive paraquat challenge experiment

Dietary paraquat (1,1'–dimethyl-4,4'–bi-pyridinium dichloride, Sigma, St. Louis, MO, USA) is able to generate superoxide anion radicals (Michaelis et al., 1933). To examine the resistance of flies against superoxide-induced stress, both OR flies (n=400 in 20 vials) and SOD^{n108} mutant flies (n=400 in 20 vials) were maintained on their corresponding control diet and experimental diet containing 5 mg BBE /ml, and incubated at 25°C. At day 25, the fruit flies in two groups were first starved for 2 hours, and then transferred into new vials containing a filter paper saturated with 1 ml of 20mM paraquat in a 6% glucose solution. Every 4-6 hours, dead flies were counted until all flies died.

5.3.6 Hydrogen peroxide (H_2O_2) challenge test

H_2O_2 is able to generate a hydroxyl radical (OH). It was therefore used to examine the resistance of flies against OH-induced oxidative stress in the present study. OR flies (n=400) and Cat^{n1} mutant flies (n=400) were maintained on their corresponding control diet or experimental diet containing 5 mg BBE /ml and incubated at 25°C. Similarly, the fruit flies in the two groups were first starved for 2 hours, and then were transferred into new vials containing a filter paper saturated with 1 ml of 30% H_2O_2 in a 6% glucose solution at day 25. Every 4-6 hours, dead flies were counted until all flies died.

5.3.7 Chronic paraquat challenge

Long term exposure to paraquat has been regarded as a potential risk factor to induce neurodegenerative diseases such as Parkinson's disease. To examine the resistance of flies against paraquat-induced mortality and locomotor deficiency, 900 newly eclosed male OR flies were randomly divided into three groups, namely blank control group (BCTL), control group (CTL), and the experiment diet containing 5 mg BBE/ml. Every 3 days, flies, after 2-hour starvation, were transferred into vials containing a filter paper had saturated with 1ml of 20 mM paraquat in a 6% glucose solution. After 24 hours, flies were moved to new vials containing only water-saturated filter paper for 2 hours before they were transferred back into vials containing respective diets. The experiment lasted 45 days until all the fruit flies in BBE group died. Another set of the experiment described above was similarly repeated and the fruit flies were killed at day 0, 10, 20, and 30 to quantify the expression of SOD, CAT, MTH, Cco subunits, ubiquitinated proteins and Rpn11.

5.3.8 Climbing assay

Locomotor function of fruit flies was assessed using a climbing assay as described in Chapter 4 with slight modification. In brief, 10 male flies were placed in a plastic vial, given 10 seconds to climb up. At the end of each trial, the number of flies that climbed up to a vertical distance of 8 cm or above was recorded. Each trial was performed three times. Flies were tested at selected time points during the chronic paraquat challenge survival assay.

5.3.9 SOD Activity

SOD activity assay was conducted as previously described in Chapter 2 (2.3.8) by using a SOD activity assay kit (Cayman Chemical, Michigan, USA).

5.3.10 CAT activity

CAT activity was measured as described previously in Chapter 2 (2.3.9) using a catalase assay kit (Sigma, St. Louis, MO, USA).

5.3.11 Real-time PCR

For real-time PCR assay details and primers information, please refer to Chapter 4 (4.3.12).

5.3.12 Western blot analysis

Protein abundance detection and related anti-bodies information were mentioned in Chapter 4 (4.3.13).

5.3.13 Statistics

Data were expressed as mean±standard deviation. The Kaplan-Meier test was employed to compare the difference between the survival curves using SPSS 15.0 (Statistical Package for the Social Sciences software, SPSS Inc, Chicago, USA). The significance of difference between means was assessed using T-test and one way ANOVA. Differences were considered significant when $p<0.05$.

5.4 Results

5.4.1 Effect of BBE on longevity of OR flies fed the basal diet and BBE diet

Fruit flies in BBE group had the longest lifespan among the three groups of OR wild type male flies (Figure 2A). The maximum lifespan increased more than 5% in BBE group with the 50% survival time being increased from 47 days to 53 days compared with that of their control flies. To be specific, the mean lifespan for the control and two BBE supplement groups were 48, 50, and 53 days, respectively (Figure 2A; Table 1). However, a significant difference in the mean lifespan was only found between 5 mg BBE/ml and the control ($P<0.01$). Meanwhile, gustatory assay did not reveal any significant difference between control and BBE-fed group (Figure 2B).

SOD1 gene was significantly up-regulated in BBE group compared with that in the control at day 35 and 45 while gene expression of SOD2 in BBE group were greater than that in the control at day 25 and 35. Similarly, expression of CAT were increased in BBE group at day 35 and 55 while Rpn11 level were up-regulated in BBE group at day 45, compared with that in the control. In contrast, MTH gene was

significantly down-regulated in BBE group than that in the control at day 15 (Figure 3).

Western blot data showed that, at day 15, BBE group had protein mass of SOD1 greater than that in the control. Otherwise, no significant differences in protein mass were observed between BBE and the control (Figure 4). The present study also investigated effect of BBE on the activity of SOD1, SOD2 and CAT in OR wild type male flies at day 0, 15, 25, 35, 45, and 55. Compared with the control group, BBE could significantly increase SOD1 activity at day 35 while it showed a greater SOD2 activity at day 25, 35; For CAT activity, BBE could significantly elevate it at day 25 than the control (Figure 5).

5.4.2 Effect of BBE on paraquat and H_2O_2-challenged OR, *Cat*^{n1} and *SOD*^{n108} flies

Results from intensive paraquat challenge test showed that BBE OR group had a longer survival time than the corresponding control ($P<0.01$). To be specific, OR wild type flies had a maximum survival time increased from 74 hr in the control to 88 hr in BBE group with mean survival time being prolonged by 16% ($P<0.01$). However, no difference was in *SOD*^{n108} mutant flies fed the control and BBE diet. Similar results were obtained in the H_2O_2 challenge assay. Supplementation of BBE prolonged the maximum survival time, 50% survival time and mean survival time only in OR wild type but not in *Cat*^{n1} mutant fly strain (Figure 6).

5.4.3 Effect of BBE on chronic paraquat challenge in OR flies

Long term exposure to 20mM paraquat could induce high mortality rate in fruit flies, shortening their maximum lifespan to 34 days and reducing their climbing

ability by more than 60% (Figure 7). Supplementation of BBE in diet could partially reverse the paraquat-induced mortality and decline in climbing ability. Results demonstrated that the maximum lifespan was 34 days in the paraquat-control group, while it was 42 days in the paraquat-BBE group (Figure 7A). At the same time, the climbing ability was less than 40% in the paraquat-control groups, while it was partially recovered to more than 50% in the paraquat-BBE flies at day 30 (Figure 7B).

Western blot data did not found any significant difference in protein abundance of SOD1, SOD2, CAT, ubiquitinated proteins, Cco subunit III and VIb among the blank control, paraquat-control and paraquat-BBE groups (Figure 8). Real-Time PCR analysis revealed that BBE could up-regulate the gene expression of CAT, SOD1, and SOD2 at day 10 or day 20 or both. Expression of Rpn11 at day 30 was significantly elevated in BBE group to the level of blank control (Figure 9). As to the enzyme activity, SOD2 showed a decreasing trend in the paraquat-control flies and BBE group could partially retain its activity with significant elevation observed at day 20 and 30; similar trends were recorded in CAT test. For SOD1 activity, no difference was found among the three groups at selected time points (Figure 10).

