

5-2016

Studies of Molecular Mechanisms of Royal Jelly Mediated Healthspan Promotion in *Caenorhabditis Elegans*

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STUDIES OF MOLECULAR MECHANISMS OF ROYAL JELLY
MEDIATED HEALTHSPAN PROMOTION IN *CAENORHABDITIS ELEGANS*

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Science

by
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May 2016

Accepted by:
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ABSTRACT

Numerous studies have demonstrated that many nutraceuticals have potential capacity to mitigate the symptoms of aging and age-related disorders. We found that Royal Jelly (RJ/eRJ) consumption could extend *C. elegans* lifespan and increase stress tolerance in an IIS/DAF-16 dependent manner. In consideration that the transactivity of DAF-16 is tightly controlled by its co-factors, our further results indicated that SIR-2.1, 14-3-3, and HCF-1 could interact with each other to fine-tune the activity of DAF-16. Additionally, these co-factors were also required in RJ/eRJ mediated stress tolerance.

Given that aging is characterized with progressively declined physiological functions, it is intriguing to investigate whether RJ/eRJ supplementation could slow down the development of neurodegenerative diseases. Strikingly, our results showed that RJ/eRJ supplementation delayed the β -amyloid ($A\beta$)-toxicity induced body paralysis in *C. elegans* AD model. The genetic analysis indicated that the RJ/eRJ mediated $A\beta$ toxicity alleviation required Insulin/IGF Signaling (IIS) pathway and DAF-16, rather than HSF-1 and SKN-1. Further research found that RJ/eRJ relied on DAF-16 to significantly improve the protein solubility in aged worms, which implied that RJ/eRJ supplementation promoted proteostasis and reduced proteotoxicity in AD worms. Additionally, RJ/eRJ also increased the solubility of $A\beta$ species. In considering that RJ/eRJ supplementation slowed down the $A\beta$ -induced paralysis in *C. elegans*, it is possible that RJ/eRJ mediated protection against $A\beta$ toxicity might be dependent on increasing the solubility of $A\beta$ species.

Overall, our findings revealed that the anti-aging function of RJ/eRJ supplementation and underscored the relationship between the improved proteostasis and the prevention of neurodegenerative disorders.

ACKNOWLEDGEMENTS

I am deeply grateful to my advisor, Dr. Yuqing Dong for all his patience, support, and help. His guidance and advice have been invaluable resource during my research. Dr. Dong taught me how to design experiment, how to solve problems, how to do presentation, and how to write scientific papers. Whether I will continue to do science or not in the future and wherever I will go, this is the biggest profit to me.

I would like to thank my committee members: Dr. Min Cao, Dr. William Baldwin, and Dr. Charlie Rice for all their help and support throughout my years at Clemson.

Thanks to Jing Li, Ye Yang, Bo Xia, Yeyun Huang, who have always been true and dependable friends and always make time to help with my experiments.

Thanks to my lab mates Ojas Natarajan and Hong Guo, who always gave me suggestions, support and help in my research, which made grad school an enjoyable and unique experience. I appreciate my colleagues in Dr. Min Cao's lab with whom I have shared and exchanged ideas and delightful conversation of science, including Joseph Angeloni, Miranda Klees, and Andrew Gitto.

I would also like to thank my former labmates, undergraduates and colleagues: Sujay Guha, Jing Li, Yao Yao, Lauren Cook, Lindsay Grasso, Jessica Dihn, Ethan Wilson and all other friends who took great care of me during my first year in Clemson.

Finally, I would like to thank my husband and parents. They gave me persistent supply of love, encouragement and support throughout my life. I would never have been able to accomplish this without them.

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LIST OF ABBREVIATIONS

FUDR.....	5-fluorouracil 1-2'-deoxyribose
WT.....	wild type
LB.....	Luria Broth
OD600.....	optical density at a wavelength of 600 nm
RNAi.....	RNA interference
RJ.....	Royal Jelly
eRJ.....	Royal Jelly (enzyme treated)
NGM.....	Nematode Growth Medium
RT-PCR.....	Realtime- polymerase chain reaction
AD.....	Alzheimer's Disease
A β	Amyloid beta
IIS Pathway.....	Insulin/Insulin-like growth factor signaling

CHAPTER ONE : INTRODUCTION

1.1 Aging is genetically controlled

Aging, a process of becoming older, is a universal inevitable process associated with declining physiological functions. Based on our knowledge, aging is characterized by declining stress resistance, increased homeostatic imbalance, and elevated risk of disease[1-3]. To date, the increase of aging population has been a big challenge in the United States. Statistical data estimates that more than 20% of the population will be over 65 years old by 2030, and the cost of medical treatment for age-related diseases will be tripled in 2050. This aging population growth could slow the nation's economic growth in the near future[4, 5]. Therefore, it is urgent for scientists to develop new therapeutic treatments to delay aging and the onset of age-related disorders.

1.1.1 Aging is regulated through genetic pathways

Most biological processes, if not all, are controlled by genes, and aging follows the same rule. It is genetically controlled by an elaborate genetic network[6-8]. Numerous publications showed that some conserved genetic pathways regulate longevity and prevent the age related disorders (Figure 1.1). Dietary interventions and genetic alterations could manipulate genes and pathways to play an important role in the aging process[9-11].

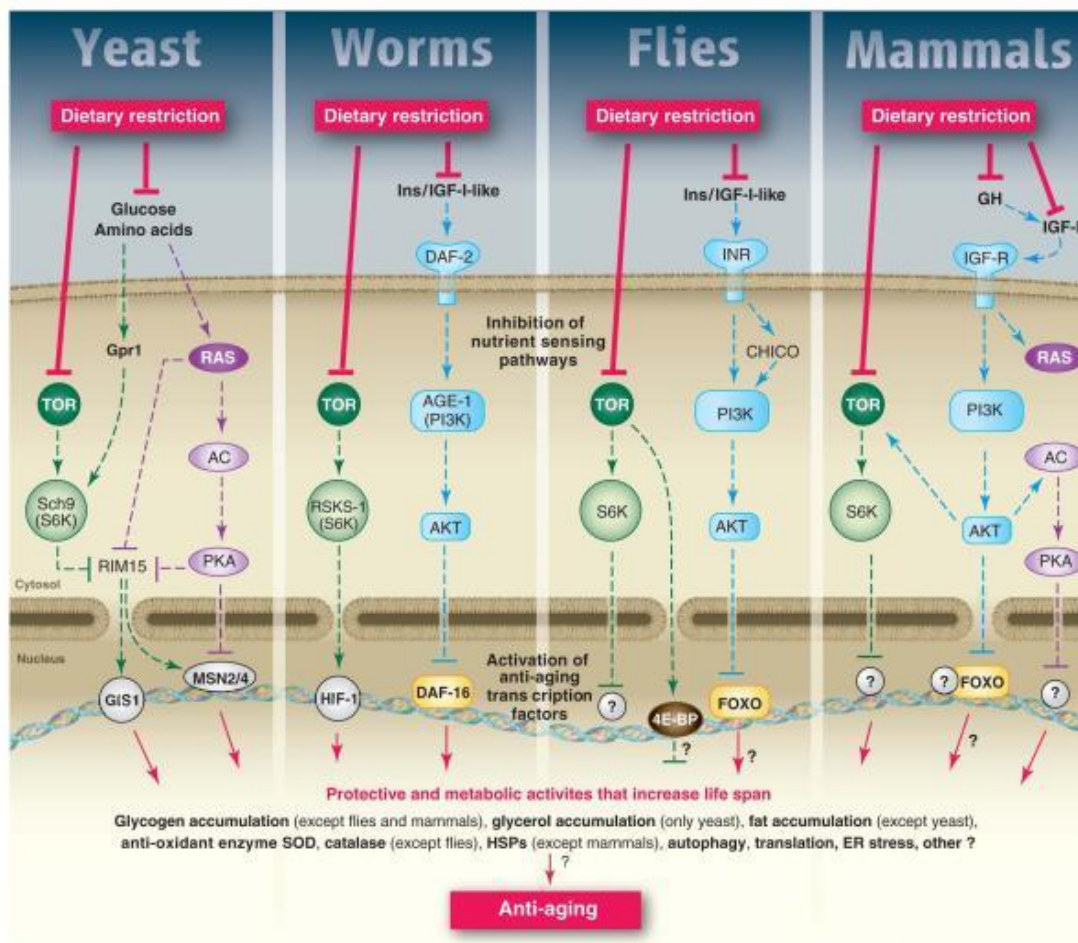


Figure 1.1 Conserved nutrient signaling pathways regulating longevity. (Fontana *et al.*, 2010)

1.1.1.1 Model Organisms are utilized to study the molecular mechanism of aging

Model organisms that mimic human responses provide us with a good platform to investigate the detailed molecular mechanism of diseases[12, 13]. Moreover, these classic model organisms offer numerous opportunities to evaluate the efficiency of drugs and therapeutic treatments[14, 15]. The well-established models in the research of aging including the yeast *Saccharomyces cerevisiae*, nematode *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster*, hydra, zebrafish, and many rodents, which were crucial in identifying the conserved pathways that regulate human aging[16-18]. In our research, roundworm *Caenorhabditis elegans* (*C. elegans*) was utilized as an animal model to investigate the molecular mechanism of aging and age-related disorders.

C. elegans is a free-living transparent multicellular animal simple enough to study the details of genetic pathways. *C. elegans* is considered a powerful model for studying aging due to its numerous advantages in culturing and genetic analysis[19, 20]. For instance, *C. elegans* is easy to culture to a bulk population, and it takes three days for the development of one generation and three weeks for the total lifespan. Moreover, these transparent animals can be used in high throughput automated experiments, which makes them an ideal tool to study the genetic regulation of aging[21]. Comparison of *C. elegans* and human genomes confirmed that nearly 80% of human disease genes and pathways are present in *C. elegans*, which indicates that *C. elegans* is a good model to study human disease[22-24]. However, the simple structure of *C. elegans* places some limitations for modeling human aging. They are multicellular organisms lacking tissues and organs, and therefore unable to regenerate their tissues, which make them limited in serving as a

model to study the aging of highly proliferative tissues[25-27]. Overall, although *C. elegans* is a primitive and simple organism, it shares many fundamental molecular structures and biological characteristics with more advanced organisms[12, 28].

1.1.1.2 Insulin/IGF Signaling (IIS) pathway and DAF-16

Insulin/IGF Signaling (IIS) pathway is the most powerful pathways for regulating longevity and is highly conserved from *C. elegans* to human beings[29, 30]. In response to insulin-like peptides (ILPs), the transmembrane receptor DAF-2 (Insulin/IGF-1) triggers the activation of many protein kinases, including AGE-1/PI3K and AKT/PKB, which leads to phosphorylation and subsequent cytoplasmic retention of the main downstream effector DAF-16/FOXO (Figure 1.2). However, in some special conditions, such as low nutrients or starving, this pathway is inactivated and thereby DAF-16 is unphosphorylated. Without phosphorylation, DAF-16 translocates from cytosol to nucleus and, in turn, elevates expression level of its target genes[31, 32]. These DAF-16 downstream genes have many essential biological functions, such as promoting stress tolerance, altering metabolic and development responses, improving immunity, and extending lifespan[33-35].

In addition to DAF-16, heat shock factor 1 (HSF-1) and SKN-1 are two other critical transcriptional factors of the IIS pathway[32]. Specifically, HSF-1 regulates a series of heat shock proteins (HSPs) under stress conditions[36, 37]. HSF-1 is phosphorylated and inactive under normal condition; upon thermal stress, HSF-1 is released and translocated to the nucleus. As a result, HSF-1 induces the expression of HSPs to protect the proteome from destruction. It has been proposed that HSF-1 plays a

key role in lifespan extension and prevention of proteopathy, diseases caused by aberrant protein structure[38, 39]. The third transcriptional factor is SKN-1, which is also located at the downstream of IIS pathway and parallel to DAF-16 and HSF-1. Noticeably, SKN-1 plays a very important role in anti-oxidative stress and metal detoxification[40, 41].

Although DAF-16, HSF-1, and SKN-1 have different biological functions and are parallel to each other, they mutually interact and some of their target genes are overlapping [38, 42, 43].

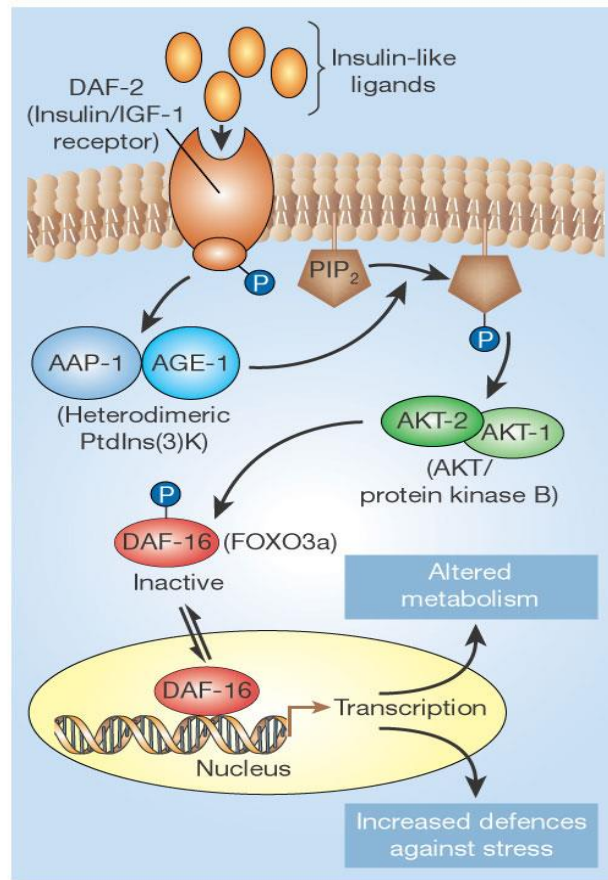


Figure 1.2 The Insulin/IGF Signaling Pathway and DAF-16/FOXO. (Nemoto *et al.*, 2004)

1.1.1.3 Important co-factors of DAF-16

Numerous studies have indicated that the precise control of DAF-16 transcriptional activity is a key regulatory step for longevity determination[44]. DAF-16 activity is regulated by post-translational modifications, nuclear/cytoplasmic translocation, and association with transcriptional co-regulators. Although necessary for its activation, translocation of DAF-16 into the nucleus is not sufficient to stimulate its transcriptional activity[45, 46]. Association with additional co-factors is also necessary for nuclear DAF-16 activation [44, 47]. It has been proposed that SIR-2.1, HCF-1 and 14-3-3 are important co-factors (binding partners) of DAF-16[3, 48, 49].

SIR-2.1, the *C. elegans* homolog of the yeast NAD⁺-dependent protein deacetylase, plays an important role in stress response and calorie restriction mediated lifespan extension[50, 51]. As an important co-factor of DAF-16, SIR-2.1 is thought to activate DAF-16 in conferring longevity as well as stress resistance. It is reported that in mammals, SIRT1(homolog of *C. elegans* SIR-2.1) can bind to and deacetylate forkhead proteins, which in turn influences the transactivity of FOXOs and thereby increases the resistance of mammalian cells to oxidative-damage induced apoptosis[52-54]. Numerous studies also demonstrated that the overexpression of SIR2 homologs in worms, yeast and flies extends lifespan, emphasizing the evolutionarily conserved role of SIR2 in longevity determination[54, 55]. Noticeably, recent studies revealed that heat stress stimulates the physical association of SIR-2.1 with DAF-16 via the scaffolding protein 14-3-3, which promotes the transactivation of DAF-16[48]. This finding indicated that SIR-2.1 could interact with other proteins to activate DAF-16.

14-3-3 proteins, also co-factors for DAF-16, function as docking proteins to bridge DAF-16 with other important co-factors. 14-3-3 proteins are small acid proteins, which could bind to diverse groups of signaling molecules to participate in a wide variety of cellular processes, including cell cycle checkpoints, DNA repair, cell differentiation and cell apoptosis[56, 57]. By binding to proteins, 14-3-3 proteins can induce the conformational change of the substrate proteins, sequester the substrates in the cytoplasm, or act as a scaffold that bridges two interacting partners as with DAF-16 and its co-factors[58, 59].

In *C. elegans*, there are two 14-3-3 proteins: PAR-5/FTT-1 and FTT-2. PAR-5 is required for cellular asymmetry in the early *C. elegans* embryo[60, 61]. FTT-2 sequesters DAF-16 in the cytosol by forming a protein complex, which in turn inactivates DAF-16[62]. Between these two 14-3-3 proteins, only FTT-2 could affect dauer formation, DAF-16 localization, and transcriptional activates; PAR-5 does not have this function[3, 62].

Counter to these DAF-16 activation co-factors, the DAF-16 co-repressors down-regulate DAF-16's transactivity. Host Cell Factor-1 (HCF-1) belongs to a family of highly conserved HCF proteins and acts as a nuclear co-repressor of DAF-16. The main role of HCF-1 is to help fine tune the regulation of a subset of DAF-16 downstream genes to modulate survival under specific conditions. By regulating the function of DAF-16, HCF-1 is essential for longevity maintenance in *C. elegans*[49].

In mammal studies, HCF-1 was reported to play a key role in cell cycle progression at the G1/S transition, M phase, and cytokinesis. In its diverse biological roles, mammalian HCF-1 acts by binding to and regulating many different transcriptional and chromatin factors and assembling appropriate protein complexes for context-dependent gene regulation. In *C.elegans*, HCF-1 mutant exhibits declined reproductive ability and low penetrance of embryonic lethality, both phenotypes consistent with a role of HCF-1 in cell proliferation[63, 64]. Similar to the results found in mammalian research, HCF-1 in *C. elegans* also functions in proper cell cycle maintenance and cell proliferation[65]. Interestingly, Li et al reported that HCF-1 is also a critical longevity determinant and transcriptional regulator of DAF-16, which is necessary for maintaining normal lifespan and stress response in *C. elegans*[49, 66]. In the absence of *hcf-1*, more DAF-16 was released and localized to the nucleus and significantly up-regulated the expression of DAF-16 target genes. This up-regulation of DAF-16 indicates that HCF-1 modulates *C.elegans* lifespan and stress response by acting as a negative regulator of DAF-16. Furthermore, Riziki et al. reported that HCF-1 could also interact with SKN-1 in stress response. Although HCF-1 is not regulated by IIS pathway, it still functions as an important factor to modulate downstream components of IIS pathway[67].

To date, a growing body of evidence indicates that DAF-16's co-factors physically interact with each other to regulate DAF-16's activity[48, 68, 69]. For instance, in mammalian cells, HCF-1 affects the expression of several SIRT1/FOXO transcriptional targets and physically associates with both FOXO3 and SIRT1[65]. This interaction also exists in *C. elegans* genetic regulation. In *C. elegans*, SIR-2.1 forms a

protein complex with DAF-16 and requires DAF-16 activity to modulate lifespan. The protein complex of DAF-16 and SIR-2.1 forms in the nucleus and requires 14-3-3 proteins as bridging molecules. Moreover, 14-3-3 proteins also physically associate with HCF-1. Based on genetic analyses, it is known that SIR-2.1 functions upstream of HCF-1, and both HCF-1 and SIR-2.1 functions upstream of DAF-16. Taken together, given certain signals, the activity of DAF-16 is regulated via the interplays between SIR-2.1, HCF-1 and 14-3-3 proteins.

1.1.1.4 Other longevity-related pathways

In addition to IIS pathway, other pathways, such as JNK, P38 MAPK, and TOR pathways, are also reported as critical modulators in aging and age-related diseases. Numerous evidence showed that some crossover molecules among these pathways contribute to form a complex genetic network to regulate longevity as well as other important biological processes (Figure 1.1) [70-73].

C-Jun N-terminal kinase (JNK) pathway has been implicated in critical biological processes such as cell development, survival, apoptosis, and cancer. The JNK family, a subgroup of the mitogen-activated protein kinase superfamily, is part of a signal transduction cascade that is activated by cytokines and external stresses, such as oxidative stress and heat shock stress[73, 74]. To date, it has been proposed that JNK pathway functions at the center of a signal transduction network that coordinates the induction of protective genes in response to oxidative stress, which can reduce the toxic effects of reactive oxygen species (ROS)[75, 76]. Moreover, under the heat shock stress, JNK-1 (an important component of JNK pathway) directly interacts and phosphorylates

DAF-16 to promote DAF-16 translocation into nucleus, which in turn extends the lifespan of *C. elegans*[77, 78].

P38 mitogen-activated protein kinase (P38 MAPK) pathway is composed of series mitogen-activated protein kinases, which are activated by variety of cellular stresses such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. It is also involved in cell differentiation, apoptosis and autophagy[79, 80]. It is well known that p38 MAPK signaling pathway works in cooperation with IIS pathway to regulate *C.elegans*' immune system in response to bacterial infection[80]. Dinh et al. reported that cranberry could protect *C. elegans* against *Vibrio cholerae* infection by inducing IIS/HSF-1 and P38 MAPK signaling pathways[81].

TOR (target of rapamycin) pathway, which regulates autophagy of proteins and lipids in response to nutrition levels, is also involved in longevity regulation. Reduced TOR signaling causes reduction in caloric intake without accompanying malnutrition, which leading to further longevity benefits. It is reported that a TOR pathway inhibitor, rapamycin, has been shown to extend lifespan in murine model[82, 83]. This finding further confirmed the lifespan extension function of TOR pathway. In addition to longevity, TOR pathway also controls several cellular processes including translation initiation and elongation, autophagy, mitochondrial respiration, and induction of stress response pathways[84-86].

1.1.2 Dietary intervention is a feasible method to modulate longevity

1.1.2.1 Dietary intervention and nutrigenomics

Although dietary intervention and genetic alteration are two essential methods for manipulating genes and pathways in the aging process, dietary intervention is more feasible and efficient to promote healthy aging[87-89].

Nutraceutical supplementation is the most popular and well-studied dietary intervention approach. A nutraceutical is defined as a product derived from food sources possessing pharmaceutical functions in addition to their basic nutritional benefits[90, 91]. To date, a growing body of evidence demonstrates how nutraceutical supplementation plays a key role in lifespan extension and alleviation of the symptoms of aging and stress[92, 93].

Nutrigenomics is provided as a research tool to investigate how nutrients affect metabolic pathways and homeostatic control and consequently how nutrients affect the aging process and disease[94]. Nutraceuticals, by regulating genetic pathways, mediate lifespan extension and stress resistance. For instance, Ginkgo Biloba extract (EGb761), a traditional Chinese medicine derived from the fruit of maidenhair tree, was reported to extend lifespan and increase tolerance of oxidative stress and thermal stress in worms and rat models. Noticeably, this anti-aging effect is dependent on Ginkgo mediated overexpression of HSP-16.2[95]. Resveratrol, a functional extraction from grape seeds, was discovered as a SIR-2.1/SIRT1 activator, which delays the progression of neurodegenerative diseases[96]. Cocoa and its derivatives, another functional food, could

improve endothelial function and protect against cardiovascular disease in mammalian studies[97]. Taken together, numerous studies indicated that nutraceutical supplementation functions as a genetic regulator to improve a healthy aging. Additionally, the nutrigenomic approach highlighted the relationship between dietary interventions and pro-longevity, revealed the molecular mechanism of age-related disorders, and served as an efficient tool to further investigate how to prevent the neurodegenerative disease. In a current study, a nutrigenomic approach is being utilized to investigate the molecular mechanism underlying Royal Jelly mediated anti-aging effects.

1.1.2.2 Royal Jelly's beneficial effects

Royal Jelly (RJ), a bee product, is secreted from the hypopharyngeal gland and mandibular gland of the worker honeybee. RJ plays a key role in the development of the queen honeybee, and it is part of the diet of honeybee larvae[98].

It is reported that Royal Jelly has numerous pharmacological characteristics, such as anti-bacterial, antioxidant, anti-fatigue, and wound-healing properties[99-101]. Moreover, several studies carried out using mammalian models revealed that Royal Jelly could maintain the balance of sugar and lipid metabolism and protect the liver from virus invasions[102]. A chemical composition analysis has shown that Royal Jelly consists mainly of proteins, sugars, lipids, vitamins, and free amino acids. Based on the previous studies, it has been proposed that the 10-HAD, royalisin and apisin are the major components contributing to the above pharmacological functions[103].

In modern diets, the use of Royal Jelly has significantly increased because of the trend toward the use of healthy, organic, nutraceutical, functional foods, and dietary supplements. Besides the merits of Royal Jelly's beneficial functions mentioned above, one phenomenon suggests that Royal Jelly also has the beneficial functions for anti-aging. In the honeybee *Apis mellifera*, queens live and reproduce for 1-4 years, but hive workers, which are derived from the same diploid genome, live for only 3 to 6 weeks. Queens are fed throughout their lives with Royal Jelly, while worker bees are fed with Royal Jelly for only a short period of time during their larval stages[98, 104]. Based on this phenomenon, it is believed that Royal Jelly might provide anti-aging agents for queens.

The recently studies demonstrated that Royal Jelly may contain the pro-longevity factors and its pro-longevity effects may depend on the insulin signaling pathway[105, 106]. However, the detailed mechanisms underlying the pro-longevity effects of royal jelly are largely unknown. Our lab focuses on the aging intervention study, which attempts to unveil the mechanism of royal jelly mediated lifespan extension. Furthermore, we will also investigate the potential correlation of pro-longevity effects and the prevention of age-related disorders by supplying royal jelly.

1.2 Pro-longevity is often associated with increased stress tolerances

Numerous studies supported that extended longevity is often correlated with increased resistance against various stressors, such as oxidative stress, heat shock, UV irradiation, osmosis stress and pathogen stress[107, 108].

1.2.1 Oxidative Stress

Oxidative stress is engendered by an imbalance between the production of free radicals and the biological system's capacity to detoxify their harmful effects via antioxidants. Free radicals are formed in mitochondria during cell metabolism under normal conditions. As a scavenger, antioxidants could attack and neutralize free radicals and, in turn, prevents the oxidative stress. However, under chronic stress conditions or in aged cells, the effectiveness of antioxidant defenses is declined and the harmful free radicals can accumulate. As a result, the production of peroxides and free radicals could cause damage to all components of a cell, including proteins, lipids and DNA. Additionally, considering some reactive oxidative species act as cellular messengers in redox signaling, the accumulation of free radicals can disrupt the normal function of several signaling pathways[109, 110].

In humans, oxidative stress is thought to be involved in the development of several aging-disorders, such as cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, and myocardial infarction. However, reactive oxygen species are not always harmful. In short-term oxidative stress, free radicals could be used by the immune system as a way to attack and kill pathogens and thereby protect organism from bacterial infections[111].