Table 1 Lifespan of OR wild type flies fed the control diet and the two experimental diet containing 2 and 5 mg BBE /ml.

	Maximum Lifespan of last fly (Day)	50% Survival (Day)	Mean Lifespan (Mean ± SE, Day)
Control	73	47	48 ± 1^{a}
2 mg BBE	73	47	50 ± 1^{a}
5 mg BBE	76	53	53 ± 1^{b}

[a,b] Means with different letters differ significantly at $p<0.01$.

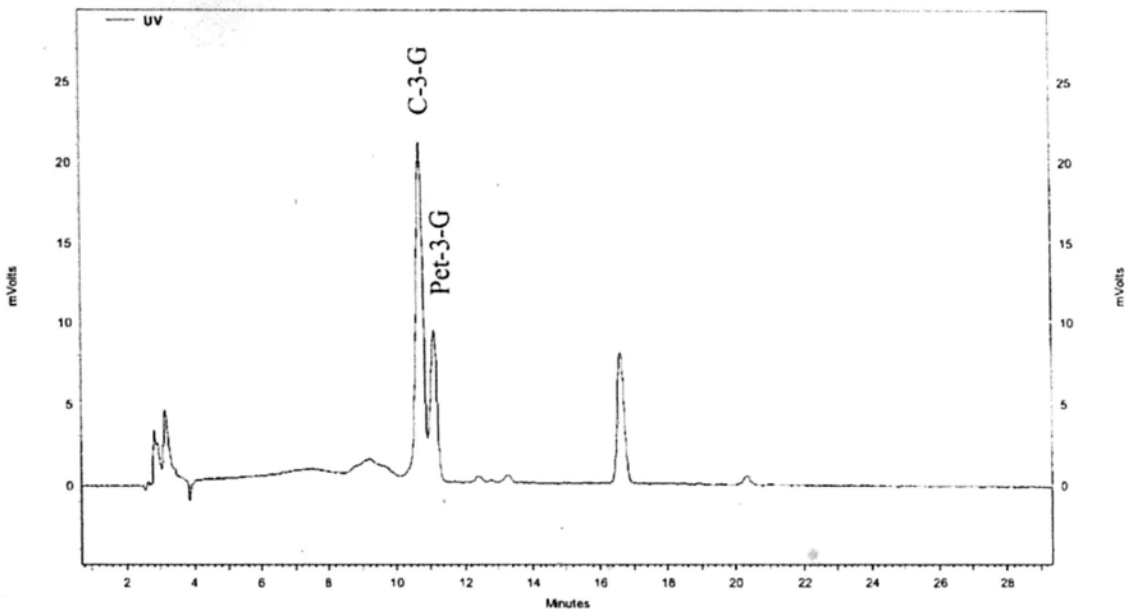


Figure 1 HPLC chromatogram of blueberry extracts (BBE)

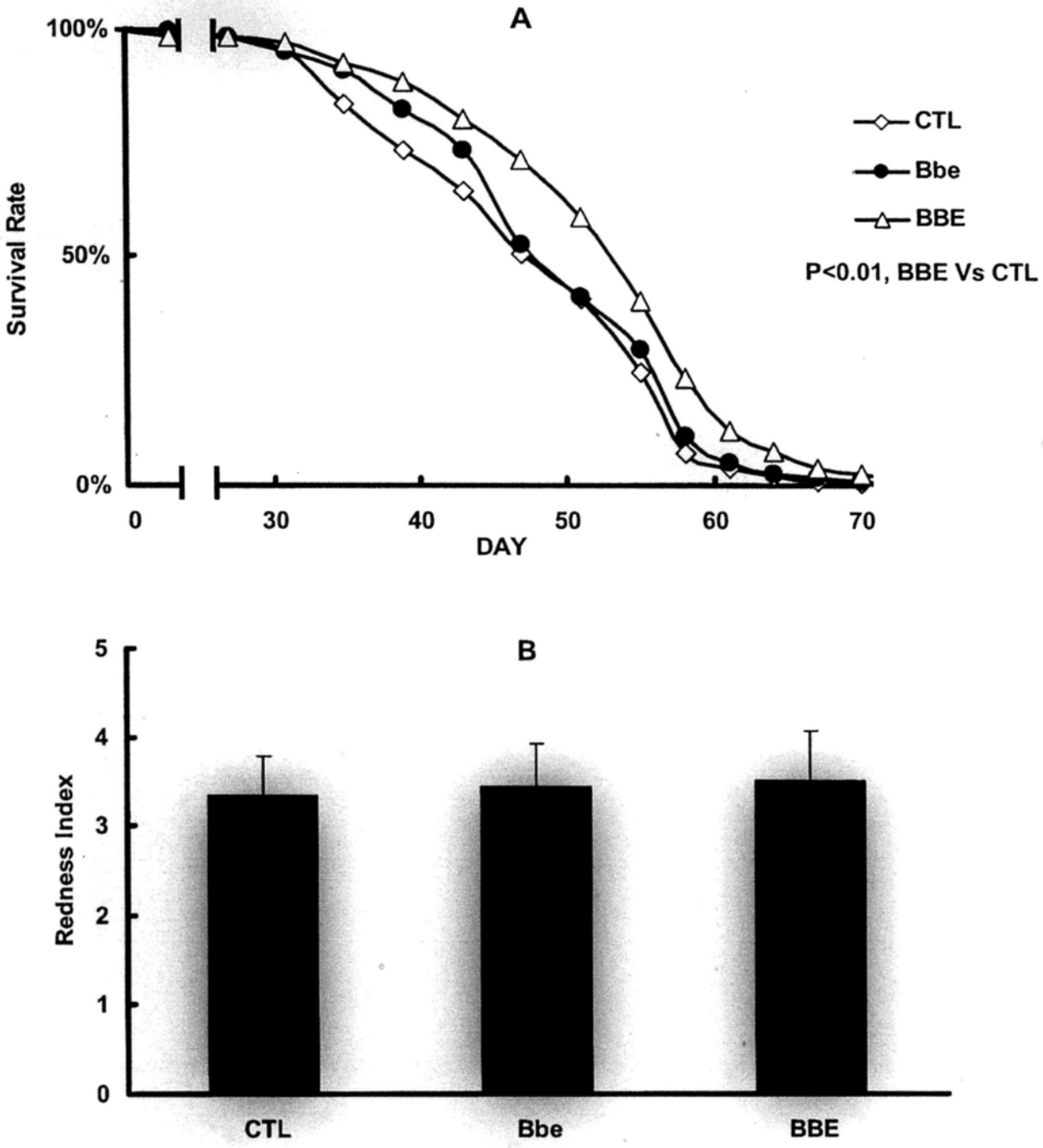


Figure 2. (A) Lifespan curve of wild type flies (OR) fed diets containing 0 mg/ml (control, CTL), 2 mg (Bbe) and 10 mg BBE (BBE) per milliliter diet. Data were expressed as the maximum lifespan of last fly, 50% survival time and mean lifespan (n=200 flies) for each group (Table 1). The Kaplan-Meier test found BBE could significantly extend the mean lifespan of fruit flies ($P<0.01$). **(B)** Gustatory Assay compared the food intake on the basis of the differences in the degree of abdomen redness among CTL, Bbe and BBE groups. Data are expressed as mean ± SD.