1.2.2 UV Irradiation

Ultraviolet (UV) irradiation is known to damage DNA through the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4-photoproducts (6-4 PPs). UV induced DNA damage triggers cell cycle arrest and apoptosis[112].

In somatic cells and tissues, the metabolism derived genotoxic agents and environmental factors (UV light or sunlight and radiation) cause DNA damage, resulting in alterations of DNA chemical structure, including a break in a strand of DNA, a base missing from the backbone of DNA, and DNA cross-linkages. These accumulative damages can give rise to mutations and cell death. In response to these negative effects, a series of DNA repair systems are activated to protect genomic integrity[113]. Once cell detects the DNA damage, the specific repair molecules could bind at or near the site of damage to fix the DNA alterations.

Nucleotide excision repair (NER), a general DNA repair system in response to UV-induced DNA damage, functions to preserve and faithfully transmit DNA to the next generation in many organisms across life domains[114]. Upon detecting an irregularity in DNA structure, NER pathway is activated to remove the abnormal DNA and synthesize new base pairs to seal the nick, which in turn to play a key role in maintaining genome integrity[114]. In humans, mutations in NER genes cause several UV sensitive hereditary disorders. For example, cockayne syndrome (CS) patients, who have defects in transcription coupled nucleotide excision repair (TC-NER), suffer neurodevelopmental problems and premature aging. Similar evidence also showed in mice model, xeroderma pigmentosum (XP), a disease caused by XPD mutation, showed many symptoms of premature aging and extreme cancer proneness[115, 116].

In addition to NER pathways, certain pathways and molecules also participate in DNA damage response and conserved in mammals and *C. elegans* to repair different

types of DNA damages, such as BER (base excision repair), NHEJ (nonhomologous end-joining), HR (homologous recombination), ICL (interstrand crosslink repair), and mismatch repair[113].

Aging is often associated with the dysfunction of the body over time, and this regressive physiological function is caused by declined cellular repair system and accumulative cellular damages. Many studies have shown that high levels of DNA oxidative damages were detected in aging cells and organisms, which might further induce the late-onset neurodegenerative diseases. Several studies showed that some nutraceuticals might protect organisms from DNA damages. For instance, Inoue and his colleagues reported that Royal Jelly could extend the C3H/HeJ mice lifespan by reducing the DNA oxidative damage[106]. This finding indicated that RJ might have the potential to maintain genomic integrity in aged animals.

1.2.3 Heat Shock Stress

Heat shock stress is produced when an organism is shifted to a higher temperature than the ideal body temperature for certain period of time. As a result, the sudden increased temperature could cause cellular damage to proteins' structure and function to a certain degree. In response to this thermal stress, the heat shock factor (HSF-1) is activated to dramatically up-regulate the expression of heat shock proteins (HSPs) to recover the destructed proteins and thereby protect organisms from this disruption[117].

It is well known that heat shock proteins (HSPs) play a critical role in protein folding, aggregation, and degradation. In addition to heat shock, upregulation of HSPs

could also be triggered by a variety of environmental stress conditions, such as inflammation, toxins (heavy metal, UV light, toxic chemicals, et al.), starvation, wound healing, and tissue remodeling. In normal condition, HSPs function as molecular chaperones to guide newly synthesized proteins folding in functional configuration and deliver them to right place at the right time, which in turn control protein quality and maintain the function of proteome[118]. Moreover, some HSPs could attach to proteins to signal their degradation via proteasome, affect their activities, and alter their cellular location. Noticeably, ubiquitin plays a key role in assisting aberrant proteins degradation, which decreases the proteotoxicity in cells[119, 120].

Given that heat shock stress and aging condition are always associated with the production of destructed proteins (unfolded and misfolded proteins), which are in an urgent need to be refolded or degraded by HSPs to recover proteins' function and thereby maintain protein homeostasis[121]. The disruption of this process could cause age-related disorders and proteopathy. Numerous studies revealed that some nutraceuticals could protect organisms from thermal stress. For instance, cranberry could protect *C. elegans* from heat shock stress by elevating the expression of HSPs[122]. Additionally, further research demonstrated that cranberry also protected *C. elegans* from A β toxicity and thereby delayed the development of Alzheimer's disease[123].

1.2.4 Osmotic Stress

Osmotic stress is caused by a sudden change in the solute concentration around a cell, causing a rapid change in the movement of water across its cell membrane. Under

conditions of high concentrations of salt, water is drawn out of the cells through osmosis. This also inhibits the transport of substrates and cofactors into the cell thus “shocking” the cell[124].

All organisms have mechanisms to respond to osmotic shock, with sensors and signal transduction networks providing information to the cell about the osmolarity of its surroundings, which activates responses to deal with extreme conditions. OSR-1 regulates survival under osmotic stress via UNC-43 and a conserved P38 MAP kinase signaling cascade and likely regulating osmotic avoidance and resistance to acute dehydration by distinct mechanisms. OSR-1 plays a central role in integrating stress detection and adaptation responses by invoking multiple signaling pathways to promote survival under hyperosmotic environments[125]. Current research also suggests that osmotic stress in cells and tissues may significantly contribute to inducing many human diseases[126].

1.3 Age-related disorders

1.3.1 Neurodegenerative diseases

Aging is one of the greatest risk factors to cause age-related disorders, and neurodegenerative disease is the most common one in the aging population[127]. Neurodegenerative disease represents a series of diseases relative to the progressive loss of neurons' structure or function, including ALS (amyotrophic lateral sclerosis),

Parkinson's disease, Alzheimer's disease, and Huntington disease. These diseases are incurable, resulting in progressive neuron cells degeneration and death[128].

Sarcopenia is the age-related loss of the muscle mass and strength, which cause functional limitations in daily living, frailty and disabilities, and finally a higher mortality rate in the elderly patients. Given that satellite cells are resident myogenic progenitors in the skeletal muscles and plays a central role in the growth and regeneration of skeletal muscles, the main pathogenesis of sarcopenia is contributed to the age-related functional disability in satellite cells[129]; Parkinson's disease (PD) is caused by the deposition of Lewy bodies in the central nervous system, which further influences the motor system. PD patients are suffering from movement-related problems, such as shaking, rigidity, and difficulty with walking. Moreover, dementia commonly arises in the advanced stages of disease[130]; Alzheimer's disease is the most common cause of dementia, which triggers the problems of communication, thinking, and memory. Numerous studies demonstrated that the etiology of AD is due to the deposition of senile plaques (tau proteins and amyloid beta proteins) in the neurons, which disrupt the neuron cells' transduction signaling induce neuron cell death[131]. Although the pathogenesis is different in these neurodegenerative diseases, based on the previous research, there are some sub-cellular level similarities shared among these diseases: the majority of neurodegenerative diseases are engendered by genetic mutations and protein misfolding problems[132]. Discovering of these molecular similarities of neurodegenerative diseases not only provided us more opportunities to better understand how these diseases are generated but also helped us to further investigate the efficient therapeutic treatments[133].

Given that AD is a major type of age-related disorder and the most common cause of dementia worldwide, our study focused on studying the molecular mechanism of AD.

1.3.2 Alzheimer's Disease

1.3.2.1 The etiology of AD

AD is a major type of neurodegenerative disease and the number one cause of dementia in the aging population. Brain atrophy and neuronal lesions are two obvious phenotypes of Alzheimer's disease, which cause problems with thinking, memory, and behavior. The worldwide statistic data demonstrated that the number of AD patients will approach 63 million by 2030 and 114 million by 2050[134]. Therefore, there is an urgent need for scientists to develop efficient therapeutic treatment of AD.

To date, although a wealth of data surrounding AD has been published, the molecular mechanism underlying this disease is still unclear. There are several accepted hypothesis trying to explain the cause of AD. First, amyloid hypothesis is the most accepted theory in aging research, which claimed that the deposits of extracellular amyloid beta ($A\beta$) are the principal cause of neuron lesions. Amyloid precursor protein (APP) is considered to be a major source of $A\beta$. In normal conditions, APP is split by α -secretase and formed soluble α APPs rather than toxic $A\beta$. However, β -secretase and γ -secretase could split APP into three fragments and, in turn, certain peptide fragments could aggregate into insoluble plaques in brain and therefore cause AD (Figure 1.3) [135]. One may note that $A\beta$ is considered as a hallmark of AD as well as an important target of anti-AD drugs.

The second hypothesis is called tau theory. The tau proteins, a group of important structural proteins in neuron cells, function to stabilize microtubules to maintain cell's cytoskeleton. It has been proposed that AD is initiated by tau protein abnormalities. Based on the previous research, tau proteins are highly overexpressed in AD patients. Specifically, the majority of these proteins are hyper-phosphorylated, which are prone to pairing with others to form neurofibrillary tangles (PHF) inside nerve cells and thus result in destroying and collapsing the neuron cells[136].

The third hypothesis, genetic heritability of AD, claimed that AD is an inherited disease and well explained the etiology of early onset familial AD. The best known genetic risk factor is the inheritance of the $\epsilon 4$ allele of the apolipoprotein E (APOE), and people carrying the APOE $\epsilon 4$ allele have higher risk to get AD. In addition to APOE, the genetic mutations in TREM2, presenillins 1 and 2, and APP have been associated with higher risk of developing AD[137]. Taken together, although the molecular mechanism of AD is intricate and complex, scientists will develop efficient anti-AD treatment in the near future.

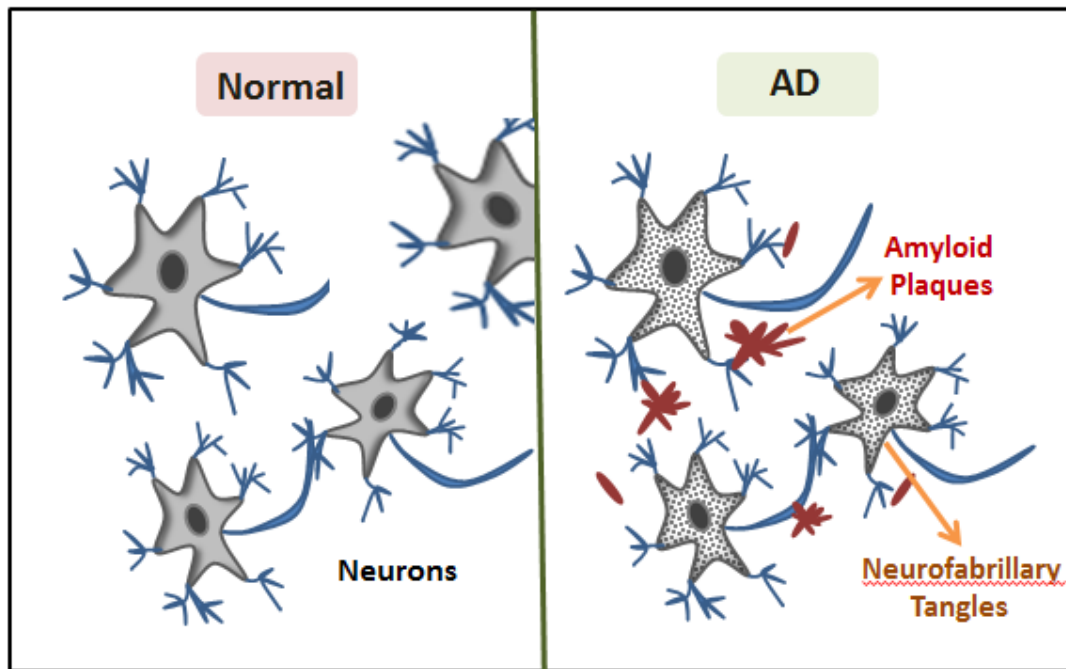


Figure 1.3 The formation of amyloid plaques and neurofibrillary tangles are thought to contribute to the degradation of the neurons. (Modified from BrightFocus Foundation, 2000)

1.3.2.2 The treatment of AD

Although AD is an incurable disease, there are several medications to temporarily mitigate the worsening of symptoms and slow the development of AD. From 1996 to 2004, the U.S Food and Drug Administration (FDA) has approved five medications to treat AD, including donepezil, galantamine, memantine, rivastigmine, and donepezil[138]. Although these drugs could improve cognitive ability, it is still unknown whether these medications could extend the lifespan of AD patients because these drugs are short-term effect cholinesterase inhibitors[139].

In addition to these five FDA approved medications, A β inhibitors are also considered as anti-AD treatments, such as A β vaccine, anti-inflammation drugs, and anti-cholesteric drugs. Clinical studies showed that these treatments are beneficial to prevent AD and treat early stages of AD patients[140, 141].

To date, a growing body of evidence suggests that dietary intervention is a good choice to slow down the age-related disorders. Numerous studies showed that anti-aging nutraceuticals could slow down the development of AD[142]. Moreover, reduction of IIS pathway could alleviate A β toxicity in *C. elegans* and mammals (Figure 1.4)[143, 144]. For instance, cocoa could mitigate the A β toxicity by reducing its aggregation in AD model, which is dependent on IIS pathway and DAF-16[97, 145]; Dostal and his colleagues reported that coffee significantly reduced A β toxicity, and this anti-AD effect required SKN-1[146]; Resveratrol, a SIR-2.1 activator, could protect neuron cells from apoptosis and thereby delay the development of AD and Parkinson's disease[147].

Collectively, nutraceutical supplementation not only extends lifespan and increases stress resistance, but also has beneficial effects to slow down the development of age-related disorders.

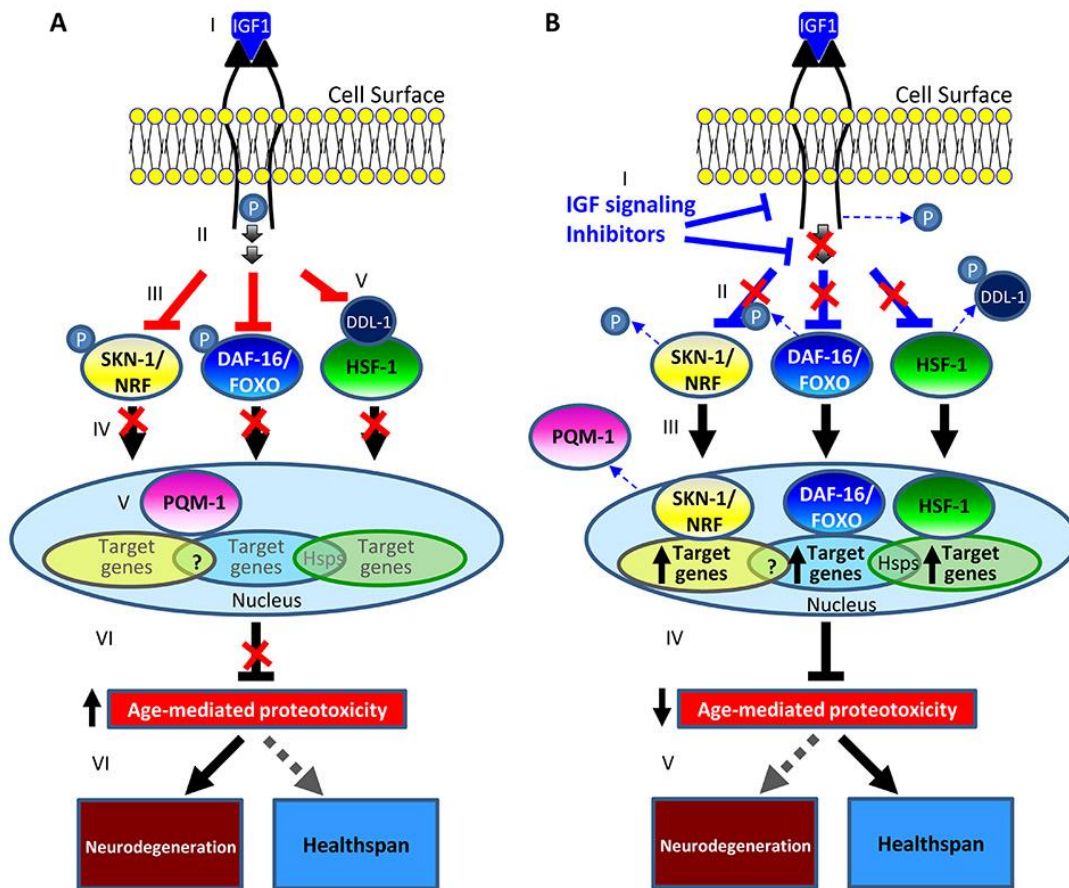


Figure 1.4 Reduction of IIS pathway prevents neurodegeneration in worms and human. (Moll *et al.*, Swiss Med Wkly. 2014;144:w13917)

1.3.3 Cellular Proteostasis

A growing body of evidence indicated that healthy aging is always associated with well-maintained proteostasis[148]. The imbalance of protein homeostasis could accelerate aging and induce neurodegenerative diseases. Proteostasis includes protein synthesis, folding, aggregation and degradation, which is the process to control the quality of cellular proteins[149]. Proteins are synthesized from ribosomes and folded to form the functional conformation. The endogenous and exogenous stresses could damage the proteins, forming the unfolded proteins. The unfolded and misfolded proteins could be refolded by heat shock proteins or be targeted by ubiquitin proteasome and lysosomal pathways to destruction and degradation[150]. This mechanical system keeps the function proteostasis in the health cells to keep the integrity of proteome and promote the cellular renewal[151]. However, in the aging cells or chronic persisting stresses conditions, this system is overwhelmed, which could form the aggregations and increase the proteotoxicity, which could increase the risk of neurodegenerative diseases. Functional proteostasis could improve healthy aging and reduce the risk of age related disorders development (Figure 1.5).

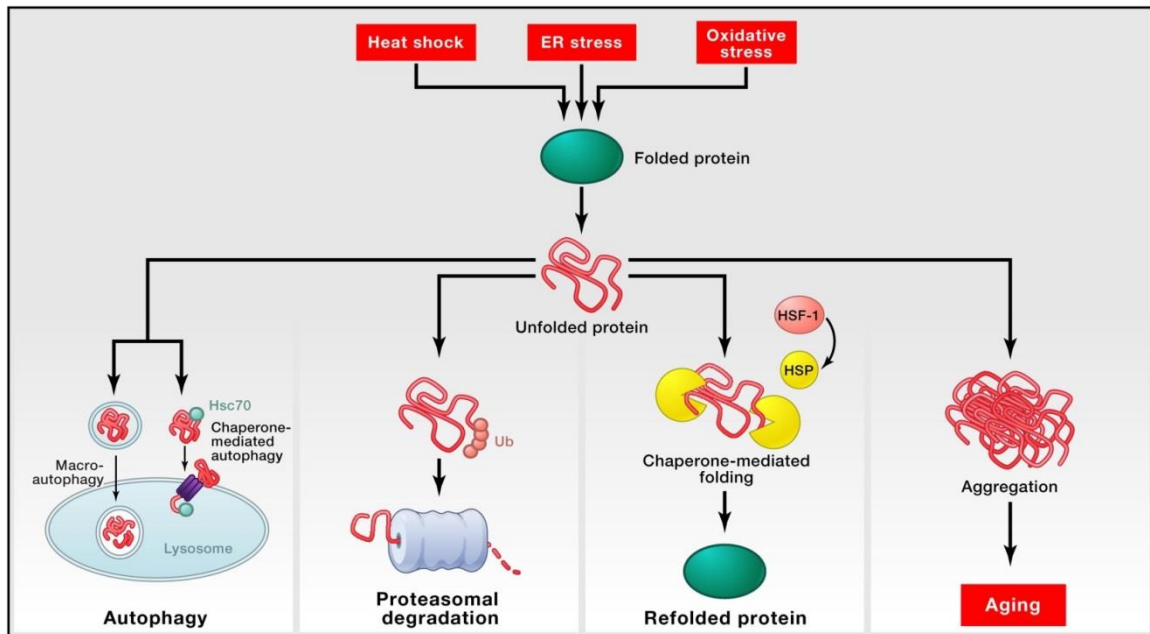


Figure 1.5 Proteostasis is the Process to Control the Quality of Cellular Proteins. (López-Otín *et al.*, 2013)

CHAPTER TWO : MATERIAL AND METHODS

2.1 Experimental Procedures- longevity and stress response research

2.1.1 Strains and Growth Conditions

All wildtype strain and mutant strains were maintained at 20 °C on NGM (nematode growth medium) seeded with *Escherichia coli* OP50 feeding strain. A 100 µl of OP50 was dropped on the center of 60 mm NGM plates, which were allowed to dry overnight before the assays were carried out. Strains used in this research were: N2 (Bristol, wild type), *daf-16* (*mgDf50*), *sir-2.1* (*ok434*), *daf-12* (*e1370*), *age-1* (*hx-546*), *ftt-2*(*n4426*), and *hcf-1*(*ok559*). All the strains were obtained from the Caenorhabditis Genetic Center (CGC), University of Minnesota, USA.

2.1.2 Preparation of RJ and eRJ

The powder of RJ and eRJ were provided by Yamada Apiculture Center, Inc., Okayama, Japan. To prepare RJ and eRJ supplemented food, appropriate amount of RJ or eRJ in powder form were dissolved in sterile distilled water and suspended into the liquid NGM one day before the assay.

2.1.3 Lifespan Assay

All lifespan assays were carried out at 25 °C. Synchronized populations were obtained by allowing 10-20 hermaphrodites lay eggs for 4 hours at 25 °C and then removing the parents. The eggs were allowed to hatch and develop. Approximately thirty L4/young adult worms per plate (NGM plate contains 50 µg/mL FUDR to prevent the growth of progeny) were used for each assay. All the assays were carried out in triplicates

and a minimum of three independent trials were performed for all conditions. The dead worms were counted starting the day after transfer and exploding, protruding, bagging, or contaminated worms were censored if applicable. We defined the day when we transferred the L4/young adult worms as day 0 of adult age. All statistical analyses were carried out using SPSS software (IBM SPSS Statistics). Kaplan-Meier lifespan analysis was carried out and p values were calculated using the log-rank test. $p < 0.05$ was accepted as statistically significant.

2.1.4 Growth Curve of E.coli OP50

The overnight culture of E.coli OP50 was 1:100 diluted in LB (200mg/mL Streptomycin), shaking at 37 °C. RJ (2mg/mL) and eRJ (1mg/mL) were added in the LB medium respectively, bacteria treated without RJ/eRJ served as controls. All the bacteria were cultured at 37 °C shaker and measured the OD600 reading every hour to compare the difference between each treatment.

2.1.5 Stress Assays

For three stress assays, wildtype N2 worms were pre-treated with 2mg/mL RJ or 1mg/mL eRJ respectively for two generations at 25 °C on OP50 seeded NGM plates. Worms on regular NGM OP50 seeded plates without RJ and eRJ served as controls. Each assay was carried out in three independent trials, and the data were pooled and analyzed using Student's t test. $p < 0.05$ was accepted as statistically significant.

For the oxidative stress assay, 5 mM paraquat (sigma-Aldrich, Corp., St Louis, MO) was used to induce the oxidative stress environment. The RJ/eRJ-pretreated

L4/young adult worms and their controls were transferred onto NGM plates containing 5mM paraquat, and survival was assessed by daily counting until all worms died.

To assay for the protective effect on UV irradiation, RJ/eRJ pretreated L4/young adult worms and controls were transferred to RJ/eRJ (2mg/mL RJ and 1 mg/mL eRJ) plates and regular NGM plates respectively, and were irradiated at 0.05 J/cm² for 20 seconds. The survival of the worms was monitored at 25 °C.

For the heat shock assay, RJ/eRJ pretreated L4/young adult stage worms and controls were transferred to RJ/eRJ supplemented (2mg/mL RJ and 1 mg/mL eRJ) plates and regular NGM plates respectively. The plates were incubated at 35 °C for 3 hours and then transferred back to 25 °C. The survival of worms was monitored daily.

2.1.6 Brood Size and Body Bend Assays

The brood size assay was carried out by counting the offspring of a single worm on an NGM plate supplemented with or without RJ/eRJ (2mg/mL RJ and 1 mg/mL eRJ). Each L4/young worm was allowed to lay eggs at 25 °C for 24 hours and was then transferred to a fresh plate until it ceased to lay eggs. The offspring were counted after they reached L3 or L4 phase. This assay was carried out in three independent trials. The data were pooled and analyzed using Student's *t* test. $p < .05$ was accepted as statistically significant.

For the motility assay, worms were pre-treated with or without RJ/eRJ (2 and 1mg/mL, respectively) for two generations on NGM plates at 25 °C. Three or four RJ/eRJ pretreated and non-treated L4/young adult nematodes were placed onto individual NGM

plates without OP50. The number of body bends performed in 3 minutes was counted, and then the number of body bends per minute was calculated. This assay was carried out in three independent trials. The data were pooled and analyzed using Student's *t* test. $p < .05$ was accepted as statistically significant.

2.1.7 DAF-16 Translocation Assay

daf-16 (mgDf47) I; xrls87 worms carrying a DAF-16-GFP fusion construct (Lee et al., 2001) were treated respectively with eRJ (1mg/mL) or RJ (2mg/mL) for two days and were then observed under a fluorescence microscope (Nikon AZ100) to monitor the nuclear translocation of DAF-16-GFP.

2.1.8 Gene Expression Analysis by Quantitative RT-PCR

The nematodes were grown on NGM plates supplemented with or without RJ/eRJ at 25 °C, until they reached the young adult stage, then collected in M9 buffer. RNA was prepared using RNazol® RT reagent (Molecular Research Center, Inc.) and stored at –80 °C. Complementary DNA was prepared by using Invitrogen Superscript first strand synthesis system for RT–PCR (Invitrogen). Quantitative PCR (qPCR) was performed using SsoFast EvaGreen Supermix and the CFX96 real-time PCR detection system according to the manufacturer suggested protocol (Bio-Rad). The qPCR conditions were: 95 °C for 3 minutes, followed by 40 cycles of 10 seconds at 95 °C and 30 seconds at 60 °C. *act-1* was used as an internal control to normalize the expression levels of target transcripts. Each qPCR experiment was repeated three times using independent RJ/eRJ treatments and RNA preparations. The data were pooled and analyzed using Student's *t* test, and $p < .05$ was accepted as statistically significant.

The qPCR primers for *sod-3* are: 5'-CCAACCAGCGCTG-AAATT CAATGG-3' (forward primer (F)) and 5'-GGAACCGAA GTCGCGCTTAATAGT-3' (reverse primer (R)). Primers for *daf-16* are 5'-CCAGACGGAAGGCTTAAACT-3' (F) and 5'-ATTCGC ATGAAACGAGAATG-3' (R). Primers for *sir-2.1* are 5'-AGAACGCGCATTTCGCC ATATTAAG-3' (F) and 5'-ATACTGACACTCCAGCGCCAG-3' (R). Primers for *hcf-1* are 5'-GCGTTTACTTGGCCGTTAAGAATC-3' (F) and 5'-GCCGTTCCCAGGTTTG ATTG-3' (R). Primers for *C32H11.4* are 5'-TACTTCCCATCGCCAAAGT-3' (F) and 5'-CAATTCCGGCGATGTATGAT-3' (R). Primers for *F21F3.3* are 5'-CCGATTCGTT CCTTTTGAAG-3' (F) and 5'-ACAACCGAATGTTCCAATCC-3' (R). Primers for *mtl-1* are 5'-ATGGCTTGCAAGTGTGACTG-3' (F) and 5'-CACATTTGTCTCCGCACTT G-3' (R). Primers for *act-1* are 5'-CCAGGAATTGCTGATCGTATGCAGAA-3' (F) and 5'-TGGAGAGGGAAGCGAGGATAGA-3' (R).

2.2 Experimental Procedures- *C. elegans* Alzheimer's Disease Model Research

2.2.1 Strains and Growth Conditions

All strains were maintained at 16 °C on nematode growth medium (NGM) seeded with *Escherichia coli* OP50 feeding strain. Strains used in this study were as follows: N2 Bristol (wild type), CL2006 (AD worm), and *daf-16 (mgDf50)*. All the strains were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota.

2.2.2 Preparation of RJ and eRJ

The powder of RJ and eRJ were provided by Yamada Apiculture Center, Inc., Okayama, Japan. To prepare RJ and eRJ supplemented food, appropriate amount of RJ or

eRJ in powder form were dissolved in sterile distilled water and suspended into the liquid NGM (2mg/mL RJ and 1mg/mL eRJ) one day before the assay.

2.2.3 RNA Interference

RNA interference (RNAi) clones were grown overnight at 37 °C on Luria Broth plate in the presence of tetracycline (12.5 µg/ml) and carbenicillin (25 µg/ml). Bacterial colonies were inoculated and grown for 8–12 hours, then induced with 2mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours at 37 °C. Ten-fold concentrated RNAi bacteria were seeded onto RNAi plates containing 25 µg/ml carbenicillin. The RNAi constructs targeting *daf-2*, *age-1*, *daf-16*, *hsf-1*, *skn-1*, *jnk-1*, *sir-2.1*, *hcf-1* and *ftt-2* were obtained from the C elegans ORFeome RNAi library v1.1.

RNAi efficiencies were checked at the same time with RNAi paralysis assay. CL2006 worms were synchronized by allowing 10–15 hermaphrodites lay eggs 6 hours on RNAi bacteria seeded NGM plates at 16 °C. The eggs were allowed to hatch and develop. The L4 /young adult worms were transferred to RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) NGM plates (containing 50 µg/mL FUDR to prevent the growth of progeny) to maintain for 6 days. CL2006 worms on regular NGM (FUDR) plates without RJ/eRJ served as controls. Worms at the 6-day old stage were collected with M9 buffer into a 50~100 µl pellet. RNA extraction and qPCR method are described in 2.1.8.

The qRT-PCR primers for *daf-16* are as follows: 5'-CCAGACGGAAGGCTTA AACT-3' (F) and 5'-ATTCGCATGAAACGAGAATG-3' (R). Primers for *hsf-1* are as follows: 5'-TTGACGACGACAAGCTTCCAGT-3'(F) and 5'-AAAGCTTGCACCAG

AATCATCCC-3'(R). Primers for *skn-1* are as follows: 5'-GTAGCCGACGACGAAGAAGA-3'(F) and 5'-GAATTGAGGTGTTGGACGAT-3'(R). Primers for *sir-2.1* are as follows: 5'-AGAACGCGCATTTTCGCCATATTAAG-3' (F) and 5'-ATACTGACACTCAGCGCCAG-3' (R). Primers for *hcf-1* are as follows: 5'-GCGTTTACTTGGCCGTTAAGAATC-3' (F) and 5'-GCCGTTCCCAGGTTTGATTG-3' (R). Primers for *ftt-2* are as follows: 5'-TCGACAAGTTCCTCATTCCA-3'(F) and 5'-TAGCTTTGCTGCGACTTC TC-3'(R). Primers for *daf-2* are as follows: 5'-CGGTGCGAAGAGAGGATATT-3'(F) and 5'-TACAGAGGTCGCCGTTACTG-3'(R). Primers for *age-1* are as follows: 5'-AGTGGATTCGGAAACAATGC-3'(F) and 5'-GGAATCGATCGACACTTTCA-3' (R). Primers for *jnk-1* are as follows: 5'-ACAGTGGAACAGGAGGAGG-3'(F) and 5'-ATACGGAAGTGGAGGTGGAG-3' (R). Primers for *act-1* are 5'-CCAGGAATTGCTGATCGTATGCAGAA-3' (F) and 5'-TGGAGAGGGAAGCGAGGATAGA-3' (R).

2.2.4 Lifespan Assays

All lifespan assays were carried out at 20 °C. Synchronous populations were obtained by allowing 10–15 hermaphrodites lay eggs overnight at 16 °C, and the parents were removed the next day. The eggs were allowed to hatch, and 30 L4/young adult worms per plate (NGM plate containing 50 µg/ml 5-fluorouracil-2'-deoxyribose to prevent the growth of progeny) were used for each assay. The dead worms were counted starting the next day, and exploding, protruding, bagging, or contaminated worms were censored if applicable. We defined the day when we transferred the L4/young adult worms as Day 0 of adult age. All the assays were carried out in triplicates, and a minimum of three independent trials were performed for all conditions. All statistical

analyses were carried out using SPSS software (IBM SPSS Statistics). Kaplan–Meier life span analysis was carried out, and *p* values were calculated using the log-rank test. *p* < 0.05 was accepted as statistically significant.

2.2.5 Worm Paralysis Assays

The assays using strain CL2006 were carried out as described by Cohen and colleagues. Synchronous populations of CL2006 worms were prepared on NGM plates by allowing 10–15 hermaphrodites lay eggs overnight at 16 °C, and the parents were removed the next day. The eggs were allowed to hatch, and 20 L4/young adult worms per plate were used for each assay. All paralysis plots were done in triplicates, and a minimum of three independent trials were performed per condition. Nematodes were scored as paralyzed if they exhibited “halos” of cleared bacteria around their heads (indicative of insufficient body movement to access food) or failed to undergo full body wave propagation upon the nose prodding. Worms were checked every day until all worms were paralyzed. The data were pooled, and the percentage of paralyzed worms was calculated and analyzed using Student’s *t*-test. *p* < 0.05 was accepted as statistically significant.

2.2.6 Western Blotting of A β Species

Rabbit polyclonal A β _{1–42} primary antibodies were from abcam (ab39377). For Western blot analysis, CL2006 worms were synchronized by allowing 10–15 hermaphrodites to lay eggs overnight on OP50 seeded NGM plates at 16 °C. The parents were removed, and eggs were allowed to hatch and develop to the L4 stage. Subsequently, worms were transferred to RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively)

containing NGM plates and continued to grow at 20 °C. CL2006 worms on regular NGM plates without RJ/eRJ served as control. After 10 days of growth, worms were transferred to micro-centrifuge tubes and washed with S-basal followed by protein immobilization on polyvinylidene fluoride (Bio-Rad) membrane. Polyvinylidene fluoride membrane was incubated with primary antibodies (1:1,000) diluted in 5% nonfat dry milk and then with secondary, HRP-conjugated goat anti-rabbit antibodies (Genscript, A00098; diluted 1:10,000). ACTIN was used as loading control, and the anti-ACTIN antibodies (MAB1501) were from EMD Millipore. Detection was undertaken with standard ECL protocol. Mean intensity of A β signals was analyzed using Image-J software (National Institute of Health).

2.2.7 Soluble Protein Extraction

The soluble protein extraction was performed as described previously with alterations. Synchronous populations of eggs were prepared by 20% alkaline hypochlorite treatment of gravid adults grown at 16 °C. Eggs were allowed to hatch and develop by transferring to OP50-seeded NGM plates containing RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) at 20 °C. Eggs hatched and developed on OP50-seeded NGM plates without RJ/eRJ served as controls. Both worms treated with or without RJ/eRJ supplementation were collected 10 days after L4 stage. Three separate replicates of each sample (about 200mg [wet weight] of worms) were collected. Total protein extracts were produced in phosphate-buffered saline by sonication on ice, and then the total protein concentration was determined by conducting a bicinchoninic acid assay. Next, the normalized protein samples were centrifuged for 10 minutes at 14,000g to remove the

insoluble fraction. The same volume of supernatants (soluble fraction) was loaded and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Mean intensities of protein bands were analyzed using Image-J software (National Institute of Health).

2.2.8 Gene Expression Analysis by Quantitative Real-time PCR

CL2006 worms were synchronized by allowing 10–15 hermaphrodites lay eggs 6 hours on OP 50 seeded NGM plates at 16 °C. The eggs are allowed to hatch and develop. The L4 /young adult worms were transferred to RJ/eRJ (2 and 1mg/mL respectively) NGM plates (containing 50 µg/mL FUDR to prevent the growth of progeny) to maintain for 6 days. CL2006 worms on regular NGM (FUDR) plates without RJ/eRJ served as controls. Worms at the 6-day old stage were collected with M9 buffer into a 50~100 µl pellet. RNA extraction and RT-PCR method are described in 2.1.8.

The qRT-PCR primers for *daf-16* are as follows: 5'-CCAGACGGAAGGCT TAAACT-3' (F) and 5'-ATTCGCATGAAACGAGAATG-3' (R). Primers for *hsp-12.6* are as follows: 5'-ATGATGAGCGTTCCAGTGATGGCTGACG-3' (F) and 5'-TTAATGCATTTTCTTGCTTCAATGTGAAGAATTCC-3' (R). Primers for *hsp-16.2* are as follows: 5'-TTGCCATCAATCT CAACGTC-3' (F) and 5'-CTTTCTTT GGCGCTTCAATC-3' (R). Primers for *hsp-70* are as follows: 5'-CGTTTCGAAGA ACTGT GTGCTGATCTATTCCGG-3' (F) and 5'-TTAATCAACTTCCTCA ACA GTAGGTCCTTGTGG-3' (R). Primers for *act-1* are as follows: 5'- CCAGGAATTG CTGATCGTATGCAGAA-3' (F) and 5'-TGGAGAGGGAAGCGAGGATAGA-3' (R). Primers for *sod-3* are as follows: 5'-CCAACCAGCGCTG-AAATT CAATGG-3' (F) and 5'-GGAACCGAA GTCGCGCTTAATAGT-3' (R). Primers for *mtl-1* are as follows: 5'-

ATGGCTTGCAAGTGTGACTG-3' (F) and 5'-CACATTTGTCTCCGCACTTG-3' (R). Primers for *sip-1* are as follows: 5'-GGTCAATCTTGAGGGACACG-3' (F) and 5'-GAGTGAACGATCTCTTGCTG-3' (R). Primers for *aip-1* are as follows: 5'-GAGCGGGATCACAGTTGTGAG-3' (F) and 5'-GATGTGATTGAATCCGTCCAG-3' (R). Primers for *F10D7.5* are as follows: 5'-GCACTAGAGGACCATTACAGTT-3' (F) and 5'-CGTTCTCGTCAATCTCGATAGG-3' (R). Primers for *ZK218.8* are as follows: 5'-TGCTACTGGCTGTGTGTTAG-3' (F) and 5'-CAATAGTTCCGGCATTACATAAT-3' (R). Primers for *T05G5.10* are as follows: 5'-GGAGACTTGGGAAACACTATCC-3' (F) and 5'-TGTATCCGAGAATAGCCTCCT-3' (R). Primers for *Y71H2AR.2* are as follows: 5'-GAGTTGTGGCAGAGGGTAATG-3' (F) and 5'-GGATGGGTTGTAGATTCCGATTT-3' (R).

CHAPTER THREE : THE MOLECULAR MECHANISM OF ROYAL JELLY MEDIATED LIFESPAN EXTENSION

3.1. Introduction

Aging is an inevitable process associated with physiological decline and higher risk to get disease. Nowadays because of the development of technology and medical treatment, the population structure changed and population aging already became a challenging issue to our society. Age-related diseases affect aging population and their families; moreover, the increasing medical cost brings concerning economic burden to the society and government. In order to solve this problem, more and more scientists are dedicated to doing research in the anti-aging field, not only study the molecular mechanism of aging, but also look for effective methods to delay the onset of age-related disorders. Although the anti-aging research field is very new, it is extremely urgent for scientists to find out efficient solutions to slow down the age-related diseases development, therefore, improving the aging populations' quality of life.

As same as other biological processes, aging is also controlled by genes[31]. Insulin like signaling (IIS) pathway is considered as a master regulator to modulate longevity and metabolism. DAF-16 is a downstream component of IIS pathway, which functions as an essential transcriptional factor to regulate cell proliferation, metabolism, and longevity[38]. Similar to other transcriptional factors, DAF-16 is tightly controlled by other co-factors. SIR-2.1, HCF-1 and 14-3-3 are reported as major DAF-16 co-factors and physically interact with DAF-16 to regulate its activity[62].

It is well known that dietary intervention and genetic alteration could manipulate genes and pathways to regulate longevity in the anti-aging research field[152]. Comparing to other methods, dietary intervention is the most efficient and safe manner to delay the development of age related disorders[88]. Royal Jelly is a popular functional food in Asian market, which has a series of beneficial effects of health. As a bee product, Royal Jelly is secreted from the pharyngeal glands of nurse worker bees (*Apis mellifera*) to feed larvae. Adult queens are fed only with Royal Jelly for the whole life and it enables her to outlive worker bees nearly thirty fold. Most people believe that Royal Jelly might have the pro-longevity factors, and the Japanese scientists confirmed this hypothesis[105]. They reported that Royal Jelly has the anti-aging beneficial effects, but the mechanism is still unclear. In my research, I will focus on investigating the molecular mechanism of Royal Jelly's anti-aging functions.

Our Royal Jelly powders were provided by Yamada Japanese Company, and there were two types of Royal Jelly powders used in our research; one was enzyme treated Royal Jelly (eRJ), and the other was regular Royal Jelly (RJ). *Caenorhabditis elegans* (*C. elegans*) was used as a model animal to investigate RJ/eRJ's anti-aging function. Our data showed that RJ/eRJ supplementation extended *C. elegans* lifespan in a dose dependent manner. IIS/DAF-16 and DAF-16's co-factors were required in this RJ/eRJ mediated lifespan extension. Our proposed model was: under the supplementation of RJ/eRJ, SIR-2.1, HCF-1 and 14-3-3 could interplay with DAF-16 to fine-tune its transactivity to extend *C. elegans* lifespan.

3.2. Experiment Results

3.2.1 Supplementation of RJ/eRJ extends *C. elegans* lifespan in a dose-dependent manner

In order to determine whether RJ/eRJ has the pro-longevity beneficial effects, we treated the wild type *C. elegans* (N2 worms) with different concentrations of Royal Jelly, ranging from 0mg/mL to 5mg/mL, and carried out the lifespan assays to observe worms' survival at 25 °C. Our results showed that N2 worms treated without RJ/eRJ (control group) showed a regular lifespan. The mean lifespan was 12.79 days. Afterwards we compared the mean lifespan of RJ/eRJ supplemented worms to control worms to investigate whether RJ/eRJ could extend the lifespan of *C. elegans*.

In RJ treatment, the mean lifespan of 0.4mg/mL RJ treatment was 0.23 day longer than non-supplemented controls, but this lifespan extension was not significant. For the higher concentration RJ treatment, 1mg/mL to 3mg/mL RJ significantly increased *C. elegans* mean lifespan from 12.79 days to 13.52 days, 15.52 days, and 14.39 days, respectively. However, the highest concentration 5mg/mL RJ hardly influenced *C. elegans* mean lifespan (Figure 3.1 A and Table 1). Based on this result, we found out the best concentration of RJ for lifespan extension was 2mg/mL, which increased 21.34% of lifespan comparing to control group.

In eRJ treatment, low concentration of eRJ (0.4mg/mL) showed a similar lifespan as the control group, which indicated that this low concentration of eRJ cannot extend N2 worms' lifespan. For the higher concentration of eRJ treatment, 1mg/mL to 3mg/mL eRJ

could extend worms' lifespan significantly, from 12.79 days to 14.44 days, 14.09 days, and 13.95 days, respectively. However, the highest concentration 5mg/mL eRJ barely affected *C. elegans* mean lifespan (Figure 3.1 B and Table 1). Based on this result, we found out that the best concentration of eRJ treatment was 1mg/mL, which increased 11.33% of lifespan comparing to control group.

Taken together, the lifespan results showed that both RJ and eRJ could extend *C.elegans* lifespan in a dose-dependent manner. A 2mg/mL RJ and 1mg/mL eRJ were selected as the optimum concentrations to prolong *C. elegans* lifespan.

Several papers published that Royal Jelly also has anti-pathogen functions. In our experiment, worms were fed with E.coli OP50 as their regular food, and we questioned whether Royal Jelly's anti-aging effect is dependent on inhibiting the growth of E.coli or attenuating the expression of its virulent factors expression. In order to answer this question, we cultured E.coli OP50 in LB medium with RJ/eRJ respectively and observed their growth curve. We observed that RJ/eRJ did not inhibit the E.coli OP50 growth at any bacterial growth phases, on the contrary, RJ/eRJ increased E.coli OP50 growth at the lag phase and log phase, which indicated that RJ/eRJ mediated lifespan extension did not act through its anti-pathogen effects (Figure 3.2).

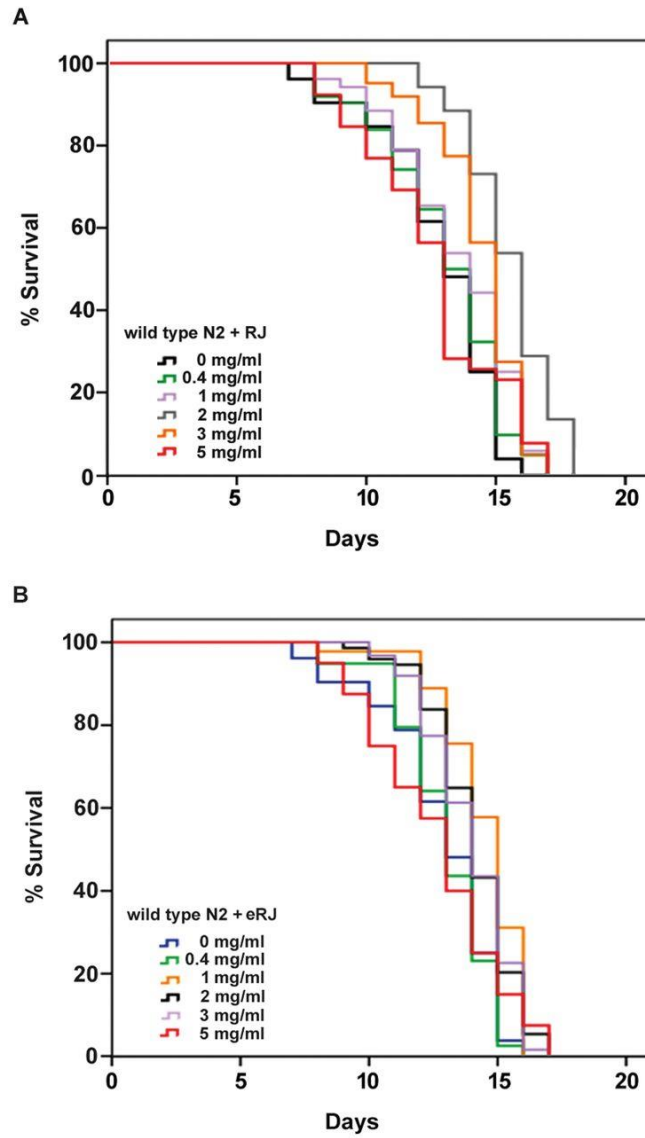


Figure 3.1 RJ/eRJ extends *C. elegans* lifespan in a dose-dependent manner.

Wild-type N2 worms were treated with RJ (A) or eRJ (B) at 0-5mg/mL. Each lifespan experiment was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments are included in Table 1.

Table 1 The Lifespan of Wildtype Worms Stain at 25 °C

Stain	Mean±SE	Median	No.of Worms	<i>p</i> value
N2	12.97 ±0.32	13.00	52	
N2+ 0.4 mg/mL RJ*	13.02 ±0.30	13.00	62	0.320
N2+ 1mg/mL RJ*	13.52 ±0.33	14.00	52	0.017
N2+ 2mg/mL RJ*	15.52 ±0.23	16.00	52	<0.001
N2 + 3mg/mL RJ*	14.39 ±0.22	15.00	62	<0.001
N2+ 5mg/mL RJ*	12.64 ±0.43	13.00	59	0.543
N2+ 0.4 mg/mL eRJ**	12.97 ±0.30	13.00	49	0.867
N2+ 1mg/mL eRJ**	14.44 ±0.24	15.00	45	<0.001
N2+ 2mg/mL eRJ**	14.07 ±0.20	14.00	74	0.001
N2+ 3mg/mL eRJ**	13.95 ±0.22	14.00	62	0.003
N2+ 5mg/mL eRJ**	12.68 ±0.40	13.00	50	0.699

Note: Lifespan and standard error are shown in days. The lifespan experiments were repeated at least three times with similar results. And the data for representative experiments were shown. The lifespan data were analyzed using the log-rank test and *p* values for each individual experiment are shown.

*Results presented in Figure 3.1 A **Results presented in Figure 3.1 B

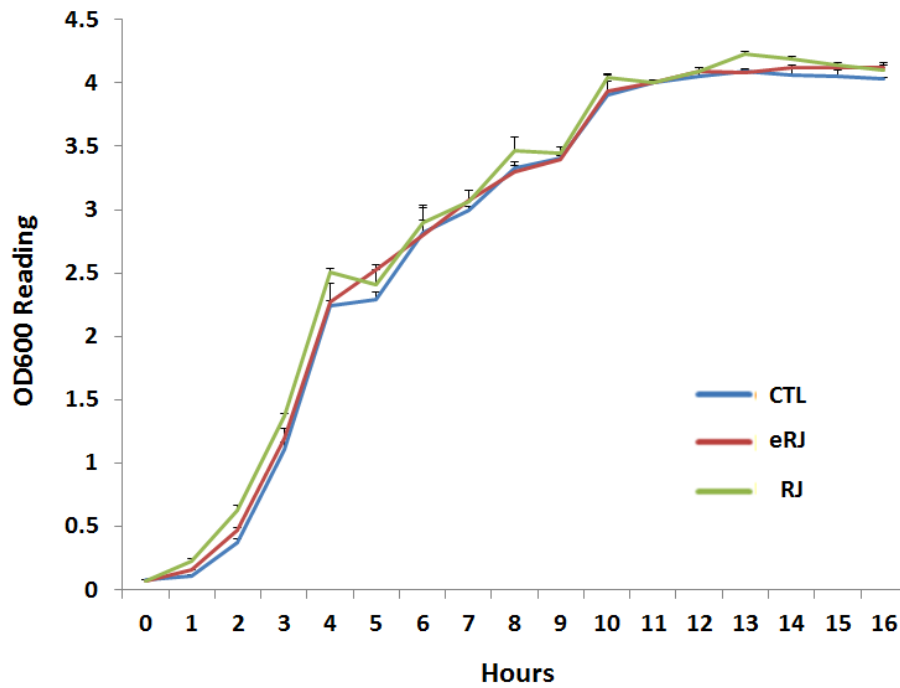


Figure 3.2 RJ/eRJ cannot affect E.coli OP50 growth. E.coli OP50 were cultured with RJ/eRJ and OD600 were measured every hour to observe the growth curve. Experiment was repeated at least three independent times with similar results. Student *t* test showed *p* value was >0.05 , quantitative data and statistical analyses were analyzed in excel.

3.2.2 RJ/eRJ barely Influences Reproductive Capacity and Motility of *C. elegans*.

Given that RJ/eRJ could extend *C. elegans* lifespan, 2mg/mL RJ and 1mg/mL eRJ were selected as the optimum concentration to promote longevity. Next, we wondered whether RJ/eRJ affected the physiological indexes of *C. elegans*. In order to address this question, brood size (reproductive parameter) and motility were tested to check worms' reproductive capacity and general fitness ability under RJ/eRJ supplementation.

In the brood size test, N2 worms were treated with or without RJ/eRJ for two generations, afterwards the total offspring numbers of wild type worms were counted under each treatment. The results showed that the average of total offspring number was 206 per worm in the control group, while the RJ/eRJ treated worms had the similar number when compared with the controls. This brood size result indicated that RJ/eRJ cannot affect worms' total offspring quantity (Table 2).

In the motility test, worms were treated with or without RJ/eRJ supplement for two generations, afterwards ten synchronized young adult worms from each treatment were transferred to non-seeded NGM plates and the body bends per minute were counted to check their moving ability. Our data showed that the average body bends per minute of one worm was 13.9 in the control group, while the RJ/eRJ treated worms had similar body bends times when compared with the controls. This result demonstrated that RJ/eRJ barely affected worms' moving ability (Table 2). Taken together, our results suggested that RJ/eRJ mediated lifespan extension is not associated with any significant changes in reproductive capacity and motility of *C. elegans*.

Table 2. RJ/eRJ Supplementation Does Not Influence Motility and Reproductive Capacity of *Caenorhabditis elegans* at 25°C

	control \pm SE	RJ-Treated \pm SE	eRJ- Treated \pm SE	<i>p</i> value	Total No. of Worms
Brood Size	206 \pm 23.6	194 \pm 18.9	203 \pm 24.7	0.37*, 0.84†	6*, 6†, 6‡
Motility	13.9 \pm 1.0	14.1 \pm 1.5	13.8 \pm 1.2	0.79*, 0.81†	10*, 10†, 10‡

Notes: These data represent the average of three independent trials for each test.

*RJ-treated worms.

†eRJ-treated worms.

‡Control worms.

3.2.3 Supplementation of RJ/eRJ Modulated *C. elegans* Lifespan through the IIS Cascade and DAF-16

Our lifespan assays revealed that RJ/eRJ could extend *C. elegans* lifespan in a dose dependent manner. Next we want to know the molecular mechanism underlying this beneficial effect. It is well known that Insulin like signaling pathway (IIS pathway) is one of the most powerful pathways to regulate cell proliferation, metabolism, and longevity. We questioned whether RJ/eRJ mediated lifespan extension is dependent on the regulation of IIS pathway. First of all, we examined whether DAF-16, an important transcriptional factor of IIS pathway, was involved in the RJ/eRJ mediated anti-aging function. We treated null *daf-16 (mgDf50)* mutant worms with RJ (2mg/mL) and eRJ (1mg/mL) respectively and counted lifespan under 25 °C. Our data showed that the mean lifespan of non-supplemented *daf-16* deletion mutants was 6.85 days, and the mean lifespan of RJ and eRJ treatment was 6.91 days and 6.74 days respectively (Figure 3.3 A and B, and Table 3). This finding suggested that DAF-16 was required in RJ/eRJ mediated lifespan extension effect.