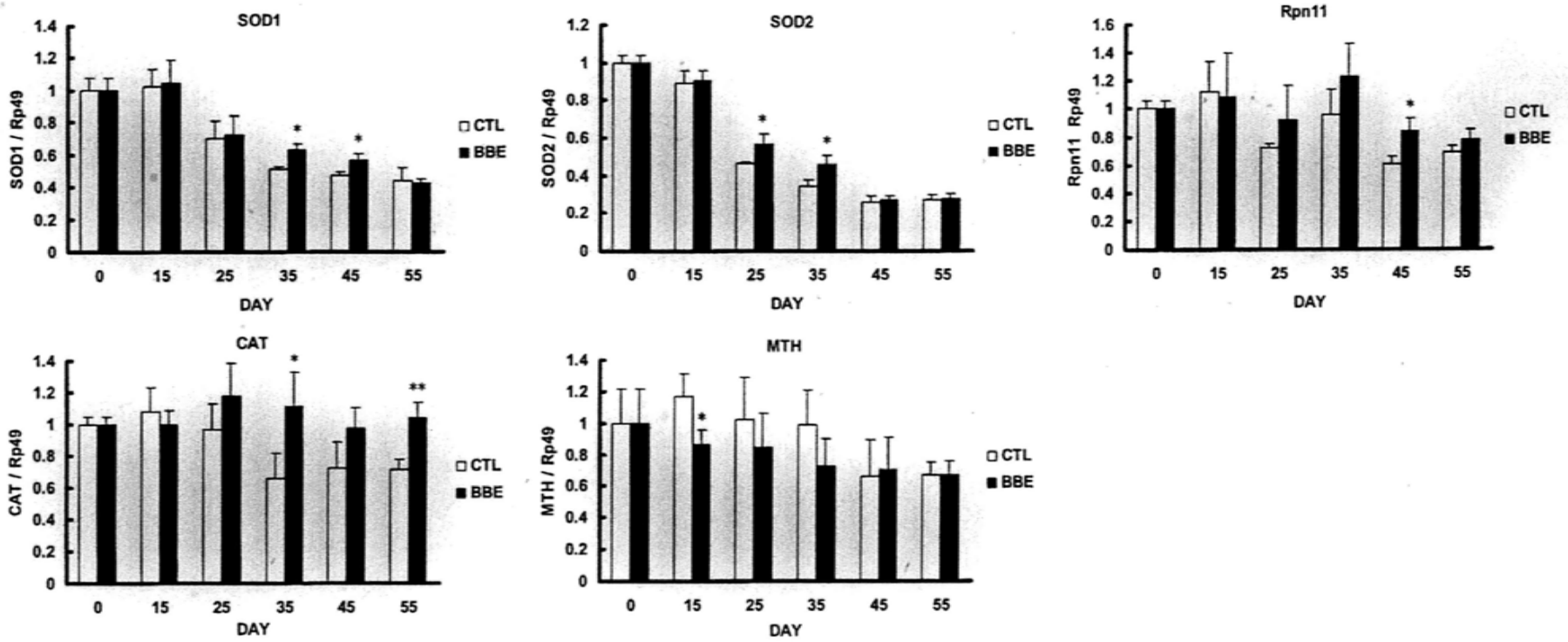


Figure 3 Effect of blueberry extracts (BBE) supplementation (5 mg/ml diet) on mRNA of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2), Rpn11, catalase (CAT) and Methuselah (MTH) compared with those in the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 15, 25, 35, 45 and 55 days. Data are expressed as mean ± S.D. * $P<0.05$; ** $P<0.01$ compared with the control value.

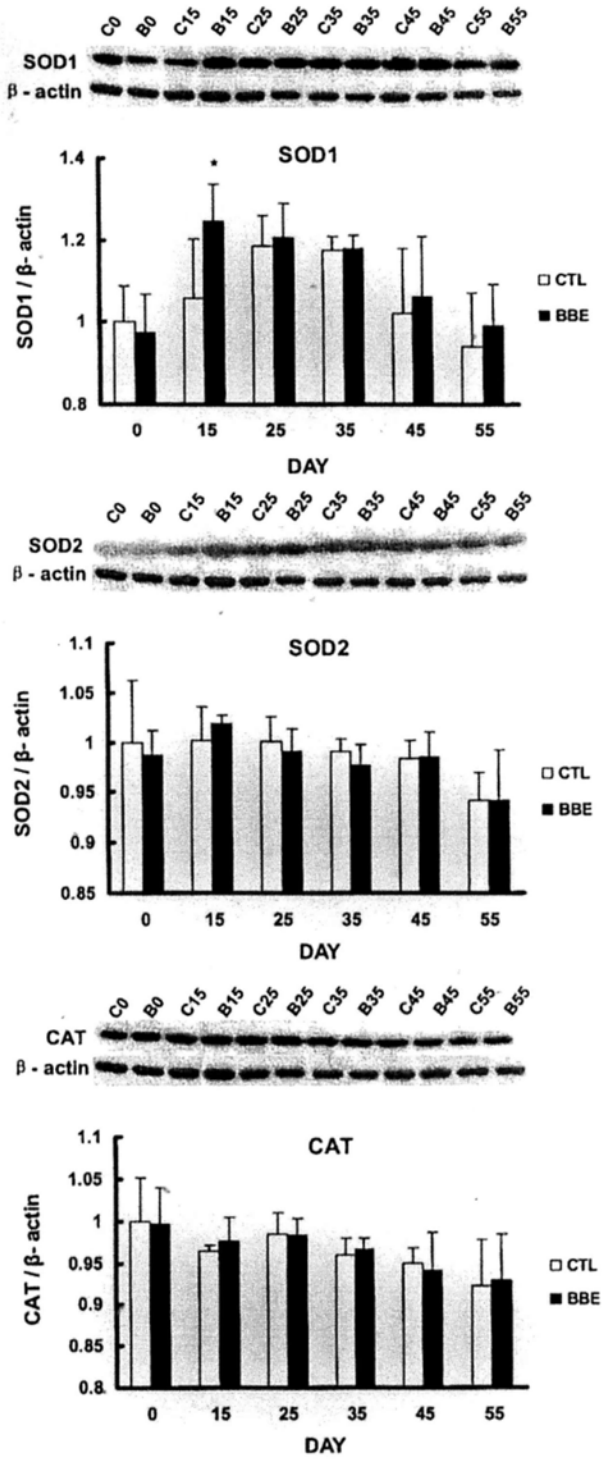


Figure 4 Effect of BBE supplementation (5 mg/ml diet) on the relative protein mass of SOD1, SOD2 and CAT compared with those in the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 15, 25, 35, 45 and 55 days. C0-C55 represented the protein bands for the control at day 0-55 days while B0-55 represented the protein bands for BBE at day 0-55. Data are expressed as mean ±SD. * $P<0.05$ compared with the control value.