Next, we tested whether DAF-2 and AGE-1, two major components of the IIS pathway, were required in RJ/eRJ mediated lifespan extension.. The mutant worms *daf-2 (e1370)* were treated with RJ (2mg/mL) and eRJ (1mg/mL) respectively and lifespan was counted under 25 °C. Our data showed that the mean lifespan of non-supplemented *daf-2 (e1370)* mutants was 26.68 days, and the mean lifespan of RJ and eRJ treatment was 26.99 days and 27.01 days respectively (Figure 3.3 C and Table 3). Similarly, the mean lifespan of non-supplemented *age-1 (hx546)* mutants was 27.08 days, and the mean

lifespan of RJ and eRJ treatment was 27.31 days and 27.00 days respectively (Figure 3.3 D and Table 3). This result indicated that RJ/eRJ acted, at least in part, through the IIS pathway and DAF-16 to increase lifespan in *C. elegans*.

Considering that the IIS pathway regulates lifespan in *C. elegans* by limiting DAF-16 nuclear trans-localization, we postulated that RJ/eRJ treatment might affect DAF-16 nuclear localization and transcriptional activities. In order to check the DAF-16 nuclear localization, we examined subcellular localization of DAF-16 using transgenic worms overexpressing DAF-16::GFP (*daf-16(mgDf47;xrls87)*), which showed GFP signal in almost all somatic cells. Specifically, the DAF-16::GFP worms were supplemented with RJ/eRJ on NGM plates from the early L1 stage until day 2 adults. Afterwards the DAF-16::GFP signal in live worms (day 2 adults) was observed under a fluorescent microscope (Nikon AZ100). Unfortunately, we did not observed a significant increase of DAF-16-GFP in the nucleus after treatment of either RJ or eRJ (data not shown).

In order to test how DAF-16 is modulated in RJ/eRJ mediated lifespan extension, we chose several DAF-16 target genes to check whether DAF-16 activity is influenced in the RJ/eRJ supplementation treatments. Wild type N2 worms were treated with or without RJ/eRJ for two generations and then the target genes expressions were tested by quantitative PCR methods. The result showed that the mRNA level of DAF-16 was not changed when compared to non-supplemented controls, but its target genes expressions were significantly affected. *sod-3* is an important biomarker of DAF-16, which could

reflect the DAF-16 activity obviously. Moreover, *mtl-1*, *C32H11.4* and *F21F3.3* are DAF-16 target genes, which were selected in this experiment to check DAF-16's transactivity. Based on our quantitative PCR results, the elevated expression of *sod-3*, *mtl-1*, *F21F3.3* and reduced expression of *C32H11.4* showed that DAF-16 activity was up-regulated in the RJ/eRJ supplementary treatments (Figure 3.3 E).

Taken together, these findings suggested that RJ/eRJ treatment prolonged *C. elegans* lifespan, which were dependent on promoting transcriptional activities of DAF-16 rather than improving the accumulation of DAF-16 in nuclei.

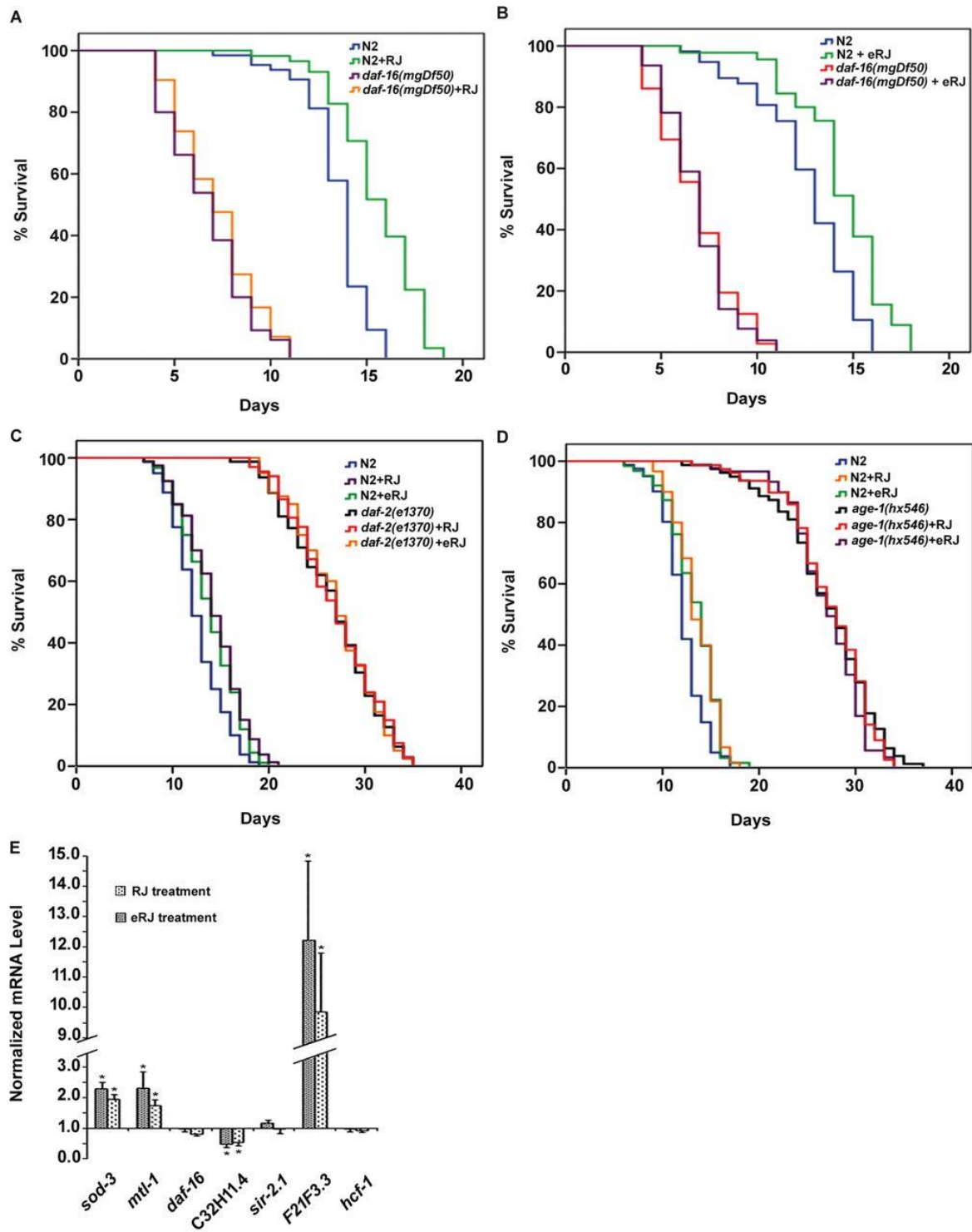


Figure 3.3 Both RJ and eRJ modulate *Caenorhabditis elegans* life span through the IIS pathway and DAF-16. (A) *daf-16(mgDf50)* mutant worms were treated with 2mg/mL RJ. (B) *daf-16 (mgDf50)* mutant worms were treated with 1mg/mL eRJ. (C) *daf-2(e1370)* mutant worms were treated with either 2mg/mL RJ or 1mg/mL eRJ. (D) *age-1(hx546)* mutant worms were treated with either 2mg/mL RJ or 1mg/mL eRJ. Each life-span experiment was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments are included in Table 1. (E) The transcript levels of *daf-16*, *sod-3*, *mtl-1*, *C32H11.4*, *F21F3.3*, *sir-2.1*, and *hcf-1* in N2 worms treated with and without 2mg/mL RJ or 1mg/mL eRJ were quantified using qRT-PCR. The data from three independent experiments were pooled to calculate the mean RNA level normalized to the internal control *act-1*. The standard errors of the mean (SEM) were shown. The normalized mean RNA level of controls in non-treated N2 worms were set to 1. * $p < .05$ compared with non-treated control.

Table 3. The Lifespan Data of Worms Strains at 25 °C

strain	Mean±SE	Median	No. of Worms	<i>p</i> value
N2*	13.48±0.22	14.00	64	
N2+ 2mg/mL RJ*	15.57±0.28	16.00	58	<0.001
<i>daf-16(mgDf50)*</i>	7.21±0.23	7.00	84	
<i>daf-16(mgDf50) + 2mg/mL RJ*</i>	6.74±0.26	7.00	65	0.216
N2**	12.65±0.34	13.00	57	
N2+ 1mg/mL eRJ**	14.40±0.37	15.00	45	<0.001
<i>daf-16(mgDf50)**</i>	6.85±0.23	7.00	72	
<i>daf-16(mgDf50) + 1mg/mL eRJ**</i>	6.91±0.19	7.00	78	0.874
N2†	12.64±0.30	12.00	80	
N2+ 2mg/mL RJ†	14.29±0.35	14.00	80	<0.001
N2+ 1mg/mL eRJ†	13.86±0.31	14.00	92	0.003
<i>daf-2(e1370)‡</i>	26.68±0.52	27.00	79	
<i>daf-2(e1370) + 2mg/mL RJ†</i>	26.99±0.55	27.00	67	0.726
<i>daf-2(e1370) + 1mg/mL eRJ†</i>	27.01±0.48	27.00	80	0.910
N2‡	12.14±0.24	12.00	81	
N2+ 2mg/mL RJ‡	13.53±0.28	14.00	63	<0.001
N2 + 1mg/mL eRJ‡	13.32±0.33	13.00	60	0.001
<i>age-1(hx546)‡</i>	27.08±0.56	27.00	79	
<i>age-1(hx546) + 2mg/mL RJ‡</i>	27.31±0.50	27.00	89	0.672
<i>age-1(hx546) + 1mg/mL eRJ‡</i>	27.00±0.42	27.00	78	0.179

Notes: Life span and standard error are shown in days. The life-span experiments were repeated at least three times with similar results, and the data for representative experiments were shown. The life-span data were analyzed using the log-rank test and *p* values for each individual experiment were shown.

*Results presented in Figure 3.3 A.

** Results presented in Figure 3.3 B.

†Results presented in Figure 3.3 C.

‡Results presented in Figure 3.3 D.

3.2.4 RJ/eRJ Mediated Lifespan Extension is Modulated by SIR-2.1, and HCF-1, as Well as FTT-2.

As an important transcriptional factor, DAF-16 is tightly controlled by several co-factors[73]. Next, we wondered whether DAF-16's co-factors are involved in this RJ/eRJ mediated lifespan extension. Previous studies reported that SIR-2.1, HCF-1, and 14-3-3 are important DAF-16 co-factors to modulate its transactivity. For instance, SIR-2.1 physically associates with DAF-16 via FTT-2, a 14-3-3 protein of *C. elegans*, to promote the transactivation of DAF-16 under heat shock stress. Moreover, genetic and biochemical studies demonstrated that, associated with 14-3-3 proteins, HCF-1 and SIR-2.1/SIRT1 physically interacted and antagonized each other to fine-tune target gene expressions DAF-16/FOXO and, in turn, to promote longevity and stress resistance[48, 62, 153].

First of all, we investigated whether SIR-2.1 was required in RJ/eRJ mediated lifespan extension. Deletion mutant worms *sir-2.1 (ok434)* were treated with RJ/eRJ to carry out lifespan assays. Our results showed that the mean lifespan of RJ/eRJ treatments were 12.82 days and 12.74 days. As compared to the mean lifespan of non-supplemented controls (12.82 days), we found out that RJ/eRJ cannot extend *sir-2.1 (ok434)* mutants lifespan. This result suggested that SIR-2.1 was required in RJ/eRJ mediated lifespan extension (Figure 3.4 A and B and Table 4).

Next, we tested whether RJ/eRJ modulated *C. elegans* lifespan dependent on another two factors: 14-3-3 and HCF-1. Deletion mutant worms *ftt-2 (n4426)* and *hcf-1 (ok559)* were treated with 2mg/mL RJ or 1mg/mL eRJ, and lifespan assays were carried

out under 25 °C. For the *fit-2* (*n4426*) mutant worms, the mean lifespan of RJ/eRJ treatments were 11.57 days and 11.51 days. As compared to mean lifespan of genotype matched non-supplemented controls (11.67 days), we found out that RJ/eRJ cannot extend *fit-2* (*n4426*) mutants lifespan (Figure 3.4 C and D and Table 4). Similarly, RJ/eRJ supplementation failed to extend *hcf-1* (*ok559*) mutant worms' lifespan. Our data showed that the mean lifespan of RJ/eRJ treatments were 15.79 days and 15.75 days, while the mean lifespan of non-supplemented controls were nearly the same (15.94 days). These results indicated that besides SIR-2.1, HCF-1, and 14-3-3 were also required in RJ/eRJ mediated lifespan extension (Figure 3.4 E and F and Table 4).

Considering that either overexpression of SIR-2.1 or attenuation of HCF-1 can extend *C. elegans* life span, we wondered whether RJ/eRJ supplementation extended lifespan by altering the expression of SIR-2.1 and HCF-1. To address this concern, we employed qPCR to measure the expression levels of *sir-2.1* and *hcf-1* with or without RJ/eRJ supplementation. Our data showed that the mRNA levels of both *sir-2.1* and *hcf-1* were not significantly altered when compared with controls (Figure 3.3 E). Overall, our findings suggested that RJ/eRJ modulated lifespan dependent, at least in part, on the sophisticated interplays of DAF-16, SIR-2.1, HCF-1, and 14-3-3 proteins.

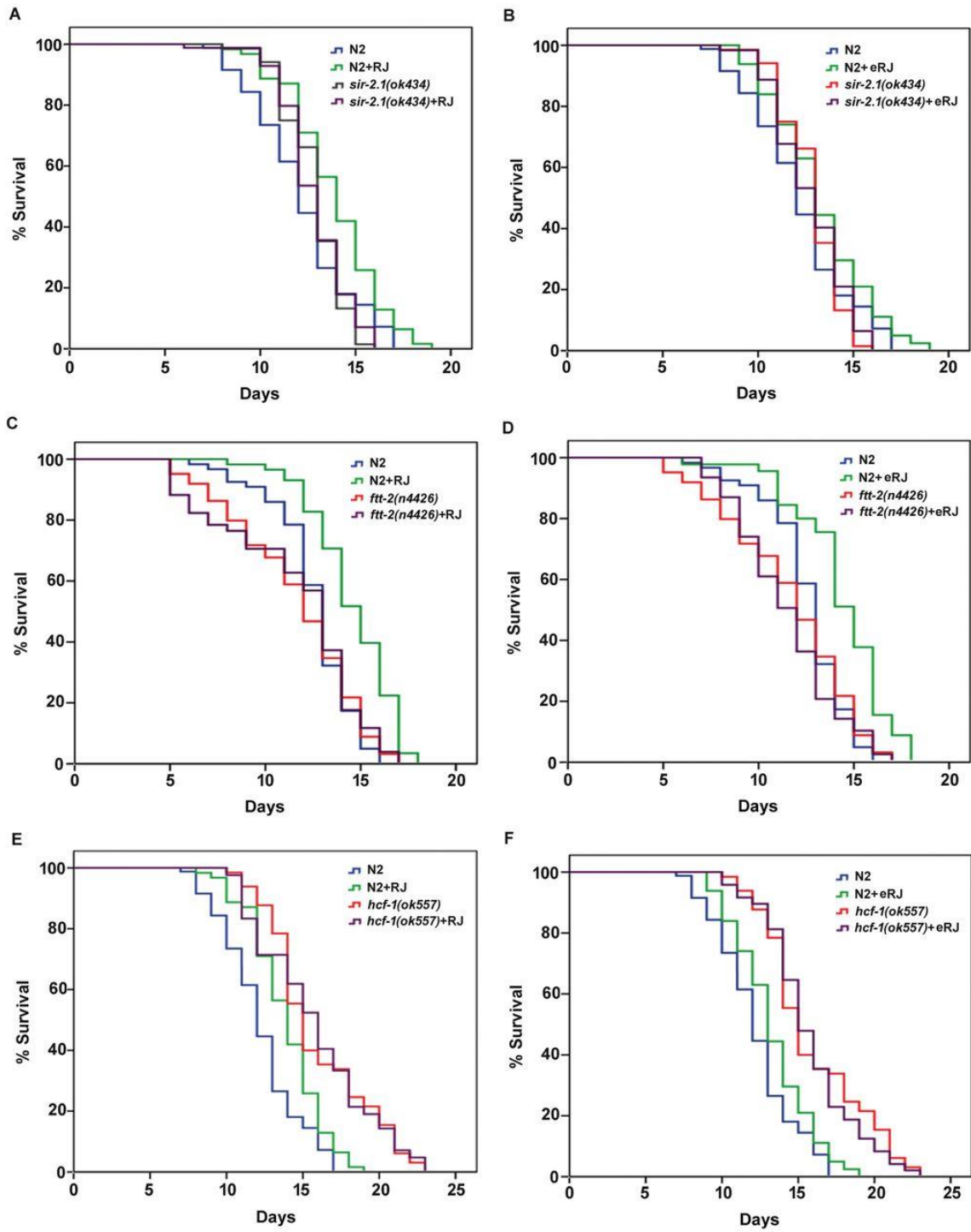


Figure 3.4 The RJ/eRJ requires SIR-2.1, HCF-1, and FTT-2 to prolong *Caenorhabditis elegans* lifespan. (A) The *sir-2.1 (ok434)* mutant worms were treated with 2mg/mL RJ. (B) The *sir-2.1(ok434)* mutant worms were treated with 1mg/mL eRJ. (C) The *ftt-2 (n4426)* mutant worms were treated with 2mg/mL RJ. (D) The *ftt-2(n4426)* mutant worms were treated with 1mg/mL eRJ. (E) The *hcf-1 (ok559)* mutant worms were treated with 2mg/mL RJ. (F) The *hcf-1 (ok559)* mutant worms were treated with 1mg/mL eRJ. Each life-span experiment shown here was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments are included in Table 4.

Table 4. The Lifespan Data of Worms Strains at 25 °C

strain	Mean \pm SE	Median	No. of Worms	<i>p</i> value
N2*	12.21 \pm 0.28	12.00	86	
N2 + 2mg/mL RJ*	13.87 \pm 0.30	14.00	62	0.001
N2 + 1mg/mL eRJ*	13.28 \pm 0.28	13.00	81	0.015
<i>sir-2.1 (ok434)</i> *	12.82 \pm 0.19	13.00	68	
<i>sir-2.1 (ok434)</i> + 2mg/mL RJ*	12.82 \pm 0.19	13.00	84	0.690
<i>sir-2.1 (ok434)</i> + 1mg/mL eRJ*	12.74 \pm 0.24	13.00	62	0.630
N2†	12.56 \pm 0.20	13.00	121	
N2 + 2mg/mL RJ†	14.57 \pm 0.28	15.00	58	<0.001
N2 + 1mg/mL eRJ†	14.40 \pm 0.37	15.00	55	<0.001
<i>ftt-2 (n4426)</i> †	11.67 \pm 0.29	12.00	124	
<i>ftt-2 (n4426)</i> + 2mg/mL RJ†	11.57 \pm 0.52	13.00	51	0.795
<i>ftt-2 (n4426)</i> + 1mg/mL eRJ†	11.51 \pm 0.30	12.00	77	0.318
N2‡	12.21 \pm 0.28	12.00	86	
N2 + 2mg/mL RJ‡	13.87 \pm 0.30	14.00	62	0.001
N2 + 1mg/mL eRJ‡	13.28 \pm 0.28	13.00	81	0.015
<i>hcf-1 (ok559)</i> ‡	15.94 \pm 0.42	15.00	65	
<i>hcf-1 (ok559)</i> + 2mg/mL RJ‡	15.79 \pm 0.57	16.00	52	0.977
<i>hcf-1 (ok559)</i> + 1mg/mL eRJ‡	15.75 \pm 0.43	15.00	48	0.629

Notes: Life span and standard error were shown in days. The lifespan experiments were repeated at least three times with similar results, and the data for representative experiments were shown. The lifespan data were analyzed using the log-rank test and *p* values for each individual experiment were shown.

*Results presented in Figure 3.4 A and B.

†Results presented in Figure 3.3 C and D.

‡Results presented in Figure 3.4 E and F.

3.3 Discussion

Aging causes the physiological decline and increases the risk of disease, which brings a challenging question to society and encourages scientists to look for efficient methods to slow down the development of age related disorders. Accumulation evidences suggested that nutraceuticals, such as cranberry, cocoa, green tea, and herbal medicines, provide a variety of health benefits to relay the aging process[91]. Several research labs published that Royal Jelly, a bee product, has the lifespan extension function in *C. elegans* model and alleviates type II diabetes disease in murine model. However, the detailed molecular mechanism of Royal Jelly's anti-aging effects remains elusive[103].

In this study, we utilized *C. elegans* as a model to investigate Royal Jelly's anti-aging function, we observed that regular Royal Jelly (RJ) and enzyme treatment Royal Jelly (eRJ) could extend *C. elegans* lifespan in a dose dependent manner. Moreover, RJ/eRJ did not affect *C. elegans* normal physiological traits, such as reproductive ability and motility. Our genetic analysis indicated that Insulin like Signaling (IIS) pathway and its important component DAF-16 were required in RJ/eRJ mediated lifespan extension. Meanwhile, three DAF-16 co-factors, SIR-2.1, HCF-1, and 14-3-3, were also involved in this beneficial effect. Our studies proposed a genetic model to elucidate the molecular basis of RJ/eRJ's pro-longevity function. Under the supplementation of RJ/eRJ, SIR-2.1, HCF-1, and 14-3-3 are activated to fine-tune DAF-16 activity, and the sophisticated interplay of these four proteins could regulate the expression of DAF-16 target genes to promote animals' health aging.

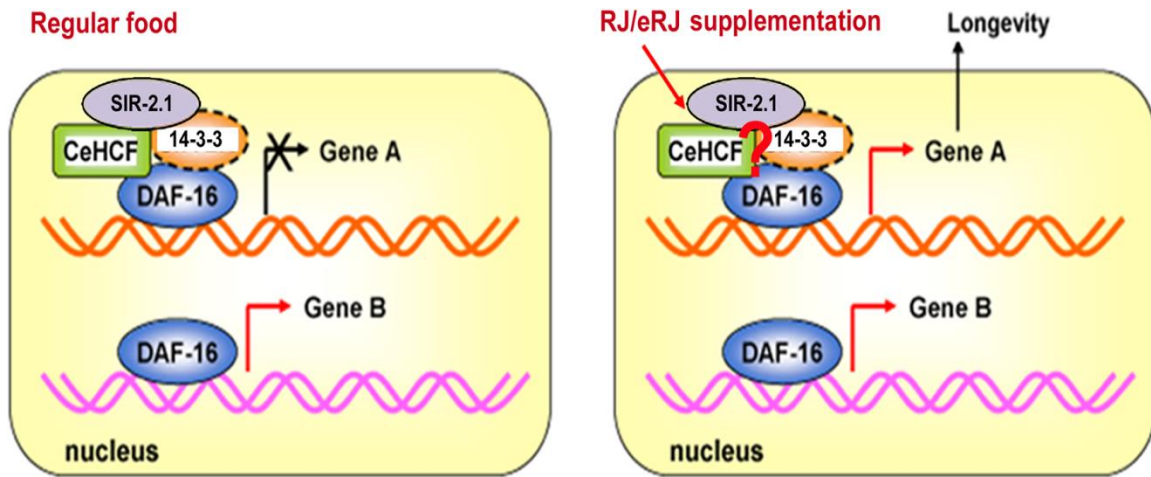


Figure 3.5 RJ/eRJ depends on the interplay of DAF-16, SIR-2.1, HCF-1 and 14-3-3 to modulate *C. elegans* lifespan. *C. elegans* lead a regular lifespan when treated with regular food (*E. coli* OP50), however, RJ/eRJ supplementation may trigger the regulatory interplays of DAF-16, SIR-2.1, HCF-1, and 14-3-3 to fine-tune DAF-16 activity in the nucleus autonomously. This sophisticated modulation induces a series of DAF-16 target genes expressions to extend *C. elegans* lifespan.

At the beginning, wild type worms were treated with different concentrations of RJ/eRJ, ranging from 0 to 5 mg/mL, to select an optimum concentration for lifespan extension by comparing their mean lifespans. The lowest concentration (0.4 mg/mL) and highest concentration (5mg/mL) barely influenced mean lifespan of wild type worms, but the concentration of 1 to 3 mg/mL RJ/eRJ extended *C. elegans* lifespan significantly. Our results indicated that RJ/eRJ extended *C. elegans* lifespan in a dose dependent manner. In consideration that Royal Jelly is composed of 67% water, 12.5% crude protein, 11% simple sugars, and a relatively high amount (5%) of fatty acids[154], it is conceivable that the effective pro-longevity factors are included in proteins and fatty acids parts. Therefore the lower concentration (0.4 mg/mL) does not contain enough functional proteins to prolong worms' mean lifespan. In contrast, even the 5mg/mL Royal Jelly includes high concentration of proteins and fatty acid, the results showed that the 5mg/mL Royal Jelly shortened worms' lifespan. It is well known that many natural substances used for therapeutic benefit have the potential to have their benefits eclipsed by toxic effects at high doses[155]. Moreover, it is reported that high concentration of sugar could shorten worms' lifespan. In agreement with this, we realized that the 5 mg/mL RJ/eRJ contains nearly 1mg/mL sugar, which might be the reason that higher concentration of RJ/eRJ cannot prolong the lifespan.

Next, we wondered whether there is any difference of anti-aging effect between the two types of Royal Jelly powders in our research. The manufacturing process of the two powders are almost the same: after the filtration, disinfection, lyophilization, and pulverization steps, fresh Royal Jelly is processed to powder, which makes Royal Jelly

easy to be stored and utilized in experiments. The only difference between the two types of Royal Jelly is the proteases treatment, which decomposes large proteins into amino acids and peptides. The aim of this enzyme treatment is to decrease the large-proteins induced allergic properties but still retain bioactive functions. In order to prepare the RJ/eRJ paltes, the RJ/eRJ powders were dissolved in sterile distilled water and vortex vigorously to dissolve adequately. Noticeably, a lot of floating bubbles were observed on the surface of RJ powder mixture, but this phenomenon was not observed in eRJ mixture, which indicated that the composition of RJ and eRJ were different and the bubbles in RJ might be cause by certain large proteins. Intriguingly, our lifespan results showed that both RJ and eRJ increased *C. elegans* lifespan, but the optimum concentrations of RJ and eRJ were different (2mg/mL RJ and 1mg/mL eRJ, respectively) (figure 3.1 and table 1). This finding suggested that the enzyme digestion might decrease the toxicity of certain RJ and, in turn, alter bioactivity and improve the immune system of *C. elegans*. As a result, the optimized concentration of eRJ for lifespan extension is reduced to 1mg/mL, only half of RJ concentration. However, there is also a problem cannot be ignored, although the enzyme treatment decreased the large-proteins induced allergic response in eRJ supplementation, the mean lifespan of 2mg/ml RJ (15.52 days) was 1.08 days longer than 1mg/mL eRJ treatment(14.44 days). This data demonstrated that certain large RJ proteins, which were deleted by protease in eRJ powder, might have the beneficial effects in anti-aging function. Further experiments need to be conducted to investigate the differences between RJ and eRJ's pro-longevity effects.

Overall, our lifespan results indicated that RJ/eRJ extended *C. elegans* lifespan in a dose dependent manner, and the optimum concentrations of lifespan extension were determined to be: 2mg/mL RJ and 1mg/mL eRJ, which were used in the further experiments to investigate the molecular mechanism underlying this beneficial effect. Moreover, RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) did not affect worms' physiological indexes, such as reproductive capacity and mobility, which indicated that RJ/eRJ was safe to be consumed as a functional product. Therefore, it is possible that RJ/eRJ might be a beneficial nutraceutical for human consumption because of its lifespan extension function and safety.

Given that RJ/eRJ extended *C. elegans* lifespan, we further investigated the molecular mechanism underlying this beneficial effect. It is well known that Insulin Like Signaling pathway (IIS) is a master regulator of longevity, and DAF-16 was an important component at downstream of IIS pathway. Honda's research group published that IIS/DAF-16 was required in Royal Jelly's anti-aging function. In agreement with their study, our genetic results also showed that IIS/DAF-16 was critical in RJ/eRJ's anti-aging function, because both RJ/eRJ cannot extend the mean lifespan of *daf-2* deletion mutants, *age-1* deletion mutants and *daf-16* deletion mutants' lifespan respectively (Figure 3.3). As an important transcriptional factor of IIS, DAF-16 plays a key role in regulating proliferation, metabolism, and longevity. Moreover, DAF-16's activity is known to be regulated by post-translational modifications, nuclear/cytoplasmic translocation and is also associated with transcriptional co-factors[156]. We further investigated whether RJ/eRJ mediated lifespan extension was dependent on modulating the activity of DAF-16.

Previous studies have demonstrated that reduction of IIS activates DAF-16 by increasing its nuclear translocation to extend lifespan. Our genetic results suggested that RJ/eRJ mediated lifespan extension, at least in part, acted through IIS pathway. This finding indicated that under the supplementation of RJ/eRJ, increased amount of DAF-16 might translocate into the nuclear. Unfortunately, we did not observe the fluorescence signal in RJ/eRJ treated DAF-16:GFP worms, indicating that RJ/eRJ barely affected DAF-16 nuclear translocation. There are two possibilities to explain this result. First, it has been reported that many obvious DAF-16 nuclear translocations are caused by severe stresses or the alteration of crucial genes alteration. In comparison, Royal Jelly is a functional food and induces mild changes in *C. elegans* growth process, which are similar to a normal physiological level. As a result, this moderation increases of DAF-16 nucleus translocation is not strong enough to be observed under regular fluorescence microscopy. Another possibility is RJ/eRJ increases the transactivity of DAF-16 rather than promoting DAF-16's nucleus translocation. It is well known that DAF-16 nucleus translocation is very important for DAF-16 activation, but translocation of DAF-16 into nucleus is not sufficient to stimulate its transcriptional activity. Therefore our hypothesis is that although RJ/eRJ did not elevate DAF-16's nuclear translocation amount, RJ/eRJ still increased DAF-16 activity to modulate the expression of its target genes. Indeed, the changes of DAF-16 target genes expressions are much more sensitive to be detected by using quantitative PCR method. . Our quantitative PCR experiments showed that RJ/eRJ did not change DAF-16 gene expression, but significantly influenced the expression of its target genes (*sod-3*, *mtl-1*, F21F3.3 and C32H11.4). Specifically, the expression of *sod-3*

and *mtl-1* (two important biomarkers of DAF-16) were increased more than two folds in RJ/eRJ treated worms. This result indicated that DAF-16 activity was up-regulated under RJ/eRJ supplementation. Although the increased DAF-16 translocation is not detected in our research, the DAF-16 target gene expression results confirmed that RJ/eRJ anti-aging function acts through increasing the DAF-16 activity (Figure 3.3 E).

DAF-16/FOXO has been reported to play an essential role in regulating cell proliferation and maintaining the energy homeostasis in organisms. DAF-16 activity is tightly controlled by genes and pathways, the regulatory specificity and precision of DAF-16 is critical to ensure several biological processes. It is reported that dysregulation of FOXO1 could cause a series of diseases, such as hyperglycemia and glucose intolerance[157]. As an important transcription factor, DAF-16 functions to regulate many important biological processes by modulating expressions of many genes, and the regulation of DAF-16's activity depends on the balance of a complicated interplay among several co-factors. It is reported that SIR-2.1, HCF-1 and 14-3-3, three important DAF-16 co-factors, have physical interactions with DAF-16 to modulate its activity. Berdichevsky and his colleagues reported that under heat shock stress, 14-3-3 functioned as a bridging protein to link SIR-2.1 with DAF-16, this interplay induced the expression of DAF-16 target genes and thereby extended the survival of *C. elegans* under stress[48]. Another important co-factor is HCF-1, which plays a key role in transcriptional processes and cell cycle control. Li and colleagues reported that HCF-1 is also a putative longevity determinant factor to modulate DAF-16 activity, independently from IIS. Their findings revealed that the reduction of HCF-1 increased DAF-16 activity to extend lifespan[49].

Moreover, their follow-up research suggested under the reduction of IIS, HCF-1, SIR-2.1, and 14-3-3 proteins might modulate DAF-16 activity in the nucleus to respond to aging and stress[65]. In our study, the results provided more evidence to confirm their results and underscored the significance of these four proteins regulatory interplays in dietary aging intervention. Our genetic results showed that besides IIS/DAF-116, DAF-16 co-factors- SIR-2.1, 14-3-3, and HCF-1- were also required to modulate DAF-16 activity in RJ/eRJ mediated aging intervention. Additionally, the quantitative PCR results showed that RJ/eRJ did not affect the expression of *sir-2.1* and *hcf-1* genes. In considering that overexpression of SIR-2.1 and down-regulation of HCF-1 could increase the *C. elegans* lifespan respectively, our results indicated that RJ/eRJ mediated pro-longevity effects were dependent on promoting the interplays of SIR-2.1, HCF-1, and DAF-16 to modulate the transactivity of DAF-16 rather than influencing the expression of *hcf-1* and *sir-2.1*. Moreover, the expression of *C32H11.4* and *F21F3.3* (target genes of both HCF-1 and DAF-16[49]) in *C. elegans* were changed by RJ/eRJ supplementation, which further confirmed our genetic data that DAF-16 and its co-factors were required in RJ/eRJ mediated aging intervention.

Numerous studies have demonstrated that Royal Jelly is a functional nutraceutical and has many beneficial effects, such as anti-fatigue effects, stimulates bone formation, regulates immune responses, and protects liver from virus induced damage in animal models[99-101, 158] In honey bees, genetically identical honey bee larvae develop into either worker bees or queen bees depending on exposure to Royal Jelly. Based on the composition of Royal Jelly, nearly half dry mass of Royal Jelly is protein, which might

be the most powerful regulator to determine a honey bee's fate and lifespan. It is possible that Royal Jelly function proteins might be beneficial to other animals through food intake. Our research highlights the pro-longevity properties of RJ and reveals the molecular mechanism of Royal Jelly's beneficial effects, which might also be applied to mammal systems, including humans. It is conceivable that Royal Jelly supplementation functions as aging-intervention method for humans to promote healthy aging and delay the age-related disorders, further clinical research remains to be conducted to confirm our hypothesis.

It is well known that IIS pathway, DAF-16/FOXOs, SIR-2.1/SIRT1, HCF-1 and 14-3-3 are evolutionarily conserved from *C. elegans* to mammals, our RJ/eRJ supplementary *C. elegans* model might be implicated mammal models to further study the genetic network of aging. In addition to SIR-2.1, HCF-1 and 14-3-3, other DAF-16 co-factors might also involve in the RJ/eRJ mediated pro-longevity function, for example, SMK-1, BAR-1, et al[47, 159]. Moreover, besides IIS pathway, other pathways might also be required in Royal Jelly mediated beneficial effects, such as JNK pathway, TOR pathway, and P38 MAPK pathway [160-162]. Further research will be carried out in the future to help us better understand the detailed mechanism of aging process.

CHAPTER FOUR : THE ANTI-STRESS CAPABILITY OF ROYAL JELLY SUPPLEMENTATION

4.1 Introduction

In Chapter three, our results demonstrated that RJ/eRJ extended *C. elegans* lifespan without affecting the physiological indexes. This beneficial effect acted through IIS/DAF-16 and its three important co-factors, SIR-2.1, HCF-1, and 14-3-3, which are essential proteins to regulate metabolism, proliferation, and longevity in *C. elegans*. Cumulative evidence indicated that the lifespan extension benefits are often correlated with increased stress resistant capability[163, 164]. Interestingly, our quantitative PCR results showed that the supplementation of Royal Jelly could affect the expression levels of certain stress response genes, such as *sod-3*, *mtl-1*, *C32H11.4*, and *F21F3.3*. Moreover, DAF-16 regulates numerous stress response genes, such as anti-oxidant and protein turnover genes, to maintain the protein homeostasis in cells. In addition to DAF-16, its three co-factors, SIR-2.1, HCF-1, and 14-3-3 are also reported to be involved in stress response and adaptations. It is highly possible that RJ/eRJ supplementation might also have anti-stress functions. In order to confirm this hypothesis, stress assays were conducted to investigate whether RJ/eRJ could protect *C. elegans* from a variety of stressors, including oxidative stress, UV irradiation, and heat shock stress.

Environmental stresses and normal cell metabolism could form Reactive Oxygen Species (ROS), which may result in significant damage to cell components[165]. In response to this damage, cells are equipped with internal protective systems, such as antioxidant molecules, DNA repair pathways, and chaperones, to repair the destructed

DNA and proteins to maintain cellular homeostasis[166]. However, under persisting stress conditions or in the aging cells, the oxidative damages are accumulated, and the cells' repair capacity is declined, which in turn increases cellular proteotoxicity to accelerate the aging process and cause several diseases, such as neurodegenerative diseases, cancer, and premature aging[149].

Several labs have demonstrated that some nutraceuticals have pro-longevity effect as well as stress resistance capacity. For example, resveratrol could affect SIR-2.1/SIRT1's activity to extend lifespan and increase resistance to oxidative stress and UV irradiation in animal models[96]. Similarly, cranberry influenced Insulin Signaling Pathway to protect *C. elegans* from thermal stress and slow down the aging process[123]. Our research revealed that RJ/eRJ also had the anti-stress functions to protect *C. elegans* from oxidative stress, UV irradiation and heat shock. Moreover, this stress resistance beneficial effect was dependent on, at least in part, the regulation of DAF-16, SIR-2.1, HCF-1, and 14-3-3.

4.2 Results

4.2.1 Supplementation of RJ/eRJ promotes *C. elegans* stress resistance to oxidative stress, UV irradiation, and heat shock stress

Extended longevity is often genetically correlated with increased resistance against various stressors, including oxidative stress, heat shock, UV irradiation, osmosis stress, and pathogen infection. In chapter three, we demonstrated that RJ/eRJ supplementation had lifespan extension benefit. Herein, we want to investigate whether RJ/eRJ supplementation could protect worms against stresses.

First of all, oxidative stress was tested to investigate whether RJ/eRJ could protect *C. elegans* from oxidative damages. Wild type worms (N2) were treated with or without RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) for two generations (5 days) under 25 °C. Then the synchronized L4/young adult worms were transferred to NGM plates containing 5mM paraquat to observe survival. Basically, paraquat is an organic compound which can produces superoxide anions to induce redox activity. In our research, paraquat was used to create oxidative stress environment. At the same time, N2 worms treated with or without RJ/eRJ on regular NGM plates (no paraquat) were set as a system control.

Based on our observation, on the second day, the survival rate of controls was lower than 80%, whereas nearly 98% of RJ/eRJ treated worms were still alive. On the fourth day, more than 50% of control worms were already dead, but the RJ/eRJ treated worms' survival rate was more than 80%. Surprisingly, on the sixth day, the survival rate

of RJ/eRJ treatment was more than two times longer than the survival rate of the controls. This experiment was continued until day 10, and the data was analyzed to compare the differences between each treatment (Figure 4.1 A and B). This result indicated that RJ/eRJ could protect *C. elegans* from oxidative stress.

Next, UV irradiation stress was tested to study whether RJ/eRJ could protect *C. elegans* from UV-light induced DNA damage. *C. elegans* were treated with or without RJ/eRJ for two generations before exposure under UV irradiation at $0.05\text{J}/\text{cm}^2$ for 20 seconds. After treated with UV irradiation, *C. elegans* were cultured at $25\text{ }^{\circ}\text{C}$ to observe survival for 10 days. Meanwhile, N2 worms treated with or without RJ/eRJ on regular NGM lifespan plates (no UV irradiation) were set up as a system control to check whether the whole system works well. The UV irradiation result showed that on the second day, the survival rate of control group was lower than 80%, whereas nearly 98% RJ/eRJ treated worms were still alive. On the fourth day, the survival rate of control group dropped to nearly 50%; by contrast, the RJ/eRJ treated worms survival rate was still 80%. Noticeably, on the sixth day, only less than 20% of control worms were still alive, and the RJ/eRJ treated worms survival rate was around two to three times higher when compared with the rate of the control group (Figure 4.1 C and D). This result suggested that RJ/eRJ supplementation increased *C. elegans* UV irradiation resistance.

Heat shock stress was tested to investigate whether RJ/eRJ could protect *C. elegans* from thermal-induced proteins damage. Worms were treated with or without RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) for two generations at $25\text{ }^{\circ}\text{C}$ (5 days).

Then the synchronized L4/young adult worms were heat shocked for three hours at 35 °C and returned back to 25 °C. Survivals were observed for ten days to compare the difference of each treatment. Meanwhile, N2 worms treated with or without RJ/eRJ on NGM lifespan plates (no heat shock) were set up as a system control. On the second day, more than 70% of the heat shocked control worms were dead, but the RJ/eRJ worms' survival rates were more than 80% (in RJ treatment heat shocked) and 60% (in eRJ treatment heat shocked). On the fourth day, the survival rate of heat shocked control worms was less than 30%, while the survivals of RJ/eRJ treated worms were nearly two to three fold higher comparing with that of the control group. Interestingly, the results of day 6, 8 and 10 showed the same trend that RJ/eRJ treated worms survival percentages were higher than those of the control group (Figure 4.1 E and F). This result demonstrated that RJ/eRJ protected *C. elegans* against heat shock stress.

Taken together, our anti-stress assays indicated that RJ/eRJ supplementation protected *C. elegans* against oxidative stress, UV irradiation, and heat shock stress.

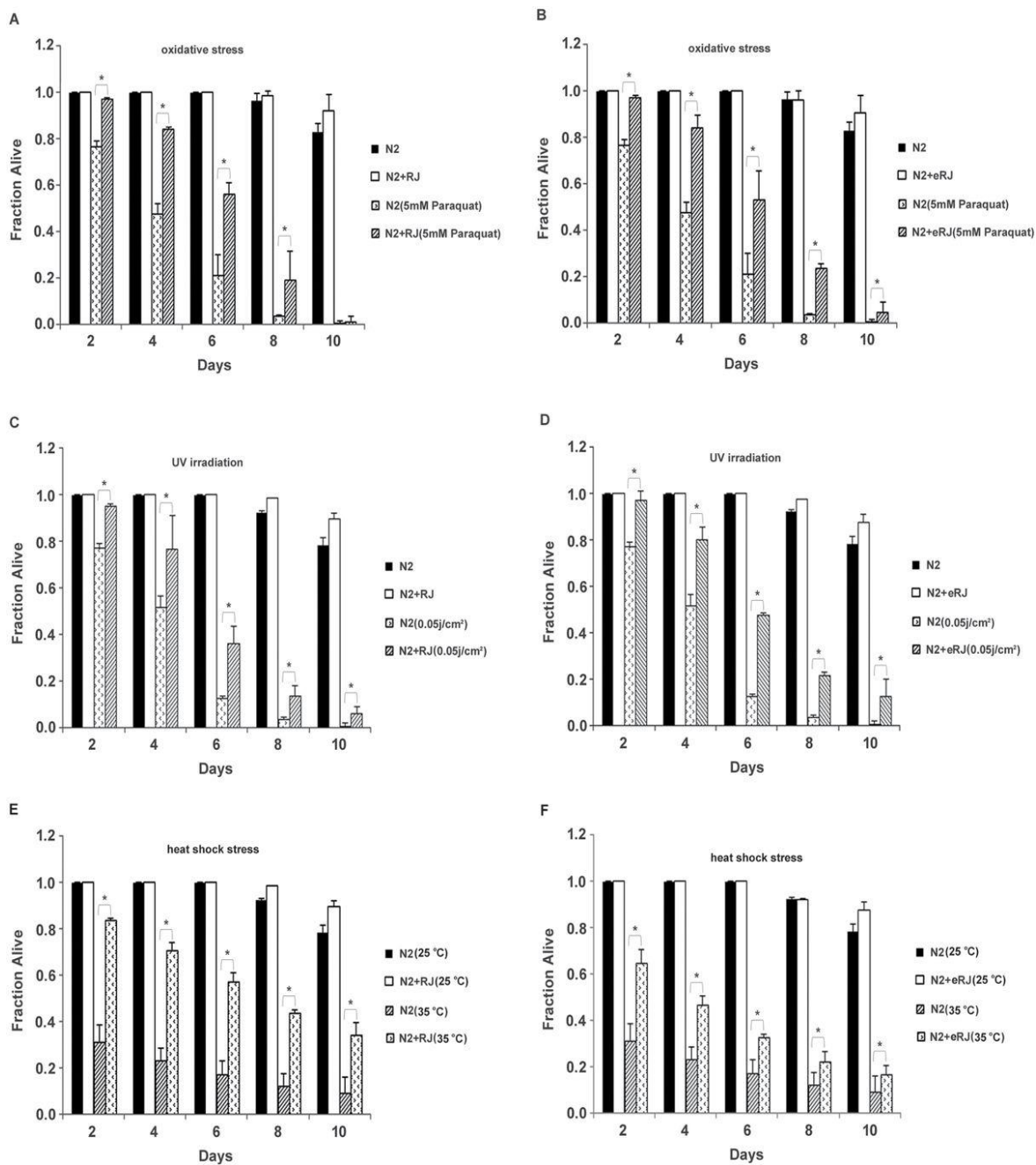


Figure 4.1 The RJ/eRJ treatment results in a promoted stress resistance to specific environmental stimuli. (A) The RJ (2mg/mL) treated N2 worms showed prolonged survival in response to 5mM paraquat compared with non-treated worms. (B) The eRJ (1mg/mL) treated N2 worms showed prolonged survival in response to 5mM paraquat compared with non-treated worms. (C) The RJ (2mg/mL) treated N2 worms showed elevated survival after ultraviolet (UV) irradiation compared with non-treated worms. (D) The eRJ (1mg/mL) treated N2 worms showed elevated survival after UV irradiation compared with non-treated worms. (E) The RJ (2mg/mL) treated N2 worms exhibited increased survival after 3 hours at 35 °C compared with non-treated worms. (F) The eRJ (1mg/mL) treated N2 worms exhibited increased survival after 3 hours at 35 °C compared with non-treated worms. Each stress assay was repeated at least three times. “Fraction alive” indicates the average survival among the multi-replicates and error bars represent the standard deviation. *p* value was calculated using Student’s *t* test. **p* < .05 compared with corresponding control. Each of the stress assays was repeated at least three independent times with similar results.

4.2.2 RJ/eRJ may promote stress resistance of *C. elegans* through, at least in part, modulating the interplays of DAF-16, SIR-2.1, HCF-1, and 14-3-3 proteins

Given that the molecular mechanisms behind lifespan elongation and stress resistance may not overlap, we questioned whether this RJ/eRJ mediated stress tolerance may also require, at least partially, the interplays of DAF-16, SIR-2.1, HCF-1 and 14-3-3 proteins.

In this study, we treated the four mutant worms, *daf-16* (*mgDf50*), *sir-2.1* (*ok434*), *hcf-1* (*ok599*), and *ftt-2* (*n4426*) with or without RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) for two generations. Then mutant worms were exposed to oxidative stress, UV irradiation, and heat shock stress to observe their stress resistant capability. The differences in survival rates were compared between RJ and eRJ treatments with controls in each stress response to analyze whether RJ/eRJ mediated stress responses share the same genetic mechanism with its pro-longevity function.

In the oxidative stress experiment, RJ/eRJ cannot protect four mutant worms under paraquat treatment, which indicated that DAF-16, SIR-2.1, HCF-1, and 14-3-3 were required in RJ/eRJ mediated anti-oxidative stress response. Interestingly, it is worth noting that the *p* value of RJ/eRJ treatment versus control group in *daf-16* (*mgDf50*) and *hcf-1* (*ok599*) were just beyond the statistical significance ($p < 0.05$), but very close to it (Table 5). These data indicated that, besides DAF-16, other transcriptional factors might also be required in RJ/eRJ mediated anti-oxidative stress.

In the UV irradiation stress assays, our data showed that RJ/eRJ cannot protect *daf-16* (*mgDf50*) and *sir-2.1* (*ok434*) mutant worms against UV irradiation, but RJ/eRJ can still increase the survival rate of *hcf-1* (*ok599*) and *ftt-2* (*n4426*) mutant worms (Table 5), which indicated that RJ/eRJ required DAF-16 and SIR-2.1, but not HCF-1 and 14-3-3 to protect *C. elegans* against UV irradiation stress.

In the heat shock stress assays, our results showed that DAF-16, SIR-2.1 and 14-3-3 were involved in RJ/eRJ mediated anti-heat shock stress. However, HCF-1 was not required because RJ/eRJ can still protect *hcf-1* (*ok599*) mutant worms from heat shock stress. Intriguingly, there is a problem cannot be ignored, the *p* values of RJ/eRJ treatment versus non-treatment controls in *daf-16* (*mgDf50*) and *sir-2.1* (*ok434*) mutant worms were just beyond the statistical significance ($p < 0.05$), but very close to it (Table 5). These data also implied that besides DAF-16, other regulators might also be required in RJ/eRJ mediated heat shock stress responses.

Overall, our findings suggested that RJ/eRJ may promote stress resistance of *C. elegans* through, at least partially, modulating the interplays of DAF-16, SIR-2.1, HCF-1, and 14-3-3 proteins.

Table 5. The Survival Time of Various Worm Strains under Stresses

	strain	Mean \pm SE	Median	No.of worms	p value
Oxidative Stress	<i>daf-16 (mgDf50)</i>	3.10 \pm 0.16	3.00	73	
	<i>daf-16 (mgDf50)</i> + 2mg/mL RJ	3.43 \pm 0.19	3.00	72	0.050
	<i>daf-16 (mgDf50)</i> + 1mg/mL eRJ	3.40 \pm 0.18	3.00	75	0.051
	<i>sir-2.1 (ok434)</i>	3.73 \pm 0.22	4.00	70	
	<i>sir-2.1 (ok434)</i> + 2mg/mL RJ	3.96 \pm 0.24	4.00	74	0.471
	<i>sir-2.1 (ok434)</i> + 1mg/mL eRJ	3.72 \pm 0.21	4.00	75	0.927
	<i>hcf-1(ok559)</i>	5.95 \pm 0.29	6.00	83	
	<i>hcf-1(ok559)</i> + 2mg/mL RJ	6.92 \pm 0.31	7.00	76	0.055
	<i>hcf-1(ok559)</i> + 1mg/mL eRJ	6.93 \pm 0.29	7.00	75	0.057
	<i>ftt-2 (n4426)</i>	3.77 \pm 0.24	4.00	68	
	<i>ftt-2 (n4426)</i> + 2mg/mL RJ	4.00 \pm 0.24	4.00	84	0.399
	<i>ftt-2 (n4426)</i> + 1mg/mL eRJ	4.25 \pm 0.26	4.00	73	0.186
	<i>daf-16 (mgDf50)</i>	2.94 \pm 0.66	3.00	63	
	<i>daf-16 (mgDf50)</i> + 2mg/mL RJ	3.17 \pm 0.08	3.00	86	0.177
	<i>daf-16 (mgDf50)</i> + 1mg/mL eRJ	3.18 \pm 0.09	3.00	76	0.181
Ultraviolet Irradiation	<i>sir-2.1 (ok434)</i>	3.57 \pm 0.11	4.00	74	
	<i>sir-2.1 (ok434)</i> + 2mg/mL RJ	3.67 \pm 0.11	4.00	78	0.474
	<i>sir-2.1 (ok434)</i> + 1mg/mL eRJ	3.77 \pm 0.13	4.00	74	0.138
	<i>hcf-1(ok559)</i>	5.07 \pm 0.29	5.00	86	
	<i>hcf-1(ok559)</i> + 2mg/mL RJ	6.28 \pm 0.28	6.00	104	0.012
	<i>hcf-1(ok559)</i> + 1mg/mL eRJ	6.02 \pm 0.32	6.00	89	0.039
	<i>ftt-2 (n4426)</i>	4.64 \pm 0.27	4.00	67	
	<i>ftt-2 (n4426)</i> + 2mg/mL RJ	5.68 \pm 0.32	5.00	56	0.022
	<i>ftt-2 (n4426)</i> + 1mg/mL eRJ	5.75 \pm 0.30	5.00	56	0.016

Table 5 (continued). The Survival Time of Various Worm Strains under Stresses

	strain	Mean \pm SE	Median	No.of worms	p value
Heat	<i>daf-16 (mgDf50)</i>	2.63 \pm 0.11	3.00	111	
Shock	<i>daf-16 (mgDf50)</i> + 2mg/mL RJ	2.95 \pm 0.12	3.00	87	0.062
	<i>daf-16 (mgDf50)</i> + 1mg/mL eRJ	2.97 \pm 0.16	3.00	68	0.063
	<i>sir-2.1 (ok434)</i>	3.35 \pm 0.31	3.00	83	
	<i>sir-2.1 (ok434)</i> + 2mg/mL RJ	4.35 \pm 0.37	4.00	69	0.051
	<i>sir-2.1 (ok434)</i> + 1mg/mL eRJ	4.19 \pm 0.34	4.00	74	0.054
	<i>hcf-1(ok559)</i>	4.25 \pm 0.36	4.00	94	
	<i>hcf-1(ok559)</i> + 2mg/mL RJ	5.76 \pm 0.38	6.00	70	0.035
	<i>hcf-1(ok559)</i> + 1mg/mL eRJ	5.55 \pm 0.39	6.00	78	0.046
	<i>ftt-2 (n4426)</i>	3.64 \pm 0.37	4.00	72	
	<i>ftt-2 (n4426)</i> + 2mg/mL RJ	4.36 \pm 0.40	4.00	81	0.166
	<i>ftt-2 (n4426)</i> + 1mg/mL eRJ	3.89 \pm 0.33	4.00	94	0.782

Notes: The life span and standard error were shown in days. The life-span experiments were repeated at least three times with similar results, and the data for representative experiments were shown. The life-span data were analyzed using the log-rank test and *p* values for each individual experiment are shown. SE = standard error.