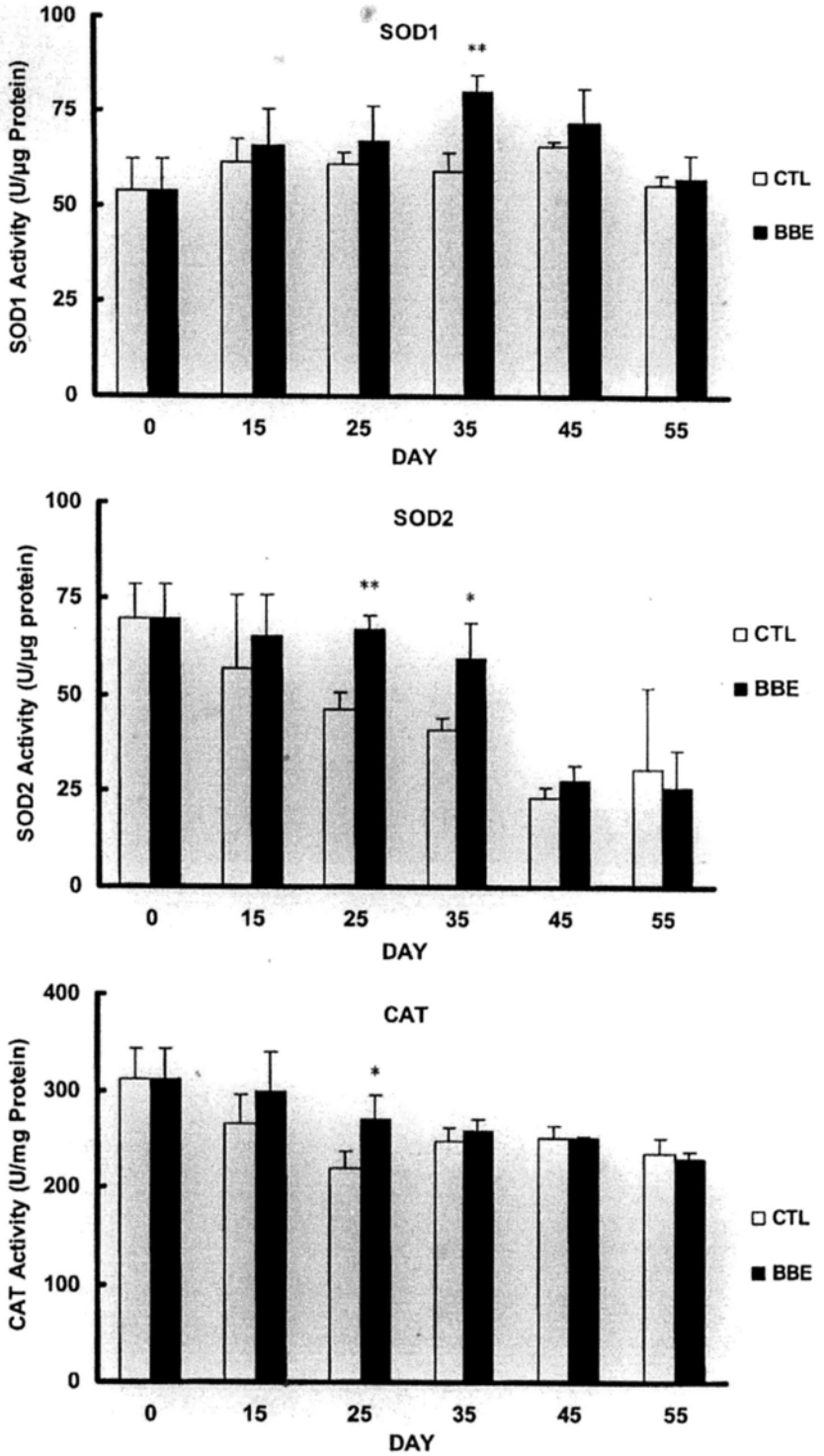


Figure 5 Effect of BBE supplementation (5 mg/ml diet) on enzymatic activity of SOD1, SOD2 and CAT compared with those in the control diet (CTL). The wild type (OR) flies (n=300 /group, n=20/vial) were incubated at 25°C for 0, 25 and 45 days. Data are expressed as mean ± SD. * $P<0.05$; ** $P<0.01$ compared with the control value.

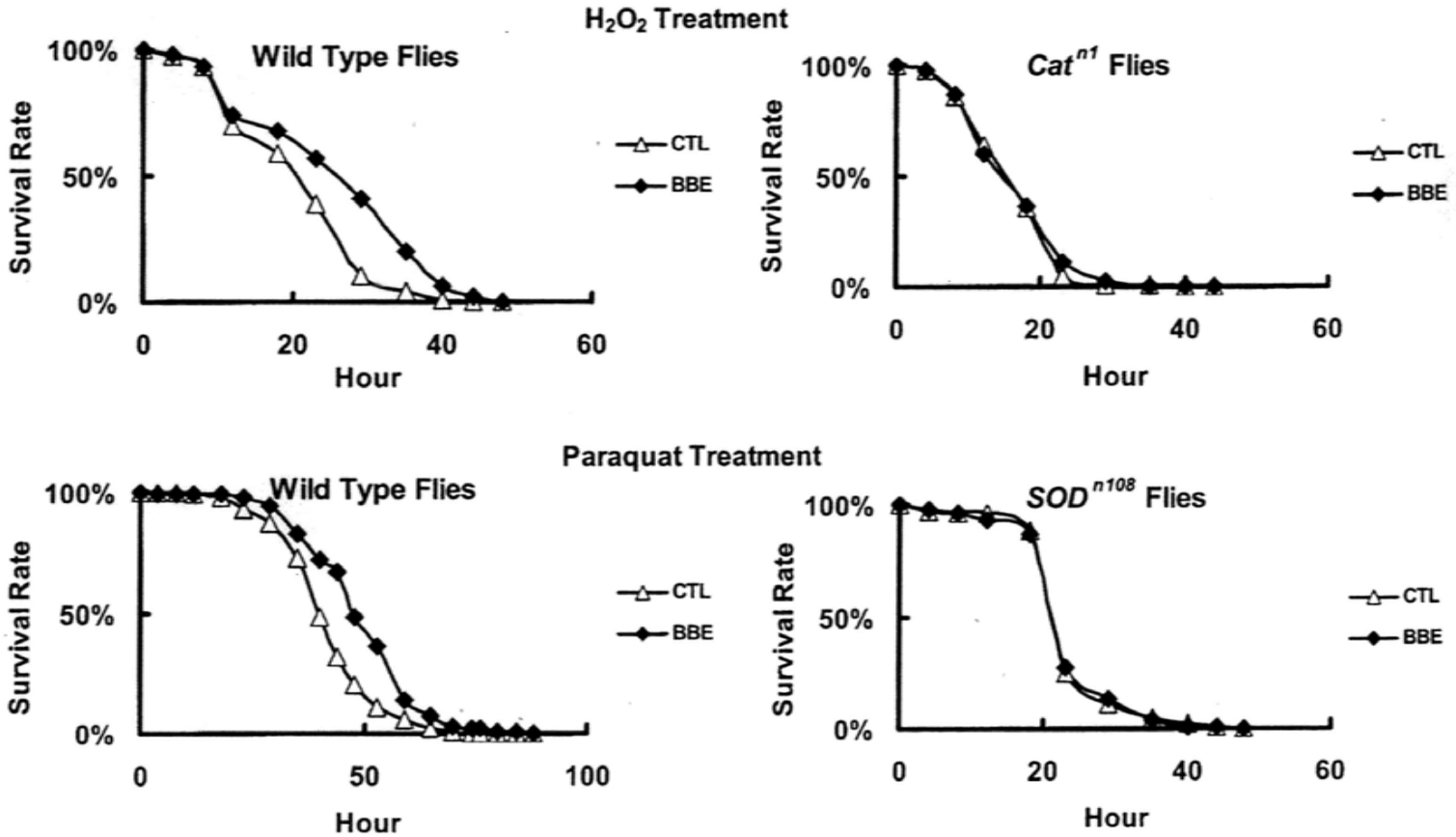


Figure 6 Effect of paraquat treatment or hydrogen peroxide treatment on the survival time of the mutant flies (SOD^{n108}) or mutant flies (Cat^{n1}) fed the diets containing 0 mg/ml (CTL) or 5 mg BBE /ml compared with that of the wild type (OR) flies. The Kaplan-Meier test found both that BBE-fed OR group survived better than its corresponding OR control ($P<0.05$) while the survival rate of BBE-fed SOD^{n108} and Cat^{n1} groups was not different from their corresponding control groups.