4.3 Discussions

Numerous studies showed that lifespan extension is often correlated with increased stress resistance[167]. Genetic manipulations in Insulin Signaling Pathways as well as feeding and dietary restrictions affected lifespan and stress resistance significantly. For instance, the deletion of *daf-2* were found to double the lifespan of wild type worms and resistant to several stressors, such as oxidative and thermal stresses[168]. Another example of this genetic manipulation is *eat-2* deletion mutant, which has a feeding problem during growth and has also showed long-lived and anti-stress phenotypes. However, the molecular mechanism underlying the link between pro-longevity and stress resistance is still unclear. One possible reason might be that some pro-longevity factors not only have the lifespan extension function but also protect organisms from the damages caused by stresses.

In our daily life, we are always exposed to complex mixtures of various environmental pollutants, which are produced by natural and human made sources such as combustion, construction, agriculture and so on. Some chemicals or pollutants could cause toxic effects to impair human's health. The accumulation of these damages could accelerate the aging process and increase the risk of cancer, diabetes, obesity, and neurodegenerative diseases. Several pieces of evidence hint that there might be a link between aging, stress, and age-related diseases[169].

Several aging research labs revealed that some functional nutraceuticals had anti-stress function. Ungvari and Jin published that resveratrol, a popular pro-longevity product, had the antioxidant effect and thereby alleviated Parkinson's disease[170].

Moreover, Guha and Guo reported that cranberry, a healthy fruit, possessed the heat shock resistant capability as well as lifespan extension. Moreover, it can delay the onset development of Alzheimer's disease[123]. In addition, numerous publications reported that nutraceuticals have lifespan extension and stress resistance, which further confirmed the link between pro-longevity and anti-stress functions.

In chapter three, our results demonstrated that RJ/eRJ had pro-longevity function and explained the molecular mechanism underlying this beneficial effect. Noticeably, our quantitative PCR results showed that RJ/eRJ regulated DAF-16 activity by affecting expressions of its target genes: *sod-3*, *mtl-1*, *C32H11.4*, and *F21F3.3*. Moreover, the elevated expressions of *sod-3*, *mtl-1*, *F21F3.3*, and repressed expression of *C32H11.4* indicated that RJ/eRJ might promote *C. elegans* stress resistance ability.

The *sod-3* gene, part of the SODs protein family that encodes an iron/manganese superoxide dismutase in mitochondrial, was found to be an anti-oxidant gene in *C. elegans* and conserved in mammals[171]. SODs are anti-oxidant enzymes that transfer superoxide radicals into hydrogen peroxide and oxygen. The *mtl-1* gene encodes one of two *C. elegans* metallothioneins and plays an important role in homeostasis and detoxification. It was discovered that the expression of intestinal *mtl-1* gene was up-regulated when worms were exposed to cadmium and heat shock[172]. It is possible that *mtl-1* functions as an essential protector in oxidative stress, DNA damage, and heat shock stress. In addition to *sod-3* and *mtl-1*, the expression of *F21F3.3* was also up-regulated in RJ/eRJ treatment. Previous research revealed that *F21F3.3* functions as a

methyltransferase to regulate protein folding and localization during the protein quality control process in the endoplasmic reticulum. Moreover, *F21F3.3* was regulated by both DAF-16 and HCF-1 and may play a role in stress resistance[49]. *C32H11.4* is one of a CUB-like protein, and its expression level was down-regulated in RJ/eRJ treated worms. Given that CUB-like proteins could affect the arsenate induced stress resistance and immune response[80], it is conceivable RJ/eRJ supplementation might strengthen worms' immune system and protect them against certain stresses. Based on these quantitative PCR results, we speculated that RJ/eRJ might protect worms from stresses through regulating the expression of DAF-16 related stress resistant genes. In order to confirm our hypothesis, we did oxidative stress, UV irradiation, and heat shock stress assays to check whether RJ/eRJ has the anti-stress ability.

First, we tested whether RJ/eRJ supplementation could protect *C. elegans* against oxidative stress. It is well known that oxidative stress is unavoidable and caused by the production of reactive oxygen species (ROS) from cellular mitochondrial respiration, which impairs a series of essential cellular components such as proteins, lipids, and DNA. In order to protect organisms from these damages, cells could produce superoxide dismutase (SOD), catalase, and glutathione peroxidases to transform the ROS to less toxic forms, which in turn detoxify the reactive intermediates and repair the cellular damages[165]. However, the balance of reactive oxygen production and consumption can be destroyed under certain conditions, such as persisting exogenous stress or in aging cells. When cells themselves cannot remove or degrade ROS efficiently, the accumulation of cellular damages will cause disease and accelerate the process of aging.

In our research, paraquat was used to mimic the oxidative stress conditions by producing a large amount of superoxide. Worms were treated with or without RJ/eRJ for two generations before being exposed to paraquat. Survival rates were observed to compare the differences in each treatment. Our results showed that both RJ and eRJ could protect *C. elegans* from paraquat induced oxidative stress and that there was no difference between RJ and eRJ's anti-oxidant ability (Figure 4.1). Moreover, further results showed that DAF-16, SIR-2.1, HCF-1, and 14-3-3 were required in this anti-oxidant response (Table 5). Noticeably, the *p* value of RJ/eRJ treatment versus that of the control group in *daf-16 (mgDf50)* and *hcf-1 (ok599)* mutants were just beyond the statistical significance ($p < 0.05$) but very close to it. However, it is worth noting that in N2 worms' oxidative stress experiment, the mean lifespan of RJ/eRJ treated worms were 7 days and 6 days respectively when compared with the mean lifespan of control (4 days) (Figure 4.1). This result demonstrated that RJ/eRJ supplementation increased survival of N2 worms under paraquat induced oxidative stress by 50%. Although the *p* value of RJ/eRJ treated *daf-16 (mgDf50)* and *hcf-1 (ok599)* versus non-supplemented genetically matched mutants was close to 0.05, RJ/eRJ only increased survival of *daf-16 (mgDf50)* mutants by 10.65% and survival of *hcf-1 (ok599)* mutants by 16.30% (Table 5). These data suggested that DAF-16 and HCF-1 also played a very important role in RJ/eRJ mediated oxidative response, and other regulators might also be required in this beneficial effect.

Accumulated evidence demonstrated that in addition to DAF-16, SKN-1, BAR-1, and SMK-1 were also important regulators in oxidative stress resistance[173, 174].

Similar to DAF-16, SKN-1 is also a transcriptional factor downstream of IIS pathway, that has elevated expression in intestinal nuclei under stress or reduced IIS signaling. Additionally, SKN-1 modulates a set of downstream genes, which contribute to detoxification, cellular repair, and pathogen resistance[40]. SKN-1 is also regulated by P38 MAPK pathway to modulate oxidative stress tolerance[175]. Our results showed that RJ/eRJ supplementation altered the expression of *C32H11.4*, and it is well known that *C32H11.4* is regulated by both DAF-16 and SKN-1 under stresses. SKN-1 may be involved in RJ/eRJ mediated anti-oxidative stress response. Another possible factor is β -catenin BAR-1, a DAF-16/FOXO co-factor, which can directly bind to FOXO to enhance its transcriptional activity in mammals. Essers and colleagues published that the interplay between BAR-1 and FOXOs was increased under oxidative stress[173]. Moreover, BAR-1 was required for the expression DAF-16 target gene *sod-3* in response to oxidative stress. Our results showed that RJ/eRJ increased the gene expression of *sod-3* approximately two fold, which raised the possibility that BAR-1 might be required in RJ/eRJ mediated anti-oxidative response. Additionally, SMK-1, another essential regulator of DAF-16, could also influence DAF-16 associated anti-aging effects by modulating DAF-16 transcriptional activity. Dillin's lab reported that SMK-1 was involved in several DAF-16 associated functions, such as UV irradiation, innate immunity, and oxidative stress response[47]. Further experiments are needed to investigate whether SKN-1, BAR-1, and SMK-1 are required in RJ/eRJ dependent anti-oxidant response; this information would greatly advance our understanding of the mechanistic details of oxidative stress.

In addition to IIS pathway and DAF-16, the well-studied but controversial sirtuin pathway was also required in RJ/eRJ mediated anti-oxidative stress (Table 5). To date, cumulative publications claim that SIR-2.1/SIRT1 plays a key role in the anti-oxidant response, but the precise role of SIR-2.1/SIRT1 is still unclear[176]. Sirtuin enzymes are a conserved family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases and ADP-ribosyl-transferases, which regulate lifespan extension and stress resistance under dietary restriction. In a study of mammals, SIRT1 was shown to deacetylate and activate FOXOs (FOXO1, FOXO3a, and FOXO4) and promote the production of DNA repair factor[177]. Moreover, under modification of ROS, SIRT1 could activate P53 to reduce cellular apoptosis and promote peroxisome proliferator activated receptor gamma coactivator 1 α (PGC-1 α) to produce mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD) in response to oxidative stress[178]. However, there are numerous contradictory results in sirtuin research, and the precise role of SIRT1 mediated oxidative resistance is still elusive. Our study revealed that SIR-2.1 was required in RJ/eRJ mediated anti-oxidant response; therefore RJ/eRJ treatment could be utilized as a model to further investigate the detailed molecular mechanism of SIR-2.1 regulation in response to oxidative stress.

Paraquat, a common herbicide, not only induces an oxidative stress but also causes toxic effect to animals[179]. In our oxidative stress assay, worms' mean lifespan was decreased from 12 days to 4 days because of the toxicity induced by the paraquat treatment (Figure 4.1). This result demonstrated that paraquat significantly shortened *C. elegans* lifespan because of its toxicity. Similarly, paraquat is also harmful to humans. In

2011, a U.S. National Institutes of Health study reported that farm workers were at a high risk for Parkinson's disease because of their frequent usage of paraquat[180].

Furthermore, other studies revealed that the accumulation of oxidizing species caused by paraquat exposure could destroy cell structure and protein function, leading to the development of Parkinson's disease in humans[181]. In addition to Parkinson's disease, oxidative stress could also increase the risk of other age-related diseases such as cancer, Alzheimer's disease, atherosclerosis, heart failure, and chronic fatigue syndrome[182].

Collectively, our results indicated that RJ/eRJ not only protected worms from oxidative stress, but also alleviated paraquat induced toxicity. It is possible that RJ/eRJ might protect *C. elegans* against cytotoxicity and we will discuss this beneficial effect in the next chapter.

It is well known that ROS is a major inducer of oxidative stress, but recent studies reported that ROS are not always harmful. Hamanaka and Chandel demonstrated that ROS also participates in cell signaling to regulate a variety of biological processes[165]. Moreover, several pathways could be activated by a short term of mild oxidative stress, which is beneficial for the immune system to kill pathogens and in turn lead a healthy aging. In 2008, Gems and Partridge published a theory of "mitohormesis," which demonstrated that a low dose of stress could stimulate immunity to fight against pathogens and harmful effects to slow down the aging process[183]. It is possible that RJ/eRJ treatment might mimic the mild stress to strengthen the immune system and

DAF-16 transcriptional activity of *C. elegans* to promote lifespan extension and oxidative stress resistance.

Next, we investigated whether RJ/eRJ could protect worms from DNA damage to maintain genome integrity. Based on our knowledge, cells maintain a good balance between ROS production and antioxidant defenses in normal conditions; however, dysregulation in this maintenance could cause mutations and cell death by high level accumulative oxidant damages to DNA[112]. In humans, a lot of environmental factors are known to induce DNA damages, such as solar UV irradiation, cigarette smoke, toxin, chemotherapeutics, and food contained chemicals. The unrepaired DNA damages could accelerate the aging process and increase the risk of cancers. In order to solve this problem, more and more scientists focus on investigating the molecular mechanisms of DNA damages and looking for effective treatment to prevent relative diseases[169].

Given that reduced oxidative damages might be correlated with increased genome integrity, it is possible that RJ/eRJ might also help *C. elegans* in DNA damage response. In order to confirm this hypothesis, UV irradiation experiment was carried out to observe worms' survival rate under RJ/eRJ supplementation and non-supplemented controls. In our study, UV irradiation was utilized to cause the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4-photoproducts (6-4 PPs), which substantially distort double helix structure of DNA to induce DNA lesions in *C. elegans*.

Our N2 worms' UV stress assay showed that RJ/eRJ prolonged the survival of N2 worms by 25% (RJ) and 50% (eRJ) under UV stress when compared with non-

supplemented control worms (Figure 4.1). In order to investigate the molecular mechanism underlying this protective function, *daf-16 (mgDf50)*, *sir-2.1 (ok434)*, *hcf-1 (ok599)*, and *14-3-3 (n4426)* mutant worms were exposed under UV irradiation with or without pre-treatment of RJ/eRJ for two generations. Surprisingly, our results showed that RJ/eRJ also increased the survival of *hcf-1(ok599)* mutants by 23.87% and the survival of *14-3-3(n4426)* mutants by 22.41%, and the *p* value of RJ/eRJ versus control was less than 0.05. However, RJ/eRJ supplementation failed to increase the survival rate of *daf-16 (MgDf50)* and *sir-2.1 (ok434)* worms (Talbe 5), which demonstrated that only DAF-16 and SIR-2.1 were required in RJ/eRJ mediated UV stress resistance.

RJ/eRJ supplementation might provide us a good model to investigate the molecular mechanism of DNA damage. A growing body of evidence indicated that there is a link between aging and age-related diseases to the loss of DNA repair capacity; the declined capacity of DNA repair resulted in accelerating the process of aging[114]. Our results showed that RJ/eRJ served as a beneficial supplementation to extend *C. elegans* lifespan as well as increase their resistance to UV stress, which correlates DNA repair capacity with healthy aging. Previous publications have revealed that the *C. elegans* mutant defective in IIS pathway lead a longer lifespan and higher DNA repair capacity[177], possibly because the reduced IIS elevated heat shock proteins (HSPs) expressions via increasing DAF-16 transcriptional activity. In the yeast model, it has been proposed that HSP90 could interplays with Ssl2, the yeast homolog of XPB, to promote enzymatic activities involved in repair of UV induced DNA lesions[184]. In a study of mammals, Mostoslavsky's lab reported that SIRT6 plays an important role in DNA

damage repair and aging[185]. Although the detailed molecular basis of DNA repair capacity is unclear, accumulating evidence indicated that heat shock proteins, IIS pathway, and surtuin pathway might be involved in this process.

In addition to IIS and surtuin pathways, other DNA repair pathways might be also involved in this RJ/eRJ mediated anti-DNA damage function. It is well known that cells are equipped with several DNA repair machineries to protect genome integrity against UV irradiation-induced cellular damage. The NER pathway, composed of numerous repair factors, is a general DNA repair machinery and displays multiple functions to repair many types of DNA damages, ranging from small base modification to double strand breaks[113]. Hyun and Ahn claimed that xpa-1, a nucleotide excision repair gene of *C. elegans*, was required in DNA repair response of IIS defective mutants[186]. This finding indicated that there are some interplays between IIS and NER pathways in DNA repair process. Moreover, it is reported that the mutations in human NER genes could cause several UV sensitive hereditary disorders, such as cockayne syndrome, premature aging, and neurodevelopmental problems, which underscored the relationship between DNA damage and aging.

Our research provided a good model to investigate the molecular mechanism of DNA damage response. Further studies are needed to determine whether IIS/DAF-16 and SIR-2.1 interplay with DNA repair pathways in providing UV resistant benefits, which could help us to develop the effective treatment for DNA damage-associated disorders, such as cancer and premature aging[153].

The last stress response tested in our research was heat shock stress. Heat stress, also called thermal stress, is designed to treat organisms at a higher temperature than the ideal body temperature for a certain period. During heat stress, high temperature could cause damage to protein structure and function in some degree. In response to this stress, the cellular internal repair system is activated to up-regulate the expressions of heat shock proteins (HSPs) to repair or degrade the destructed proteins. As a master regulator to control the quality of proteins, HSPs function as molecular chaperones and play a critical role in protein folding and intracellular trafficking, additionally, and denatured proteins repair in stress and aging conditions[117].

Numerous studies have shown that some nutraceuticals have anti-heat stress functions. In a *C. elegans* model, it is reported that under thermal stress, the first larval stage (L1) or early L2 stage worms were induced to enter a dauer stage, which showed long lived phenotype and survival under harsh conditions. However, for the well-developed young adult worms, heat shock stress could shorten their lifespan and cause reproductive problems. Numerous studies demonstrated that several antioxidant compounds could alleviate this heat shock induced protein damage. For example, α -lipoic acid and trolox, which were discovered as functional supplementation to increase *C. elegans* lifespan and heat shock resistance. In our previous study, our results showed that RJ/eRJ supplementation strengthened worms' oxidative stress tolerance; thus it is highly possible that RJ/eRJ may also have thermal stress resistance.

In our study, wild type worms were pre-treated with RJ/eRJ for two generations under 25 °C. Then the synchronized young adult worms were heat shocked at 35 °C for three hours and returned back to 25 °C to observe the survival of the worms receiving different treatments. Our results showed that heat shock shortened the mean lifespan of non-treated N2 worms from 12.97 days to 1.5 days. However, RJ/eRJ could increase worms' survival rate nearly 6 fold (RJ) and 3 fold (eRJ) when compared with the survival rate of the controls (Figure 4.1). This result indicated that RJ/eRJ might protect *C. elegans* from heat shock stress, possibly because that some bioactive factors in RJ/eRJ could promote cells' internal protein repair system to maintain protein structure and function in response to heat shock stress.

Next, we compared the differences in thermal stress resistance in two types of Royal Jelly treated worms. Noticeably, although both RJ and eRJ could protect *C. elegans* from heat shock stress, the mean lifespan of heat shocked worms were significantly different in these two treatments: the mean lifespan of RJ treatment was around 9 days, whereas the eRJ treatment was only 4 days (Figure 4.1). This result indicated that RJ's anti-thermal stress function was much better than that of eRJ. As we mentioned before in chapter three, the difference between RJ and eRJ was the enzyme pre-treatment, which removed some large proteins to reduce the immune response in eRJ powder. These removed large proteins might play an important role in heat shock resistance by stimulating the immune system. Overall, our data suggested that besides anti-oxidant and DNA repair capacity, RJ/eRJ also protected *C. elegans* from heat shock stress.

Next, we wondered whether DAF-16, SIR-2.1, HCF-1, and 14-3-3 are required in RJ/eRJ mediated heat shock resistance. Herein, *daf-16 (mgDf50)*, *sir-2.1 (ok434)*, *hcf-1 (ok599)*, and *14-3-3 (n4426)* mutant worms were supplemented with or without RJ/eRJ for two generations before being treated with heat shock stress. The results showed that RJ/eRJ cannot prolong the survival of *daf-16 (mgDf50)*, *sir-2.1 (ok434)*, and 14-3-3 (*n4426*) mutant worms under heat shock stress, which indicated that except for HCF-1, the other three proteins, DAF-16, SIR-2.1, and 14-3-3, were required in RJ/eRJ mediated heat shock resistance. Noticeably, the *p* value of RJ/eRJ treated *hcf-1 (ok599)* versus genetically matched controls was 0.046, less than the statistical significance. However, RJ/eRJ only increased the *hcf-1 (ok599)* mutants worms' mean lifespan by 1.31 fold when compared with controls, which is far less than the increasing rate (6 fold in RJ and 3 fold in eRJ) of mean lifespan elongation in heat shocked N2 worms (Table 5). This data indicated that HCF-1 was partially required in RJ/eRJ's thermal stress resistance.

Numerous papers published that DAF-16 could protect cells from various stresses by transcriptional up-regulation of its target stress response genes, especially heat shock proteins. Our quantitative PCR results showed that RJ/eRJ treatment increased the expression level of *F21F3.3* (Figure 3.3), which functions in membrane proteins folding. Besides *F21F3.3*, DAF-16 also regulates a series of protein-turnover genes. The majority of these genes belong to heat shock protein (HSP) family, which modulate protein folding, trafficking, aggregation, and degradation to maintain proteostasis[144]. As an essential regulator to control protein quality, HSPs play a key role not only in normal conditions but also in stress environments and aging cells. During the cell growth, HSPs guide the

newly synthesized and misfolded proteins to fold into their correct and functional conformations, and the well folded proteins are shuttled to the right place by chaperones inside the cells. Moreover, the misfolded and unfolded proteins can be targeted by ubiquitin, a small group of HSPs, and proteases for degradation. In addition to this protein folding function, HSPs also served as a “cell monitor” to help the immune system detect the infected cells by presenting pieces of proteins or antigens on the cell surfaces[187]. Considering that our previous data showed that RJ/eRJ increased DAF-16 transcriptional activity, it is highly possible that RJ/eRJ could also promote the gene expression levels of DAF-16 targeted heat shock proteins in heat shock resistance.

As shown above, in addition to DAF-16, SIR-2.1 and 14-3-3 were also required in RJ/eRJ mediated heat shock tolerance; however, other factors, such as HSF-1, might also be involved in this anti-heat stress function. The Guarente lab proposed a model to explain the mechanism of heat shock stress: following the heat shock stress, SIR-2.1 can bind and activate DAF-16 in a 14-3-3 dependent manner[48]. It is conceivable that RJ/eRJ supplementation triggered the interplay of DAF-16, SIR-2.1, and 14-3-3 to activate DAF-16 downstream stress resistant genes, and thereby protected *C. elegans* from heat shock. However, it is worth noticing that in our mutant worms’ heat shock stress data, the *p* value of RJ/eRJ treated *daf-16 (mgDf50)* and *sir-2.1 (ok434)* versus their genetically matched control was 0.062 and 0.054 respectively, which indicated that besides DAF-16 and SIR-2.1, other factors might also be involved in this anti-heat shock resistance. It is highly possible that heat shock factor (HSF-1) is involved in this anti-stress effect. As same as DAF-16, HSF-1 is also an important transcriptional factor

located at the downstream of IIS, and it is well known that DAF-16 and HSF-1 could act together to turn on a specific subset of genes to promote longevity[37]. Moreover, Morimoto's lab claimed that SIRT1 deacetylated HSF-1 to up-regulate its target gene HSP70 expression in mammals study. As an important HSPs, HSP70 not only assist in the establishment of proper protein conformation, but also presents antigens and proteins across membranes to strengthen immune response, which is mainly regulated by HSF-1[188]. Taken together, it is highly possible that HSF-1 might interplay with DAF-16 and SIR-2.1 to activate HSPs function in RJ/eRJ mediated heat shock stress resistance. Further experiments are needed to test our hypothesis.

It is well known that heat shock response is implicated in a series of conditions and diseases, such as neurodegenerative diseases, ischemic diseases, infection, and aging. Under stress conditions, proteins can be unfolded, misfolded, and aggregated[189]. Similarly, in the aging cells, the accumulated stresses and physiological decline associated with aging cause damages to cells by promoting protein aggregation to disrupt proteostasis[190]. In order to protect cells from this negative effect, cells themselves are equipped with an internal repair system, which could produce more HSPs to refold or degrade the destructed proteins and thereby assist the cell recovery in response to stresses. Overall, our studies revealed that RJ/eRJ supplementation protected *C. elegans* from heat shock stress, and this protection role reflected that RJ/eRJ possessed capacity to assist *C. elegans* in the repair of protein damage.

In consideration that most neurodegenerative disorders are caused by protein misfolding, studying the molecular mechanism of heat shock stress could further help us to develop the therapeutic treatment of neurodegenerative diseases. RJ/eRJ supplementation provided us with more opportunities to further investigate the detailed molecular mechanism of heat shock resistance and age-related disorders. Additional studies are needed to further understand the relationship between stress resistance and pro-longevity.

In summary, our results demonstrated that RJ/eRJ can protect *C. elegans* from a variety of stressors, such as oxidative stress, UV irradiation and heat shock stress. At least in part, these beneficial effects might act through modulating the interplays of DAF-16, SIR-2.1, HCF-1, and 14-3-3. Moreover, our quantitative PCR results showed that RJ/eRJ induced the overexpression of several stress resistant genes under normal conditions, which indicated that RJ/eRJ's stress resistance capacity might be dependent on its hermetic effects. It is conceivable that RJ/eRJ functions as a mild stress inducer to ameliorate stress and promote longevity.

CHAPTER FIVE : THE EFFECT OF ROYAL JELLY ON ALZHEIMER'S DISEASE

5.1 Introduction:

Aging is the greatest cause of neurodegenerative diseases, and a sizable aging population is already a major challenge for the healthcare system in American society. The statistical data estimated that more than 20% of the population will be over 65 years old by 2030, and the healthcare cost will reach \$1.1 trillion by 2050[136].

Alzheimer's disease (AD) is the best known and the most studied neurological disorder in the aging population. AD is characterized by impairment of thinking, communication, and memory[127]. Two obvious phenotypes of AD patients are brain atrophy and neuropathological lesions, which exacerbate over time to interfere with daily activities. Numerous studies demonstrated that in all AD patients the depositions of neurofibrillary tangles (NFT) and extracellular amyloid plaques might disintegrate the neuron's signaling transduction systems and thereby induce neural cell death as well as cognitive impairment[135].

A growing body of studies has been published to elucidate the molecular mechanism of AD. Although the detailed molecular mechanism underlying AD is still unclear, AD is often considered as a "protein misfolding disease", because it is caused by A β peptide aggregation and accumulation outside or inside of the neuronal cells[191]. It is well known that free radicals are generated by cell metabolism and stressors during cell growth, which cause damaging effects to the integrity of the proteome, leading to protein misfolding, aggregation, and amyloid formation. In order to combat this negative effect,

cells are equipped with protective machinery comprising a series of heat shock proteins (HSPs) that refold or degrade the misfolded and unfolded proteins and thus maintain a functional proteostasis. Unfortunately, the persisting harsh stresses and aging conditions overwhelm this protective mechanism, leading to aberrant proteins aggregation and cellular toxicity. It has been proposed that the aggregation and accumulation of these aberrant proteins are common features of many neurodegenerative diseases[131].