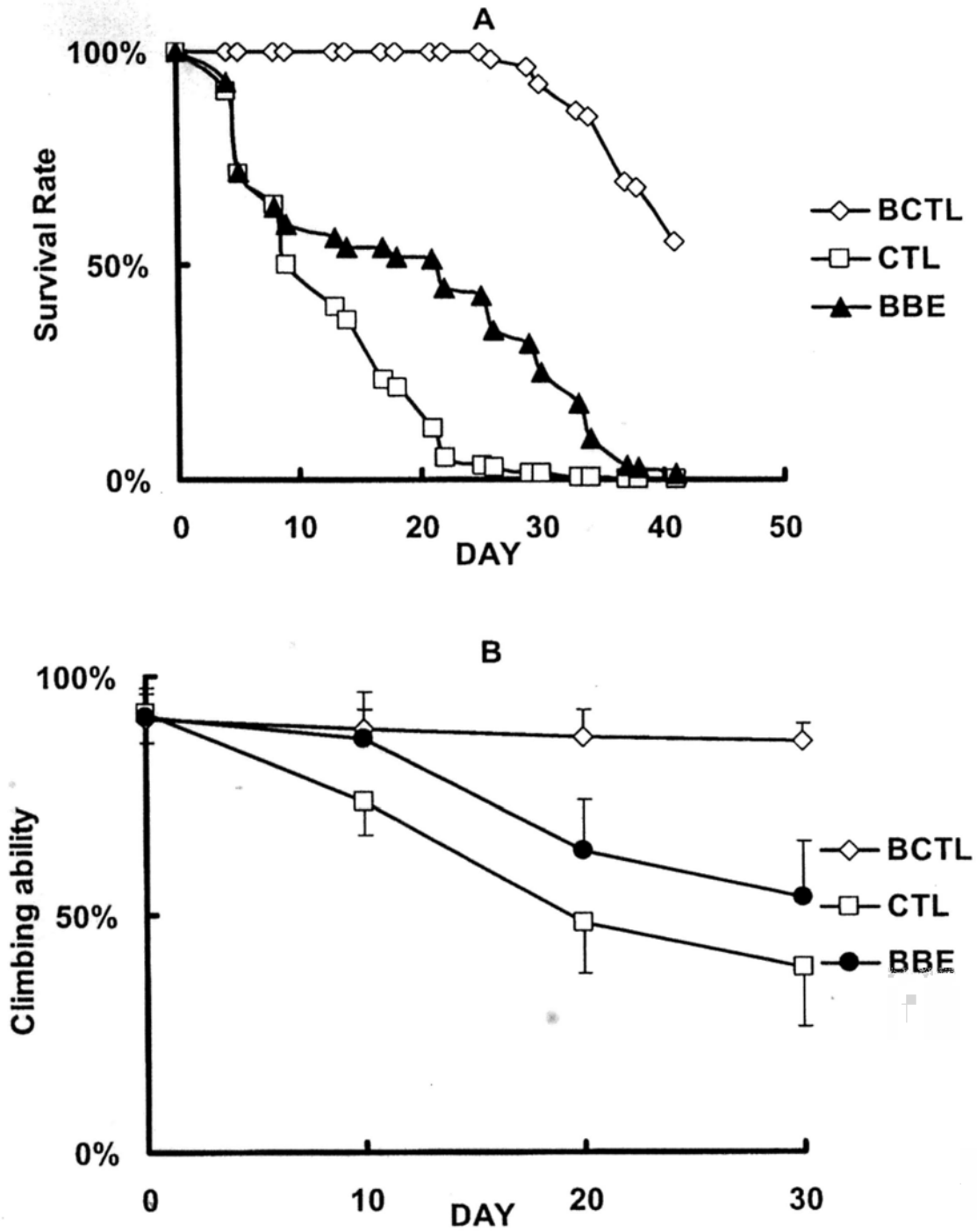
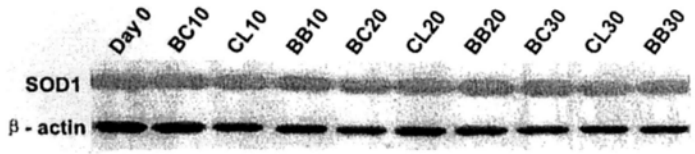
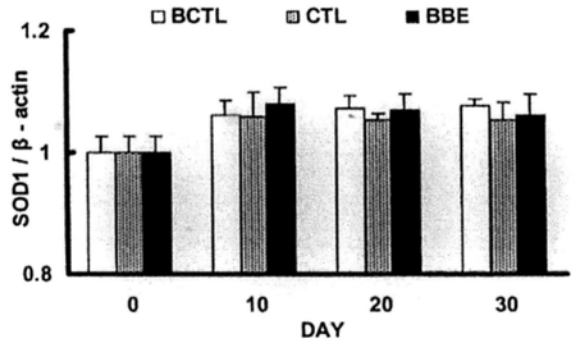
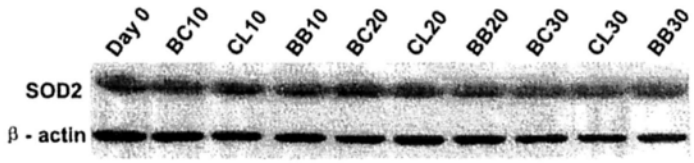
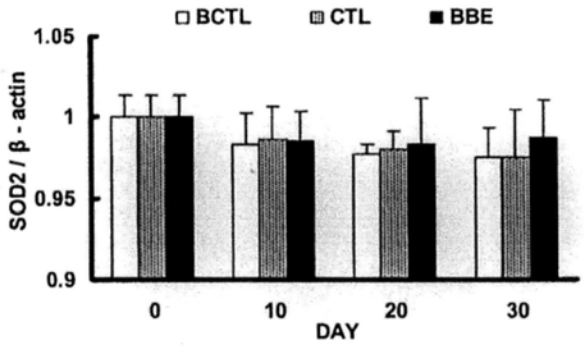


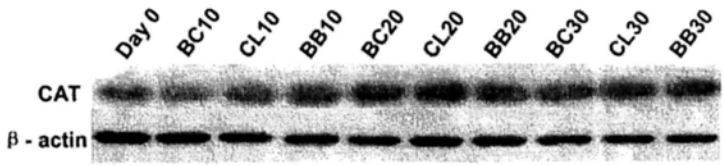
Figure 7 (A) Lifespan of OR fruit flies fed either a basal diet without exposure to paraquat (BCTL) or a basal diet with the paraquat chronic exposure (CTL) with addition of 5 mg / ml BBE (BBE) at 25°C. The Kaplan-Meier test found BBE group survived better than the CTL ($P<0.01$). **(B)** Effect of BBE on the climbing ability in BCTL, CTL, and BBE groups. BBE group could significantly alleviate locomotor deficiency in CTL group.