AD, one of the costliest chronic diseases to society, has no efficient treatment; thus there is an urgent need for scientists to investigate the pathologic mechanism of AD and explore efficient therapeutic treatments to delay the progress of age-related disorders. Because nearly 80% of genes and pathways related to human diseases are present and conserved in *C. elegans*, it is believed that *C. elegans* is a good model to study the human diseases and evaluate the efficiency of novel drugs and treatments. Specifically, the *C. elegans* CL-2006 worm strain is reported as a classic AD model in many publications. CL-2006 worms transgenically express human A β ₃₋₄₂ peptides in body wall muscle and neuron cells. During the aging process, the aggregation and accumulation of A β species in muscle cells result in a progressive or rapid paralysis phenotype, which can be used as a direct indicator of A β toxicity[192].

To date, numerous findings have shown that *C. elegans* AD model provided an excellent platform to investigate the beneficial anti-AD effects of nutraceuticals as well as molecular mechanisms of AD[22]. For instance, recent studies using the *C. elegans* model showed that curcumin, a phenol extracted from turmeric, could slow down the

development of Alzheimer's disease and Parkinson disease by alleviating the proteotoxicity[193]. Guo et al reported that cranberry could protect worms against A β toxicity in AD; IIS/HSF-1 was required in this beneficial effect[123]. Moreover, LWDH (Liu Wei Di Huang), a traditional Chinese medicine, was discovered to delay AD development by increasing the level of heat shock proteins (HSPs) in *C. elegans* AD model[194]. Collectively, these studies show that dietary intervention provides a feasible and efficient method to combat age-related disorders through the IIS pathway.

Our research focused on investigating the anti-aging effects of Royal Jelly. As a functional nutraceutical product, Royal Jelly (RJ) and its derived products have antimicrobial, anti-inflammation, immune-modulation, and anti-oxidation effects. The RJ, a bee product, is secreted by worker bees and used for developing and maintaining the queen bees. Enzyme treated RJ (eRJ) is produced by digestion of RJ with a hydrolytic enzyme to decrease the allergenic properties of RJ[103]. In chapter four, our studies demonstrated that RJ/eRJ extended *C. elegans* lifespan and protected them against several stressors. Additionally, our genetic analysis showed that these beneficial effects required IIS pathway and DAF-16, which has been reported to play a key role in attenuating A β toxicity in *C. elegans*. To this end, it is highly possible that RJ/eRJ might have a protective effect to slow down the development of AD in *C. elegans* model.

Herein, AD model CL-2006 worms were supplemented with RJ/eRJ to test this hypothesis. Strikingly, our results showed that RJ/eRJ significantly slowed down the progression of A β toxicity induced paralysis by reducing the A β species. Afterwards, the

genetic analysis showed that this beneficial effect was dependent on IIS/DAF-16, rather than HSF-1 and SKN-1. Our results consistently demonstrated that RJ/eRJ increased the protein solubility to maintain a healthy proteostasis in aged worms. Given that AD is a protein misfolding disease and caused by proteotoxicity, our western blot results showed that RJ/eRJ could alleviate the cytotoxicity in AD worms by increasing the solubility of A β species, which indicated that RJ/eRJ might influence the conformation of A β species and thus slow down the development of AD.

Taken together, our findings not only implied the molecular mechanism of RJ/eRJ mediated protection against A β toxicity but also shed light on the relationship between proteostasis and neurodegenerative disorders.

5.2 Results:

5.2.1 RJ/eRJ supplementation prolongs the lifespan of AD model *C. elegans*

It has previously been showed that RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) significantly increased the lifespan of wild type *C. elegans*, herein we want to test whether RJ/eRJ also has the same beneficial effect on trans-genetic AD worms. CL-2006 strain, engineered transgenic worms which express human A β proteins in body muscles and neurons, were treated with or without RJ/eRJ from young adult stage, and their lifespan were measured at 20 °C. In consistent with our previous study, our data showed that RJ/eRJ significantly increased the lifespan of CL-2006 worms. Comparing with non-supplemented control, the mean lifespan of RJ/eRJ treated CL-2006 worms averagely increased from 14.780 days to 17.000 days (RJ) and 17.188days (eRJ), resulting in an increase of 15.02% and 16.29%, respectively (Figure 5.1 and Table 6). Because of numerous studies revealed that lifespan extension is often associated with increased stress resistance and anti-age related disorders function, this finding implied that RJ/eRJ supplementation might be effective to AD treatment.

5.2.2 RJ/eRJ delays the progression of paralysis in AD worms by reducing the amount of A β species

Paralysis is a major marker to directly reflect A β toxicity in AD worms. Briefly, we investigated whether RJ/eRJ could slow down the progression of AD worms' paralysis. Meanwhile, the amount of A β species was tested to determine whether RJ/eRJ could influence A β production to affect A β -induced paralysis in AD worms.

5.2.2.1 RJ/eRJ supplementation delays the progression of A β toxicity induced paralysis in AD worms

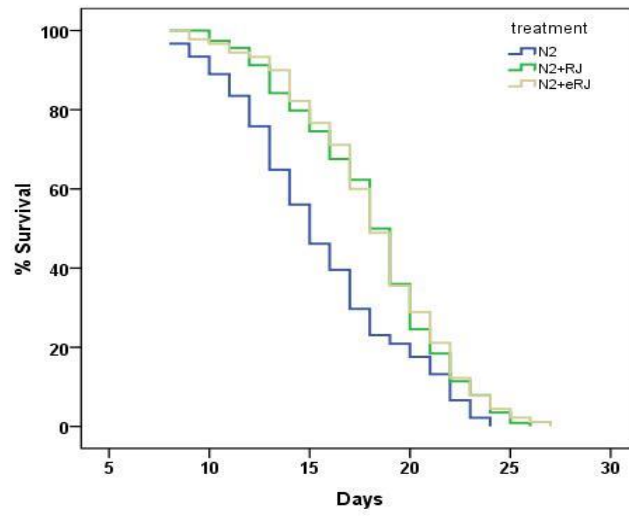
Synchronized CL-2006 young adult worms were treated with or without RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ respectively) supplementation at 20 °C and paralysis were measured every day until all worms were paralyzed. Our data indicated that RJ/eRJ supplementation dramatically delayed the progression of CL-2006 worms' paralysis when compared with the non-supplemented controls (Figure 5.2). Specifically, RJ/eRJ extended the mean time of paralysis from 10.901 days to 12.656 days and 12.091 days, displaying an average increase of mean paralysis time by 16.10% and 10.92% when compared with control group, respectively (Table 7). In considering that paralysis is a direct indicator of A β toxicity, our finding suggested that in addition to lifespan extension, RJ/eRJ also alleviated A β toxicity in AD model worms.

5.2.2.2 RJ/eRJ supplementation reduces A β species amount in AD worms

Given that AD worms' paralysis is caused by A β toxicity, numerous studies reported that many nutraceuticals possessed the ability to slow down the AD development by mitigating A β toxicity through decreasing A β species. We questioned whether RJ/eRJ followed the same rule to delay the development of AD. In order to address this concern, synchronized CL-2006 young adult worms were treated with or without RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) for 10 days at 20 °C. Afterwards, 10-day aged worms were collected to conduct a dot blot assay to test whether RJ/eRJ supplementation influenced the amount of A β species. Image-J software was used to analyze the data. Our dot blot assay result showed that RJ/eRJ displayed a decrease of

A β species amount by 13.61% and 21.90% comparing with non-supplemented controls, which was statistically significant ($p<0.05$) (Figure 5.2). Taken together, given the progression of CL-2006 worms' paralysis is driven by A β toxicity and the amount of A β species contributes to A β toxicity, our finding demonstrated that RJ/eRJ attenuated A β toxicity by reducing the level of A β species amount.

A



B

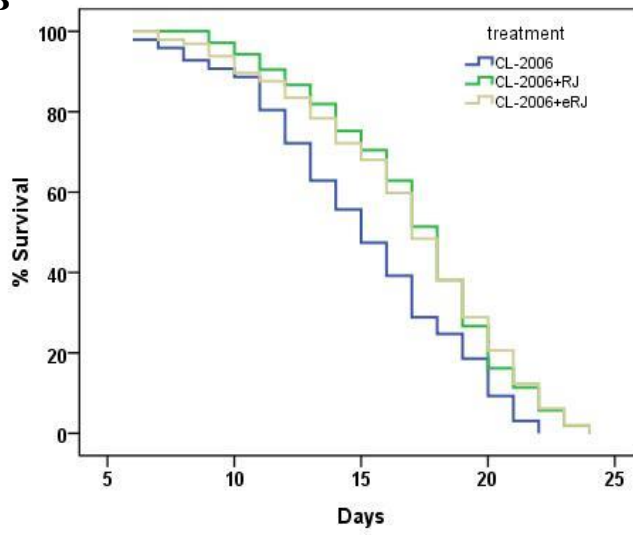
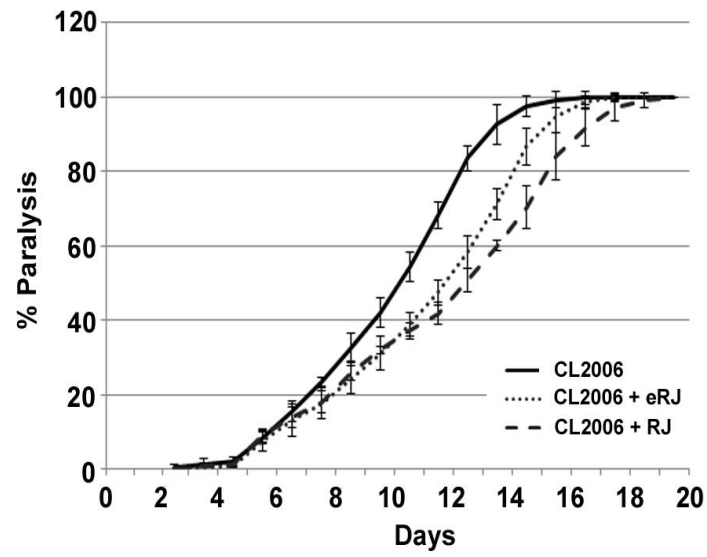


Figure 5.1 RJ and eRJ extend the lifespan of transgenic *C. elegans* expression A β peptides. (A). Wild type N2 worms were supplemented with RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively). (B). CL-2006 worms, transgenic *C. elegans* constitutively expressing A β , were treated with RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively). Each lifespan experiment was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments were included in Table 6.

A



B

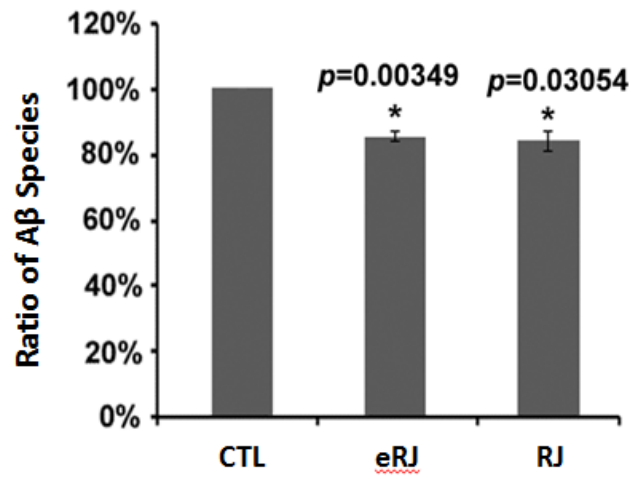


Figure 5.2 RJ/eRJ treatment mitigates A β toxicity in *C. elegans* by reducing A β species amount. (A). CL-2006 worms were treated with RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) to carry out paralysis assays. The RJ/eRJ treated CL-2006 worms (dashed line) showed delayed progression of body paralysis as compared to control worms (solid line). Each paralysis assay was conducted in triplicates and repeated at least three times with similar results. “% paralysis” indicated the average paralysis among the multi-replicates and error bars represent the standard deviation. (B). Dot blot analysis was carried out to measure the total amount of A β species using 10-day old CL-2006 worms. Total A β species were quantified by Image-J software. A dramatic reduction of total A β species were observed in RJ/eRJ treated CL-2006 worms. The graph showed the mean intensity of A β species and was the result of three independent experiments. The *p* value was calculated by using Student’s *t*-test. “*” indicated *p*<0.05 when comparing to controls.

Table 6. The lifespan Data of N2 and CL-2006 worms at 20 °C

strain	Mean±SE	Median	No. of Worms	<i>p</i> value
N2*	14.892±0.401	15.00	84	
N2 + 2mg/mL RJ*	17.848±0.401	18.00	86	0.002
N2 + 1mg/mL eRJ*	18.221±0.438	19.00	88	0.001
CL-2006 **	14.780±0.361	15.00	97	
CL-2006 + 2mg/mL RJ**	17.000±0.406	18.00	97	0.001
CL-2006 + 1mg/mL eRJ**	17.188±0.312	17.00	105	0.001

Note: Lifespan and standard error are shown in days. The lifespan experiments were repeated at least three times with similar results, and the data for representative experiments are shown. The lifespan data were analyzed using the log-rank test and *p* values for each individual experiment are shown.

*Results presented in Figure 5.1 A **Results presented in Figure 5.1 B

Table 7. The Paralysis Data of CL-2006 Worms at 20 °C

strain	Mean±SE	Median	No. of Worms	<i>p</i> value
CL-2006 [†]	10.901±0.143	11.00	394	
CL-2006 + 2mg/mL RJ [†]	12.656±0.149	12.00	449	0.001
CL-2006 + 1mg/mL eRJ [†]	12.091±0.196	13.00	389	0.001

Notes: Paralysis and standard error were shown in days. The paralysis experiments were repeated at least three times with similar results, and the data for representative experiments were shown. The paralysis data were analyzed using the log-rank test and *p* values for each individual experiment are shown.

[†]Results presented in Figure 5.2 A.

5.2.3 RJ/eRJ requires IIS/DAF-16, rather than HSF-1, SKN-1, and JNK-1, to delay the development of body paralysis in *C. elegans*

Moll demonstrated that reduction of IIS prevented neurodegenerative diseases in mammals and worms by increasing the transactivities of its transcriptional factors: DAF-16, HSF-1, and SKN-1[143]. Moreover, it has been proposed that, in parallel to IIS, JNK pathway also plays an important role in Alzheimer's disease and regulate the activities of these IIS pathway downstream transcriptional factors (DAF-16, SKN-1, and HSF-1). Thus we wondered whether RJ/eRJ requires IIS pathway and JNK pathway to slow down the development of AD in *C. elegans*.

5.2.3.1 Insulin Signaling Pathway and DAF-16 are required in RJ/eRJ mediated protection from A β toxicity

In chapter three, our genetic analysis showed that IIS/DAF-16 was involved in RJ/eRJ mediated lifespan extension. Considering the potential link between pro-longevity and anti-AD function, we questioned whether IIS/DAF-16 also plays a key role in RJ/eRJ mediated protection against A β toxicity. In order to address this concern, genetic epistasis assays were carried out in CL-2006 worms treated with and without RJ/eRJ supplementation to monitor their progression of paralysis.

Briefly, RNA interference (RNAi) was utilized to distinctively knock down the gene expression of major IIS pathway components (*daf-2* and *age-1* respectively) and an important IIS pathway downstream transcriptional factor (*daf-16*). CL-2006 worms were synchronized on NGM plates seeded with particular RNAi or a paired RNAi control (L4440 empty vector for *age-1* RNAi and *daf-16* RNAi, PAD-12 empty vector for *daf-2*

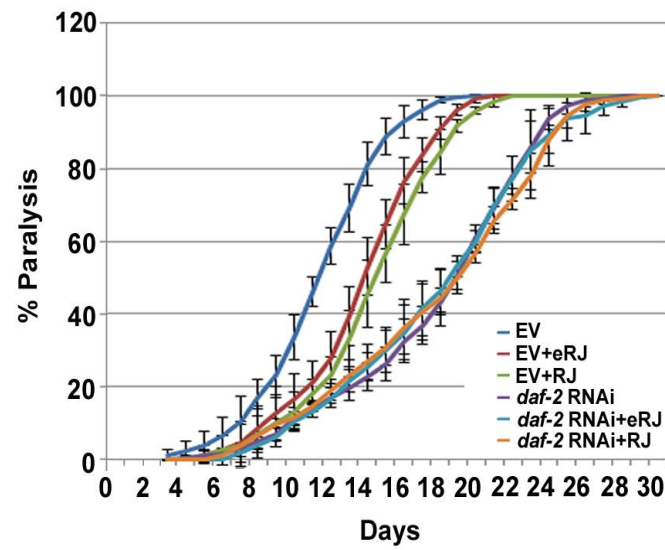
RNAi) bacteria. Afterwards, the synchronized young adult worms treated with or without RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) were fed with particular RNAi bacteria to carry out paralysis assays. Worms' paralysis was observed every day until all worms were paralyzed. Meanwhile, another set of young adult worms supplemented with or without RJ/eRJ were fed with particular RNAi (*daf-16*, *age-1*, and *daf-2*, respectively) and paired RNAi control bacteria for 6 days. Then the 6-day aged worms of different treatments were collected to conduct a quantitative PCR experiment to check the RNAi efficiency.

In our paralysis assay results, CL2006 worms fed with two RNAi paired control bacteria were set up as system controls. When compare with non-treated controls, RJ/eRJ supplementation dramatically delayed the progression of paralysis in CL-2006 worms fed with two RNAi paired control bacteria, which confirmed that our experimental system and RJ/eRJ treatment method worked well. Then we analyzed the paralysis assay data in each RNAi (*dat-16*, *age-1*, and *daf-2* respectively) bacteria fed worms. Interestingly, our data showed that the beneficial effects of RJ/eRJ on delaying the progression of paralysis was completely withdrew in CL-2006 worms fed with *age-1* RNAi, *daf-2* RNAi and *daf-16* RNAi bacteria comparing with RNAi matched non-supplemented controls, respectively (Figure 5.3 A, B, and C). Moreover, the quantitative PCR results showed that the RNAi successfully inhibited almost 80-90% gene expressions (*age-1*, *daf-2*, and *daf-16*), respectively (Figure 5.3 D). This result indicated that RNAi method worked efficiently to repress selected gene expression in genetic epistasis assays, and further confirmed that our genetic epistasis assay results were reliable. Summarily, our findings

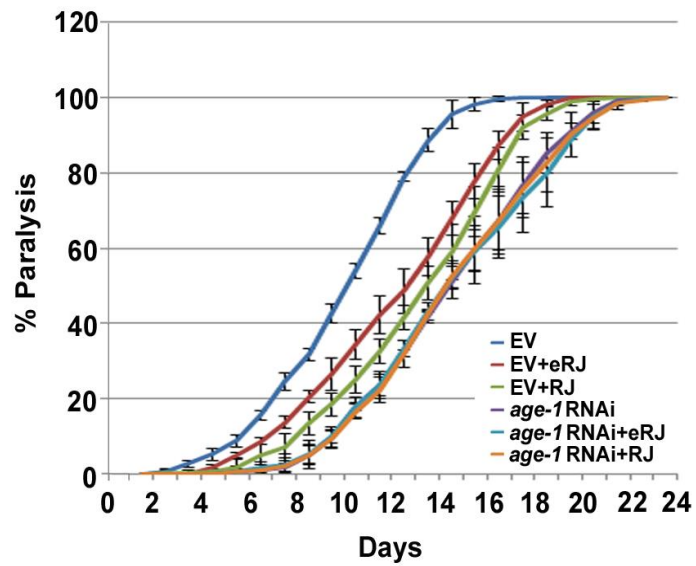
showed that IIS and DAF-16 were required not only in RJ/eRJ mediated pro-longevity effect but also in protection against A β toxicity.

In chapter three, we reported that RJ/eRJ extended *C. elegans* lifespan through regulating the trans-activity of DAF-16. In consideration of DAF-16 was also involved in RJ/eRJ mediated protection of A β toxicity, we questioned whether this beneficial effect also acts through activating the DAF-16 target genes. To this end, synchronized CL-2006 young adult worms were treated with or without RJ/eRJ for 6 days, and several representative DAF-16 target genes were selected to measure their expression levels in each treatment. Our results showed that *sod-3*, *mtl-1*, *Y71H2Ar.2*, *hsp-16.2*, and *hsp-12.6* were up-regulated in CL-2006 worms treated with RJ/eRJ when compared with non-supplemented controls (Figure 5.4). This finding indicated that RJ/eRJ mediated protection against A β toxicity might be dependent on elevating the transactivity of DAF-16.

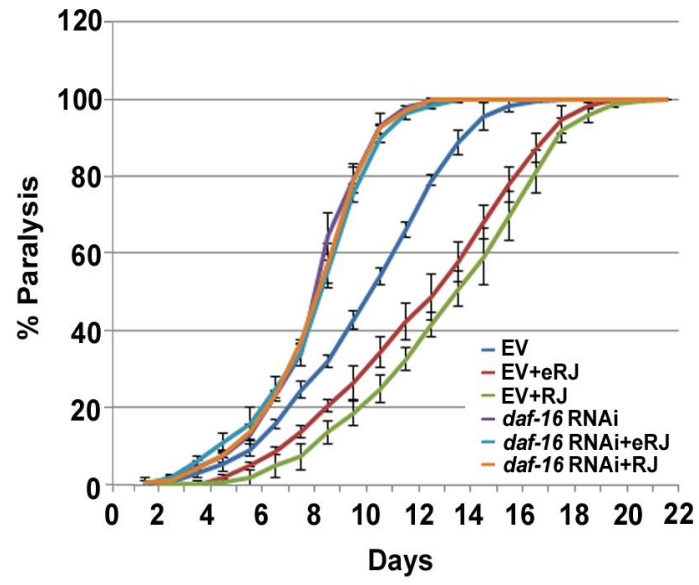
A



B



C



D

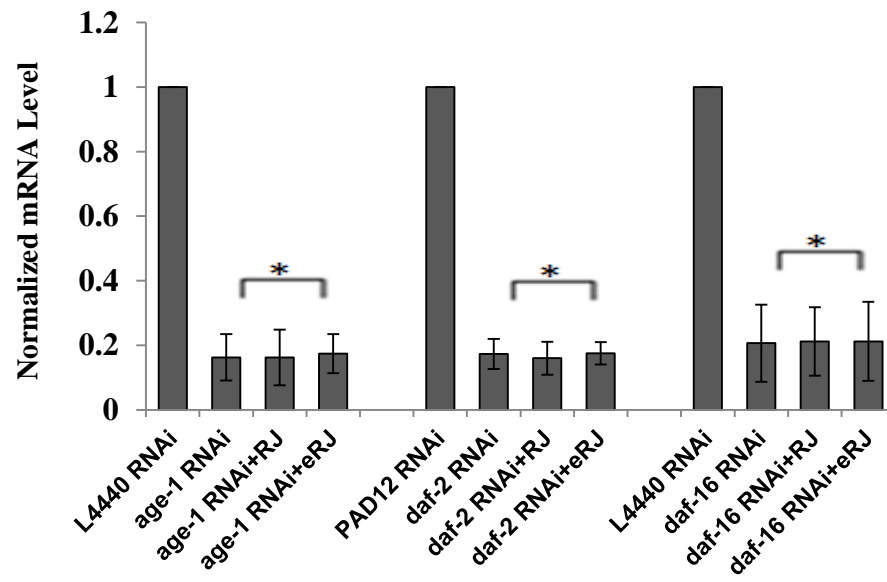


Figure 5.3 RJ/eRJ requires IIS/DAF-16 to protect against A β toxicity in CL-2006 worms. (A). RJ/eRJ cannot slow down the progression of body paralysis in CL-2006 worms with reduced IIS by *daf-2* RNAi. (B). RJ/eRJ cannot delay the progression of A β toxicity-induced paralysis in CL-2006 worms with reduced IIS by *age-1* RNAi. (C). RJ/eRJ cannot delay the progression of A β toxicity-induced paralysis in CL-2006 worms with reduced DAF-16 level by *daf-16* RNAi. Each paralysis assay was repeated at least three independent times with similar results. “% paralysis” indicated the average paralysis among the multi-replicates and error bars represent the standard deviation. (D). RNA interference efficiently knocked down selected gene expression (*age-1*, *daf-2*, and *daf-16* respectively) in CL-2006 worms’ genetic epistasis assays. The expression levels of *age-1*, *daf-2*, and *daf-16* were measured with qRT-PCR in CL-2006 worms supplemented with RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively). CL-2006 worms feeding with paired RNAi control (L4440 empty vector for *age-1* and *daf-16*, PAD-12 empty vector for *daf-2*) served as controls to check gene knock down efficiency. The data from three independent experiments were pooled to calculate the mean mRNA level normalized to the internal control *act-1*. The standard errors of the mean (SEM) were shown. “*” indicated $p < 0.05$ when compared to paired RNAi bacteria controls.

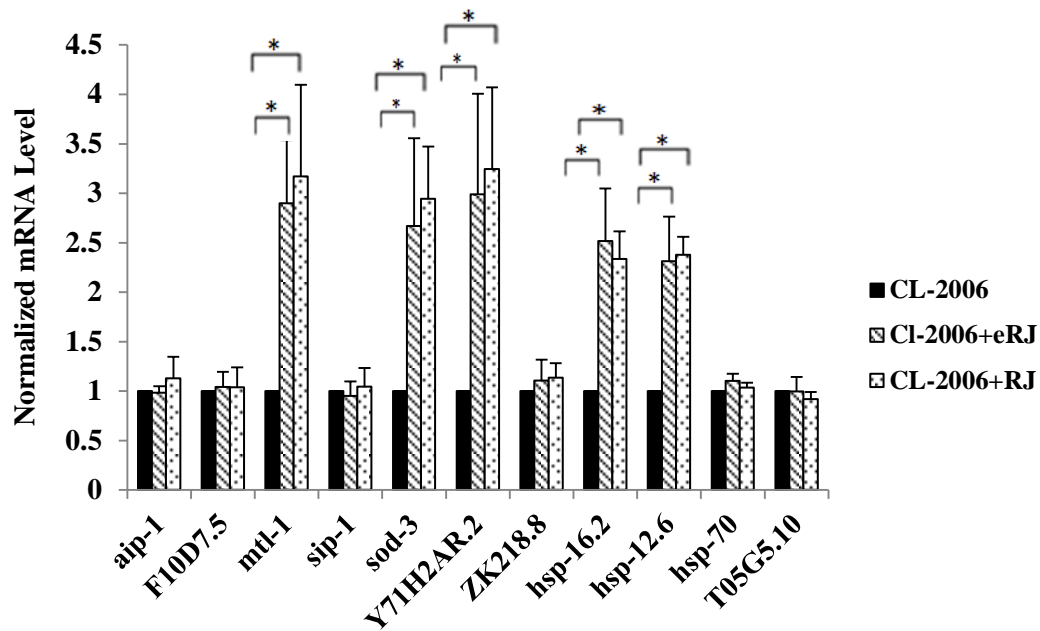


Figure 5.4 The transcript levels of *mtl-1*, *sod-3*, *Y71H2AR.2*, *hsp-12.6*, and *hsp-16.2* are up-regulated through RJ/eRJ mediated elevation of DAF-16's trans-activity. The expression levels of *aip-1*, *F10D7.5*, *mtl-1*, *sip-1*, *sod-3*, *Y71H2AR.2*, *hsp-12.6*, *hsp-16.2*, *hsp-70*, and *T02G5.10* were measure with qRT-PCR in CL-2006 worms supplemented with RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively). The data from three independent experiments were pooled to calculate the mean mRNA level normalized to the internal control *act-1*. The standard errors of the mean (SEM) were shown. “*” indicated $p < 0.05$ when compared to non-treated controls.