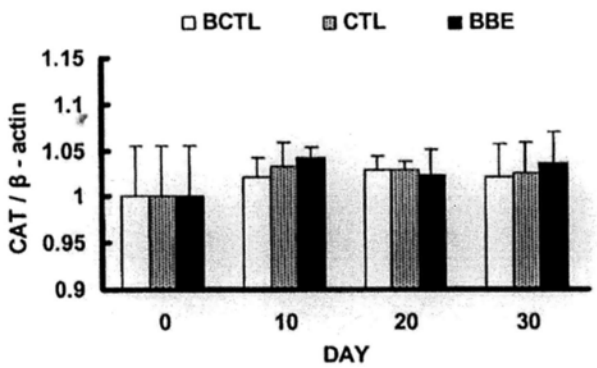

Day 0
BC10
CL10
BB10
BC20
CL20
BB20
BC30
CL30
BB30
SOD1
β - actin


BCTL
CTL
BBE
1.2
1
0.8
SOD1 / β - actin
0
10
20
30
DAY


Day 0
BC10
CL10
BB10
BC20
CL20
BB20
BC30
CL30
BB30
SOD2
β - actin


BCTL
CTL
BBE
1.05
1
0.95
0.9
SOD2 / β - actin
0
10
20
30
DAY


Day 0
BC10
CL10
BB10
BC20
CL20
BB20
BC30
CL30
BB30
CAT
β - actin


BCTL
CTL
BBE
1.15
1.1
1.05
1
0.95
0.9
CAT / β - actin
0
10
20
30
DAY

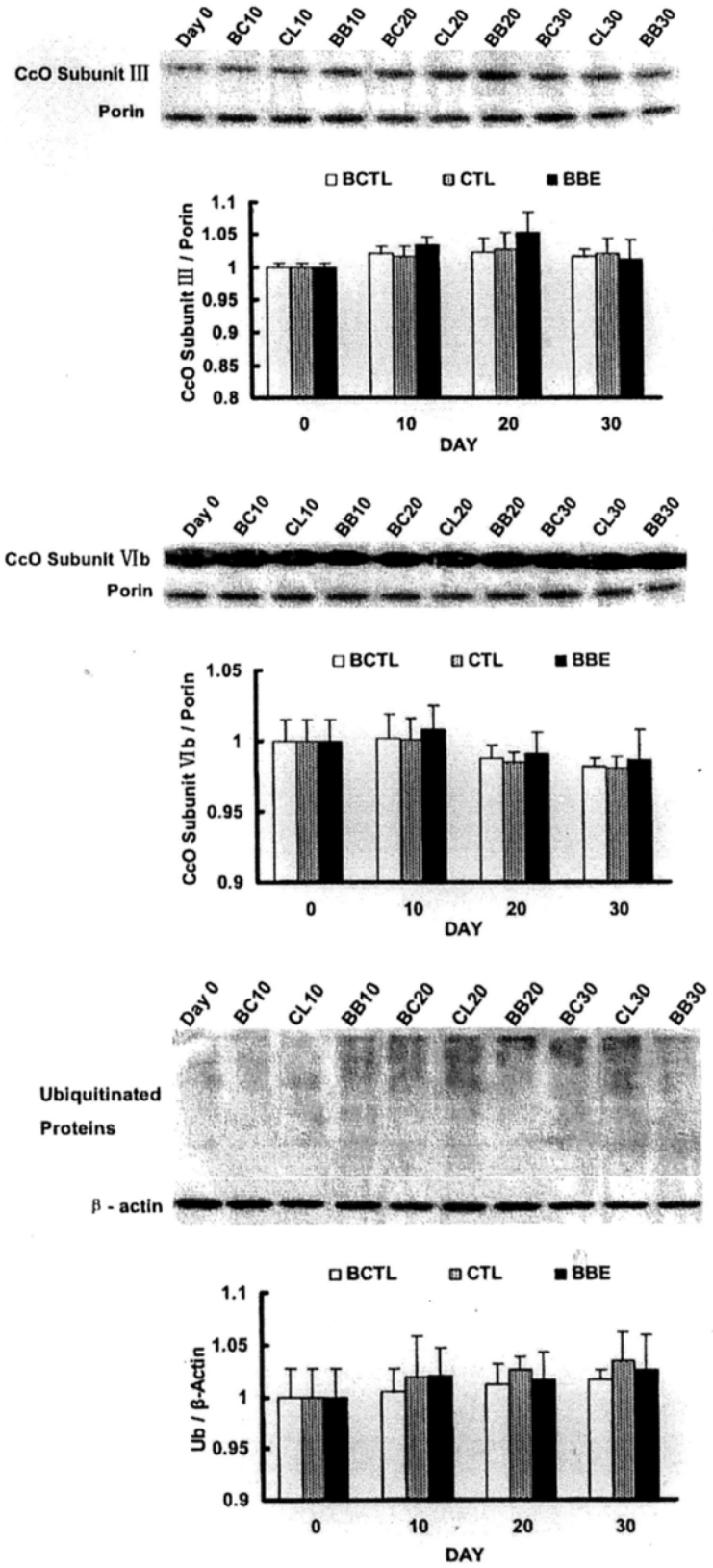


Figure 8 The relative immunoreactive mass of SOD1, SOD2, CAT, CcO Subunit III, CcO Subunit VIb, and Ubiquitinated Proteins in OR fruit flies fed either a basal diet without going through the paraquat challenge cycle (BCTL; BC10-BC30) or a basal diet with the paraquat chronic exposure (CTL; CL0-CL30) with addition of 5mg / mL BBE (BBE; BB10-BB30).

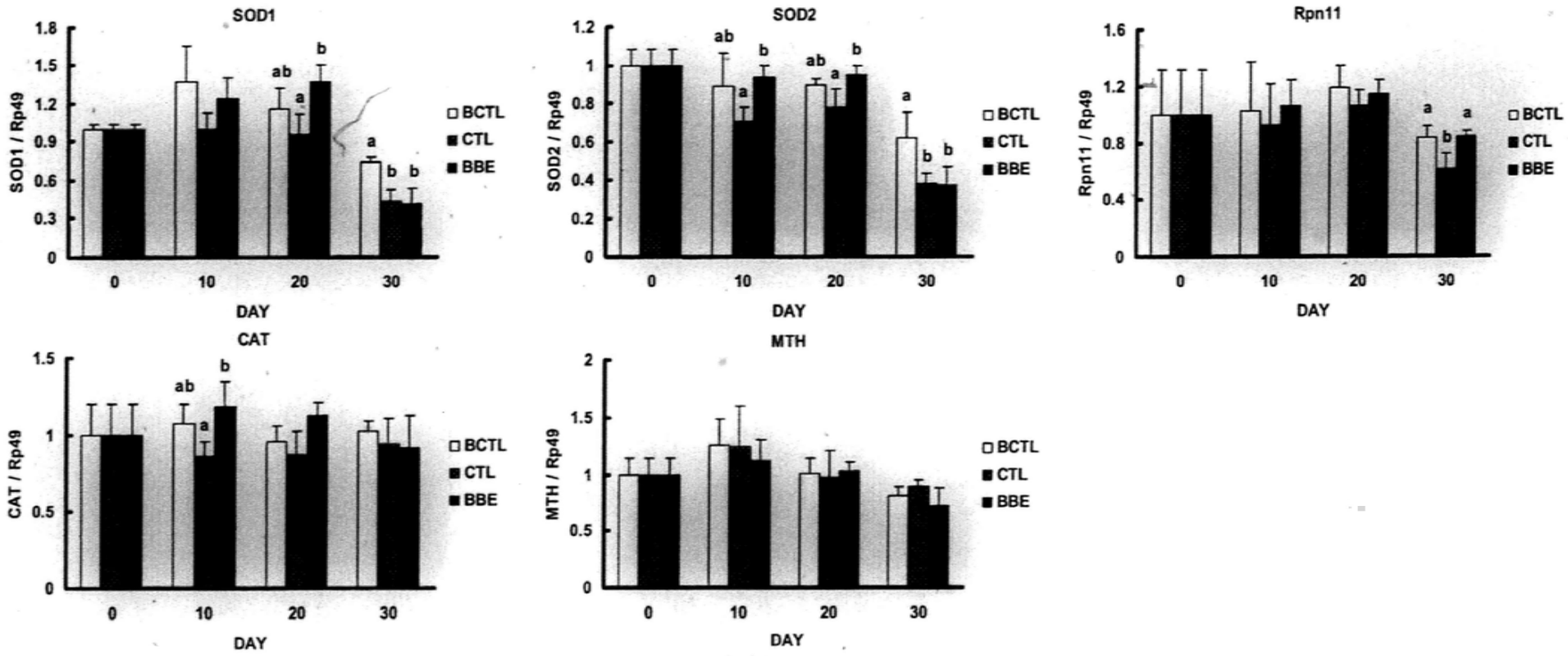


Figure 9 mRNA of copper-zinc containing superoxide dismutase (SOD1), manganese containing superoxide dismutase (SOD2), Rpn11, catalase (CAT) and *methuselah* (MTH) in OR fruit flies fed either a basal diet without going through paraquat exposure (BCTL) or a basal diet containing with paraquat challenge cycle (CTL) with addition of 5 mg / mL BBE (BBE) at 25°C. [a,b] Means at the same time point differ significantly at $P<0.05$

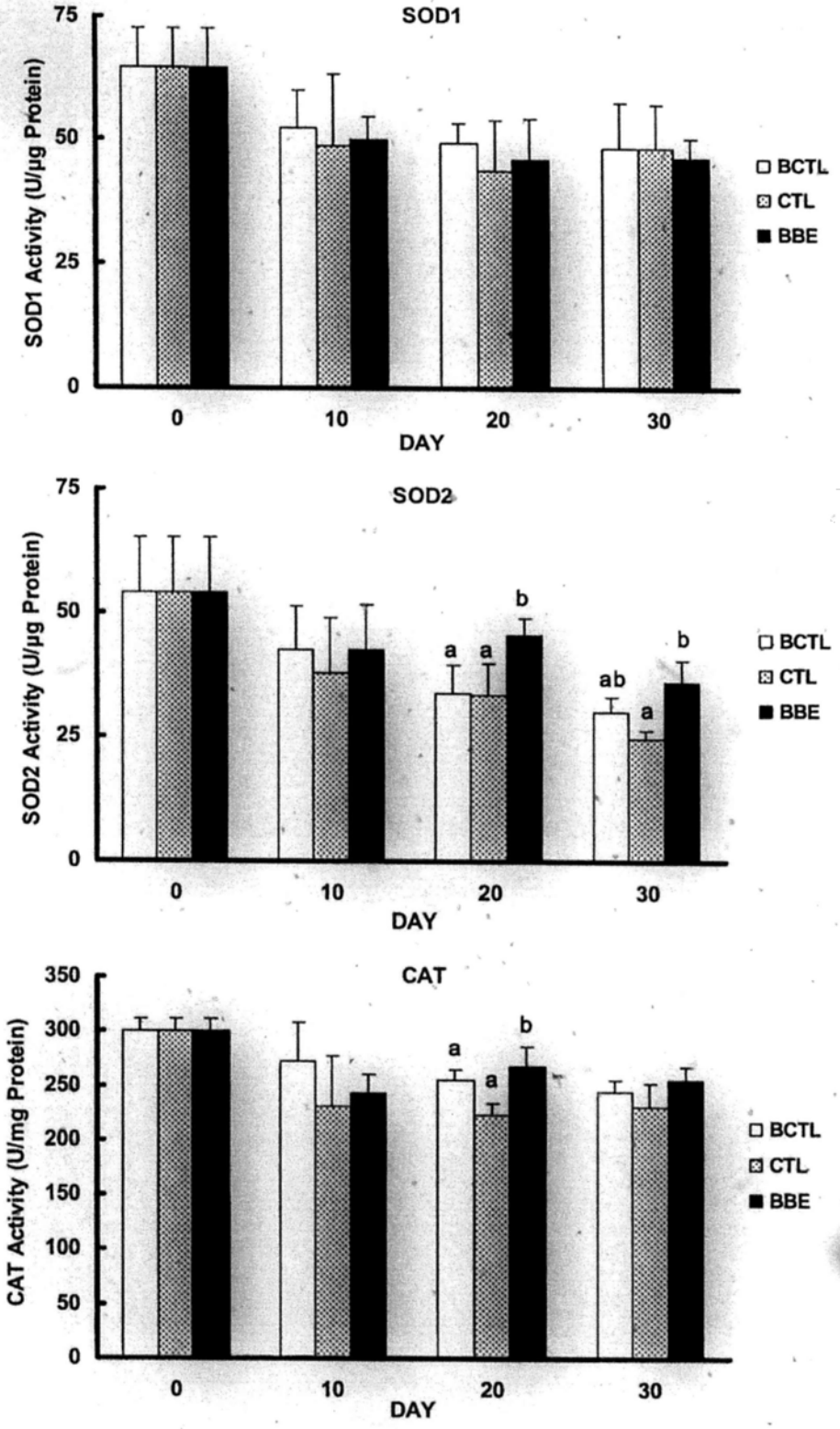


Figure 10 Enzymatic activity of SOD1, SOD2 and CAT in OR fruit flies fed either a basal diet without going through paraquat exposure (BCTL) or a basal diet containing with paraquat challenge cycle (CTL) with addition of 5 mg / mL BBE (BBE) at 25°C. [a,b]Means at the same time point differ significantly at $P<0.05$

5.5 DISCUSSION

This study was the first report demonstrating BBE's anti-aging activity in *Drosophila melanogaster*. Results showed that 5 mg BBE/ml in diet could prolong the mean lifespan of fruit flies by more than 10%, compared with the control. Meanwhile, BBE's effect on maximum lifespan of fruit flies on a population level was minor (Figure 2A, Table 1). This is in agreement with the study conducted by Wilson et al. (2006), who demonstrated that blueberry extract, mainly the fraction enriched in proanthocyanidin compounds, in diet could increase life span and slow aging related declines in *C. elegans* (Wilson et al., 2006). Clinical data addressing anti-aging activity of BBE are still lacking. However, recent studies provided inspiring results. A human trial study conducted by Krikorian et al. (2010) evaluated the health benefits of blueberry supplementation, revealing that daily consumption of wild blueberry juice for 12 weeks would improve memory function in older adults with early memory decline (Krikorian et al., 2010). In addition, the results in this study were consistent with our previous work on green tea extracts (Li et al., 2007), black tea extracts (Peng et al., 2009), soybean isoflavones (Chapter 4), and apple polyphenols (Peng et al., 2011), indicating that natural nutraceuticals with the anti-aging activity might be mainly conducive to mean lifespan, rather than maximum lifespan, of a living organism.

The present study found that supplementation of BBE was associated with elevated mRNA level of SOD1, SOD2 and CAT starting from the middle age of *Drosophila*'s lifespan (day 25 or 35); on the contrary, MTH was significantly reduced in BBE group compared with that in the control at day 15 (Figure 3), suggesting that anti-aging activity of BBE was mediated at least in part by

up-regulation of endogenous antioxidant enzymes as well as down-regulation of longevity-determined gene MTH. However, protein abundance detected by western blot did not reveal as much difference between the control and BBE group as real-time PCR data did (Figure 4). Two main reasons were proposed for this discrepancy. Firstly, western blot assay is generally less sensitive than real-time PCR technique. Secondly, it might be posttranslational modifications that lead to this discrepancy.

The involvement of SOD and CAT associated with BBE's anti-aging activity were further confirmed in intensive paraquat / H_2O_2 challenge assays, which showed that BBE could only prolong the survival time in OR wild type flies but did not affect that of SOD^{n108} or Cat^{n1} mutants, in which gene of either SOD or CAT was partially knocked out (Figure 6), supporting that BBE extended the mean lifespan of fruit flies at least mediated in part by interaction with genes of SOD and CAT.

Furthermore, BBE in diet could partially reverse the chronic paraquat exposure-induced high mortality rate and decline in climbing ability (Figure 7), demonstrating that superoxide anion radicals generated by paraquat could accelerate while dietary BBE could delay the aging process. It has been suggested that chronic paraquat exposure may be one of factors contributing to neurodegenerative disorder such as Parkinsonian syndrome (Dinis-Oliveira et al., 2006). Data from survival and locomotor activity in this assay implied that BBE could help ameliorate the neurodegenerative disorder. With chronic paraquat exposure, gene expression of SOD1, SOD2, CAT, and Rpn11 were down-regulated at selected time points. BBE supplementation could partially and significantly restore the decreasing trend. Rpn11 is a suppressor of progressive neurodegeneration and knocking out of it could lead to formation of excess amount of ubiquitinated proteins and shorten life span. This

result together with the previous data suggests an additional mechanism by which BBE prolonged the mean lifespan of fruit flies in addition to its effects on endogenous antioxidants, SOD and CAT.

In conclusion, the present study demonstrated that BBE in diet could prolong the mean lifespan, alleviate paraquat-induced mortality rate and partially reverse the decline of locomotor deficiency in fruit flies. The anti-aging activity and anti-neurodegenerative disorder were believed to be at least partially mediated by its interaction with genes SOD, CAT, Rpn11, and MTH. In addition, this study, along with my work described in previous Chapters, suggested that nutraceuticals rich in antioxidants have the potential to be anti-aging and anti-neurodegenerative candidate compounds by extending the mean lifespan of fruit flies.

Chapter 6

General Discussion and Conclusion

Excess production of free radicals has been regarded to be one of the most vital risk factors inducing aging and age-linked diseases. By enhancing the endogenous antioxidant system, oxidative stress to living cells would be less severe. This could theoretically lead to a longer lifespan. Nutraceuticals rich in antioxidants have been demonstrated to be efficient in preventing and treating certain chronic diseases, such as, hypercholesterolemia, diabetes, and various cancers. However, to date, few reports exist demonstrating their anti-aging activity in vivo. Their influence on endogenous antioxidants and various predictive biomarkers for aging and age-related diseases, such as, MTH and Rpn11, remains poorly understood.

This study is the first report demonstrating the anti-aging activity of BTE, SIF, AP and BBE in *Drosophila melanogaster*. Their influence on a specific batch of flies might vary. The regulation of related molecular biomarkers was diverse at selected time points as well. However, despite all these divergences, the nutraceuticals in this study actually share a lot of common features in terms of lifespan extension. For example, either one of the nutraceuticals in my study could significantly prolong the mean lifespan of fruit flies, compared with the corresponding control ones, which had two indications. First, it implied that extending fruit flies' lifespan by nutraceutical-supplemented diet might be universal phenomena rather than random coincidences, indicating that compounds rich in antioxidants can serve as potential anti-aging candidates in vivo. Second, the lifespan prolonging effect might have an upper limit by this means. Specifically speaking, in my study, the mean lifespan of

fruit flies could hardly be prolonged by more than 10%. Meanwhile, effects on flies' maximum lifespan were minor.

Paraquat or H_2O_2 exposure would lead to higher superoxide or hydroxyl radical level in vivo, resulting in severe oxidative stress to living cells. All nutraceuticals tested in this study could significantly reduce the high mortality rate in wild type fruit flies when challenged intensively by paraquat or H_2O_2. However, the lifespan prolonging effects were not retained in the SOD^{n108} or Cat^{n1} mutant fly strain, indicating that those nutraceuticals might induce the higher resistance to intensive oxidative challenge through SOD and CAT rather than reacting with harmful molecules directly in vitro.

The gene expression of SOD1, SOD2, CAT, MTH, and Rpn11 was in general characterized by a decreasing trend in fruit flies with physiological aging, suggesting the ability of scavenging free radicals declined as fruit flies got older. Nutraceuticals employed in this study could elevate the expression level at selected time points. Specifically speaking, the administration of BTE or SIF could significantly up-regulate gene expression of SOD and CAT at selected time points. However, they had no major effect on the gene expression of MTH, indicating that life prolonging activity of BTE and SIF in fruit flies was associated with their effect on endogenous antioxidants SOD and CAT, yet unlikely related to the longevity determined gene MTH. As to AP and BBE, supplementation of either compound was associated with elevated mRNA level of SOD, CAT, and Rpn11 (Rpn11 up-regulation was a response exclusively to BBE treatment) starting from the middle age of Drosophila's lifespan (day 25 or 35); on the contrary, MTH was significantly reduced in AP and BBE groups compared with that in the control, suggesting that anti-aging activity of AP and BBE was mediated at least in part by up-regulation of endogenous antioxidant

enzymes as well as down-regulation of longevity-determined gene MTH. What's noteworthy is that almost all up-regulation started from the middle age of fruit flies, suggesting that middle age might be a crucial time point at which the endogenous antioxidant system is still able to be reinforced by exogenous sources. It would be interesting in the future to design experiments testing the anti-aging properties of nutraceuticals starting implemented from different phase of the whole lifespan.

However, the protein abundance detected by western blot did not always reveal as much difference between the control and nutraceutical supplemented groups as Real-Time PCR data did. Two main reasons were proposed here for this discrepancy. Firstly, the anti-bodies employed in protein mass study were not all specific to the very species of *Drosophila melanogaster*, so there were the chances that the bands detected in western blot assay might not be purely what it should represent. Secondly, there were way more experimental and artificial interfering factors in western blot assay than Real-Time PCR technique, such as film development, band intensity detection, which would disturb the sensitivity and reproducibility of the results.

Besides physiological aging, this study also focused on age-related diseases, especially the ones induced by environmental toxins. Chronic paraquat exposure, which has been recognized as one of the critical environmental risk factors inducing neurodegenerative diseases, drastically increased the mortality rate and impaired the locomotor activity of fruit flies. AP or BBE in diet could significantly reverse the high mortality rate and partially retain the climbing activity of those fruit flies. With chronic paraquat treatment, gene expressions of SOD, CAT, and Rpn11 were down-regulated at selected time points. AP and BBE supplementation could partially and significantly restore the decreasing trends. This result together with the previous data obtained from normal aging study suggested an additional mechanism by which

AP and BBE prolonged the mean lifespan of fruit flies in addition to their effects on endogenous antioxidants, SOD and CAT.

In conclusion, the present study demonstrated that BTE, SIF, AP, and BBE in diet could significantly prolong the mean lifespan in fruit flies. Therein, AP and BBE could alleviate the paraquat-induced mortality rate and partially reverse the locomotor deficiency. The anti-aging and anti-neurodegeneration activity were believed to be most likely mediated by their interaction with genes SOD, CAT, MTH, and Rpn11, suggesting that nutraceuticals rich in antioxidants in general have the potential to be the anti-aging and anti-neurodegeneration candidate compounds. Furthermore, it was unlikely that these effects were associated with any variations in food intake, as body weight tracking and gustatory assay found no difference in average body mass and stomach redness index between the basal diet and nutraceutical fed fruit flies.

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