Table 8. The Paralysis Data of Worms at 20 °C

strain	Mean±SE	Median	No. of Worms	<i>p</i> value
EV(L4440) RNAi ^{†,‡}	10.708±0.188	11.00	253	
EV(L4440) RNAi+RJ ^{†,‡}	13.008±0.234	13.00	261	5.68E-05
EV(L4440) RNAi+eRJ ^{†,‡}	12.379±0.223	12.00	256	1.41E-04
<i>age-1</i> RNAi [†]	15.391±0.201	15.00	335	
<i>age-1</i> RNAi+RJ [†]	15.463±0.201	15.00	348	0.222
<i>age-1</i> RNAi+eRJ [†]	15.438±0.214	15.00	331	0.095
<i>daf-16</i> RNAi [†]	8.821±0.113	9.00	340	
<i>daf-16</i> RNAi+RJ [†]	9.053±0.115	9.00	318	0.53
<i>daf-16</i> RNAi+eRJ [†]	8.909±0.128	9.00	339	0.47
EV(PAD12) RNAi*	12.734±0.181	13.00	323	
EV(PAD12) RNAi+RJ*	15.699±0.205	16.00	316	1.31E-04
EV(PAD12) RNAi+eRJ*	15.006±0.194	15.00	315	5.81E-05
<i>daf-2</i> RNAi*	19.253±0.273	20.00	348	
<i>daf-2</i> RNAi+RJ*	19.389±0.313	20.00	314	0.712
<i>daf-2</i> RNAi+eRJ*	19.296±0.305	21.00	301	0.728

Notes: Paralysis and standard error are shown in days. The paralysis experiments were repeated at least three times with similar results, and the data for representative experiments were shown. The paralysis data were analyzed using the log-rank test and *p* values for each individual experiment are shown.

[†]Results presented in Figure 5.3 A

*Results presented in Figure 5.3 B

[‡] Results presented in Figure 5.3 C

5.2.3.2 HSF-1 and SKN-1, two other important transcriptional factors in IIS pathway are dispensable in RJ/eRJ mediated protection against A β toxicity

Besides of DAF-16, SKN-1 and HSF-1 are another two important transcriptional factors at the downstream of IIS pathway, and also play a key role in regulating A β aggregation to alleviate A β toxicity in *C. elegans*. To this end, we examined whether SKN-1 and HSF-1 were required in this RJ/eRJ mediated beneficial effect. CL-2006 worms supplemented with or without RJ/eRJ were fed with RNAi bacteria of *skn-1* and *hsf-1*, respectively. CL-2006 worms fed with RNAi control (L4440 empty vector) bacteria served as a system control. Meanwhile, another set of young adult worms treated with or without RJ/eRJ were fed with particular RNAi (*skn-1* and *hsf-1* respectively) and paired RNAi control bacteria for 6 days. Then the 6-day old worms in each treatment were collected to carry out a quantitative PCR experiment to check the efficiency of each RNAi.

Our results showed that RJ/eRJ still delayed the progression of paralysis in CL-2006 worms fed with *skn-1* and *hsf-1* RNAi bacteria respectively, comparing with matched non-supplemented controls (Figure 5.5 A and B). Based on the analysis, RJ/eRJ treated worms displayed an average increase of the mean paralysis time by of 24.67% (RJ) and 22.18% (eRJ) when fed with *hsf-1* RNAi bacteria. Similarly, RJ/eRJ supplemented worms showed an average increase of mean paralysis time by 17.74% (RJ) and 16.56% (eRJ) when fed with *skn-1* RNAi bacteria, which were spastically significant when comparing with non-treated control group (Table 9). Considering that in paired RNAi control bacteria group, RJ/eRJ supplementation delayed 21.48% (RJ) and 15.61% (eRJ)

of mean paralysis time when compared with non-supplemented controls, which were nearly the same in *hsf-1* and *skn-1* RNAi bacteria fed worms. This comparison indicated that SKN-1 and HSF-1 might be not dispensable in RJ/eRJ mediated protection against A β toxicity. Moreover, RNAi efficiency was needed to be checked before we further confirmed the genetic data. Our quantitative PCR results showed that the RNA interference applied in this experiment successfully repressed almost 50% of *skn-1* gene expression and 90% of *hsf-1* gene expression, which indicated that RNAi method worked successfully to knock down selected gene expression in genetic epistasis assays and our genetic results were reliable. Taken together, our findings suggested that SKN-1 and HSF-1 were not required in RJ/eRJ mediated protection against A β toxicity.

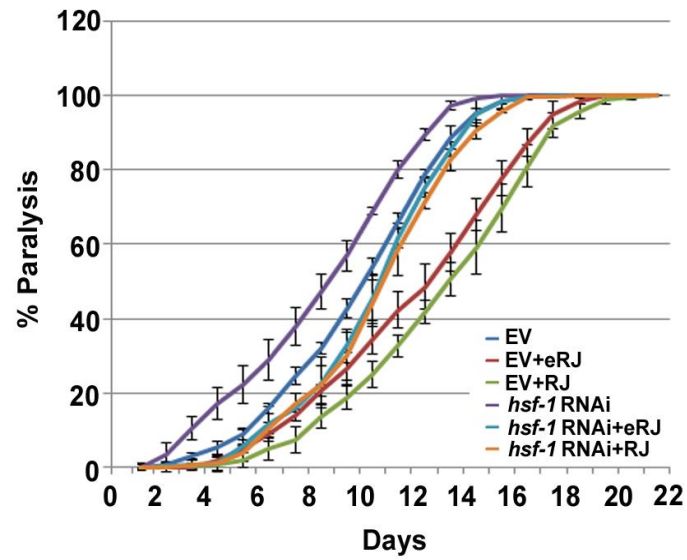
5.2.3.3 JNK pathway is not required in RJ/eRJ mediated protection against A β toxicity in AD worms

Our previous results showed that RJ/eRJ required IIS/DAF-16 to delay the progression of paralysis in AD worms. It is well known that in addition to IIS pathway, JNK pathway could also regulate the activity of DAF-16. Moreover, JNK pathway also plays a key role in the development of neurodegenerative diseases. Next, we tested whether *jnk-1*, a major component of JNK pathway, is required by RJ/eRJ in the protection of A β toxicity. Following the same method, CL-2006 worms treated with or without RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively) were fed with *jnk-1* RNAi and control RNAi (L4440 empty vector) bacteria to observe paralysis. Meanwhile, another set of worms treated with or without RJ/eRJ were fed with *jnk-1* RNAi and

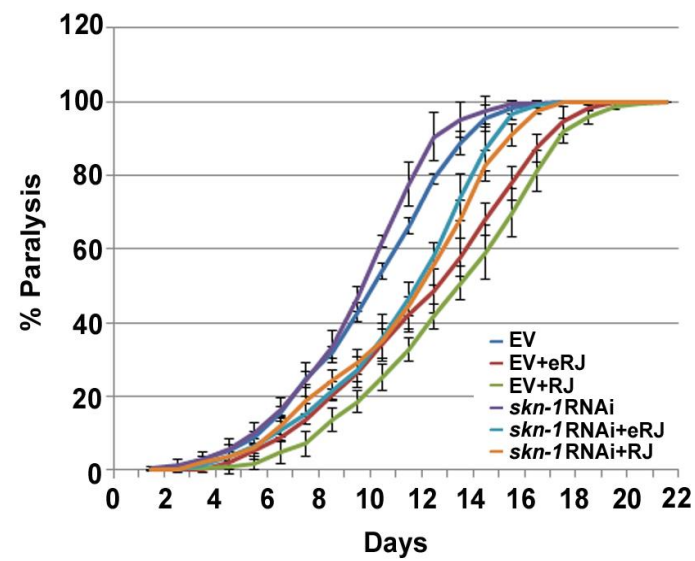
control RNAi for 6 days. Then the 6 day old worms in each treatment were collected to conduct a quantitative PCR experiment to check the efficiency of RNA interference.

Our results showed that RJ/eRJ still slowed down the progression of paralysis of CL-2006 worms fed with *jnk-1* RNAi bacteria (Figure 5.5 C). Specifically, RJ/eRJ increased the mean paralysis time by 22.43% (RJ) and 22.59% (eRJ) when compared with non-treated controls, and this increased rate was almost as same as RNAi control bacteria (Table 9). Moreover, the quantitative PCR result showed that *jnk-1* RNAi applied in this experiment repressed almost 70% of *jnk-1* gene expression, which confirmed that *jnk-1* RNAi efficiently knocked down *jnk-1* gene expression in genetic epistasis assays and our genetic results were reliable (Figure 5.5 D). Taken together, the results suggested that JNK pathway were not required in RJ/eRJ mediated protection against A β toxicity.

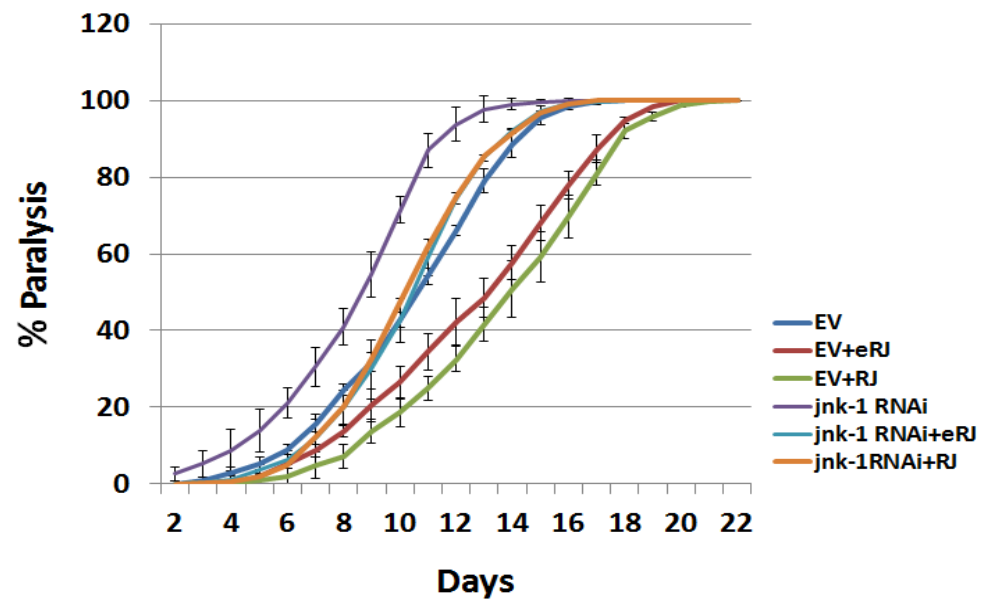
A



B



C



D

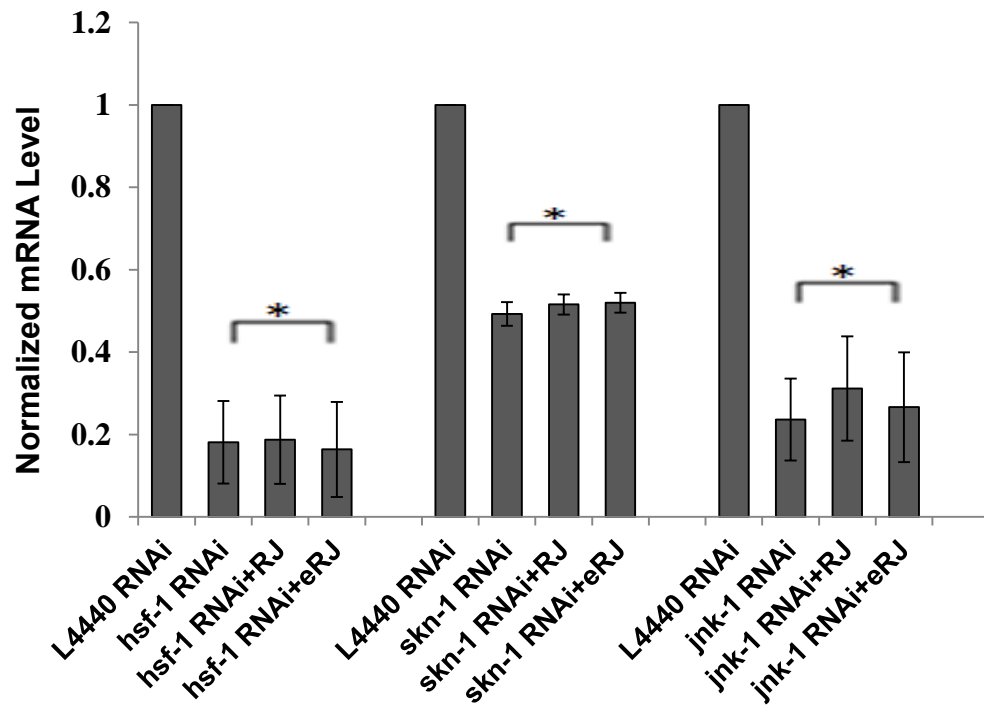


Figure 5.5 RJ/eRJ mediated protection against A β toxicity is dispensable on HSF-1, SKN-1 and JNK-1. (A). RJ/eRJ slowed down the progression of body paralysis in CL-2006 worms with reduced HSF-1 level by *hsf-1* RNAi. (B). RJ/eRJ delayed the progression of toxicity-induced paralysis in CL-2006 worms with decreased SKN-1 level by *skn-1* RNAi. Each paralysis assay was repeated at least three independent times with similar results. “% paralysis” indicated the average paralysis among the multi-replicates and error bars represent the standard deviation. (C). RJ/eRJ mediated protection against A β toxicity was dispensable on JNK pathway. RJ/eRJ slowed down the progression of body paralysis in CL-2006 worms with reduced JNK-1 level by *jnk-1* RNAi. Each paralysis assay was repeated at least three independent times with similar results. “% paralysis” indicated the average paralysis among the multi-replicates and error bars represent the standard deviation. (D). RNA interference efficiently knocked down the selected gene expression (*hsf-1*, *skn-1*, and *jnk-1*, respectively) in CL-2006 worms’ genetic epistasis assays. The expression levels of *hsf-1*, *skn-1*, and *jnk-1* were measured with qRT-PCR in CL-2006 worms supplemented with RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively). CL-2006 worms feeding with paired RNAi control (L4440 empty vector) served as controls to check gene knock down efficiency. The data from three independent experiments were pooled to calculate the mean mRNA level normalized to the internal control *act-1*. The standard errors of the mean (SEM) are shown. “*” indicated $p < 0.05$ when compared to paired RNAi bacteria controls.

Table 9. The Paralysis Data of CL-2006 Worms at 20 °C

strain	Mean±SE	Median	No. of Worms	<i>p</i> value
EV(L4440) RNAi ^{*,†,‡}	10.708±0.188	11.00	253	
EV(L4440) RNAi+RJ ^{*,†,‡}	13.008±0.234	13.00	261	5.68E-05
EV(L4440) RNAi+eRJ ^{*,†,‡}	12.379±0.223	12.00	256	1.41E-04
<i>jnk-1</i> RNAi [*]	8.737±0.159	9.00	308	
<i>jnk-1</i> RNAi+RJ [*]	10.697±0.151	11.00	304	1.37E-04
<i>jnk-1</i> RNAi+eRJ [*]	10.771±0.147	11.00	328	6.27E-05
<i>hsf-1</i> RNAi [†]	9.401±0.187	10.00	309	
<i>hsf-1</i> RNAi+RJ [†]	11.720±0.166	12.00	307	2.65E-05
<i>hsf-1</i> RNAi+eRJ [†]	11.486±0.157	12.00	313	2.65E-05
<i>skn-1</i> RNAi [‡]	10.435±0.167	11.00	294	
<i>skn-1</i> RNAi+RJ [‡]	12.286±0.197	13.00	311	0.001
<i>skn-1</i> RNAi+eRJ [‡]	12.163±0.183	13.00	301	0.001

Notes: Paralysis and standard error are shown in days. The paralysis experiments were repeated at least three times with similar results, and the data for representative experiments were shown. The paralysis data were analyzed using the log-rank test and *p* values for each individual experiment are shown.

*Results presented in Figure 5.5 C

†Results presented in Figure 5.5 A

‡Results presented in Figure 5.5 B

5.2.3.4 RJ/eRJ partially requires SIR-2.1, rather than HCF-1 and 14-3-3, to protect AD worms against A β toxicity

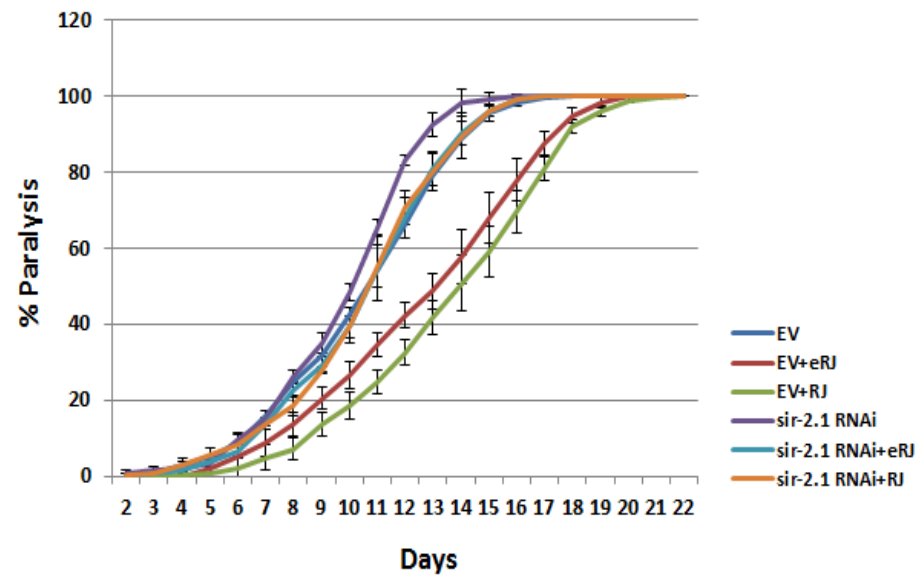
In chapter three, we reported that SIR-2.1, HCF-1, and 14-3-3, three important DAF-16 co-factors, modulated DAF-16 activity to extend the lifespan of *C. elegans*. Considering that the link between pro-longevity and anti-AD functions, herein we wondered whether these three proteins were involved in RJ/eRJ mediated protection of A β toxicity. CL-2006 worms supplemented with or without RJ/eRJ were fed with RNAi control (L4440 empty vector) bacteria and particular RNAi bacteria of *sir-2.1*, *hcf-1*, and *ftt-2*, respectively. Meanwhile, another set of young adult worms treated with or without RJ/eRJ were fed with particular RNAi (*sir-2.1*, *hcf-1*, and *ftt-2*, respectively) and paired RNAi control bacteria for 6 days. Then the worms were collected to conduct a quantitative PCR experiment to determine the efficiency of RNAi. Based on the analysis, our results showed that RJ/eRJ still delayed the progression of paralysis in CL-2006 worms fed with *sir-2.1*, *hcf-1*, and *ftt-2* RNAi bacteria respectively, when compared with matched non-supplemented controls (Figure 5.6 A, B, and C). It is worth noticing that RJ/eRJ supplemented worms displayed an increase of mean paralysis time by 7.07% (RJ) and 7.03% (eRJ) when fed with *sir-2.1* RNAi bacteria comparing with the non-treated controls fed with the same RNAi bacteria. However, in the paired RNAi control bacteria group, RJ/eRJ supplementation extended 21.48% (RJ) and 15.61% (eRJ) of mean paralysis time when compared with non-supplemented controls, which were nearly 2 to 3 times longer than the increased rates in *sir-2.1* RNAi bacteria group (Table 10). These

results demonstrated that SIR-2.1 was partially required in RJ/eRJ mediated protection of A β toxicity.

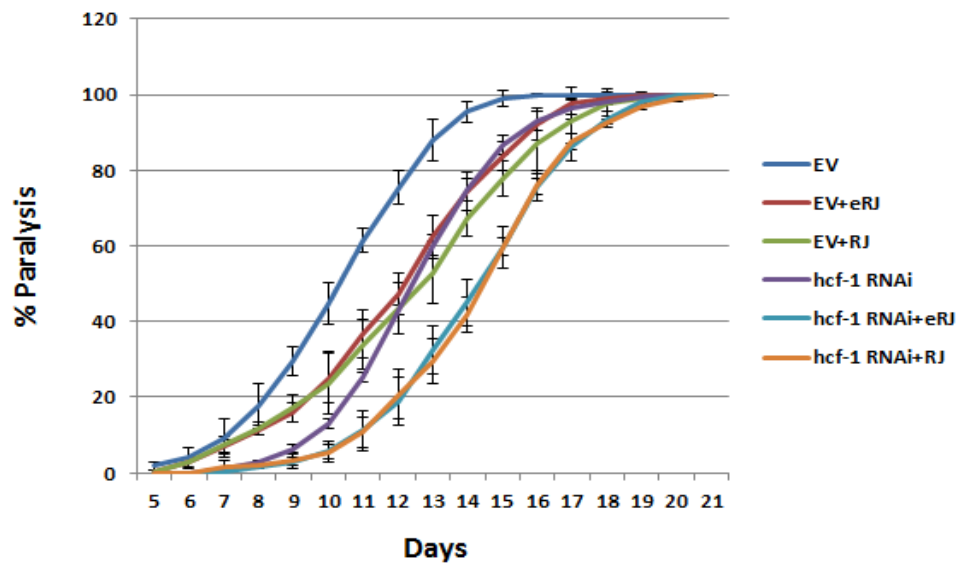
Next, the same strategy was utilized to analyze whether another two proteins, HCF-1 and 14-3-3, are involved in this RJ/eRJ mediated beneficial effect. Specifically, RJ/eRJ supplemented CL-2006 worms showed an average increase of mean paralysis time by 20.19% (RJ) and 14.84% (eRJ) when fed with *ftt-2* RNAi bacteria. Similarly, RJ/eRJ supplemented worms showed an average increase of mean paralysis time by 13.31% (RJ) and 12.98% (eRJ) when fed with *hcf-1* RNAi bacteria. Interestingly, unlike *sir-2.1* RNAi treatment, these two genes RNAi paralysis assays showed the RJ/eRJ mediated mean paralysis extended rates were almost the same in worms fed with *hcf-1* RNAi and *ftt-2* RNAi bacteria groups, when compared with worms fed with paired RNAi control bacteria (Figure 5.6 B and C). These data implied that HCF-1 and 14-3-3 were dispensable in RJ/eRJ mediated protection of A β toxicity.

In order to confirm our genetic data, a quantitative PCR experiment result was carried out to check the efficiency of RNAi. As we had expected, the RNAi applied in this experiment repressed almost 80% of *sir-2.1* gene expression, 75% of *ftt-2* gene expression, and 85% of *hcf-1* gene expression (Figure 5.6 D). These results indicated that RNAi successfully knocked down the selected genes expression in paralysis assays and further confirmed that our genetic analysis data were reliable. Collectively, our results suggested that SIR-2.1 was partially required in RJ/eRJ mediated protection against A β toxicity, whereas HCF-1 and 14-3-3 were dispensable in this beneficial effect.

A



B



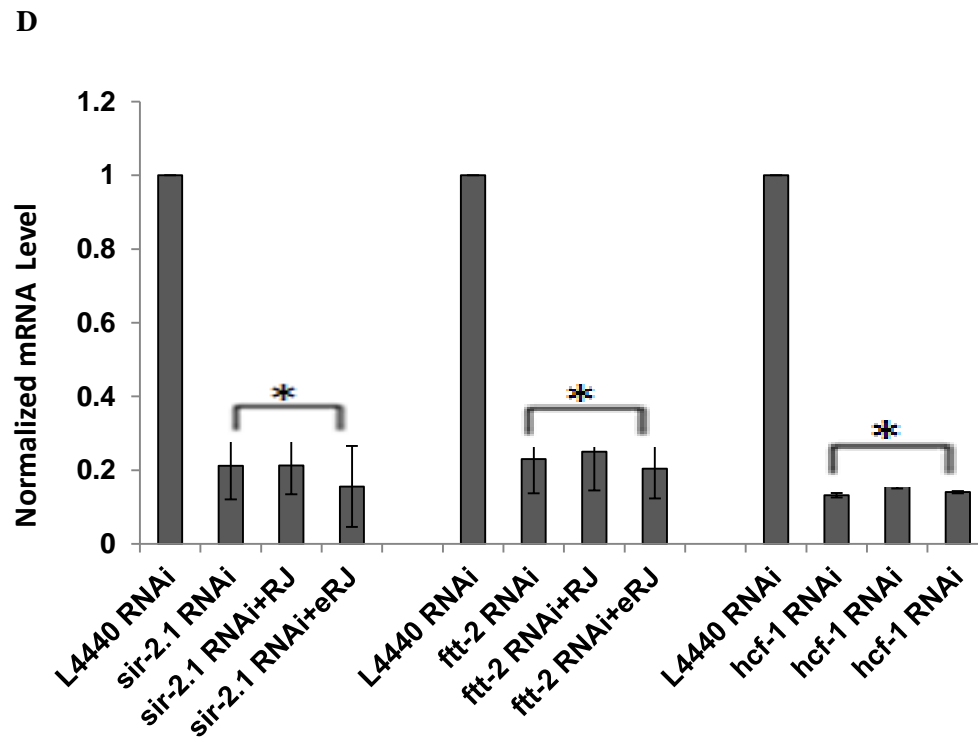
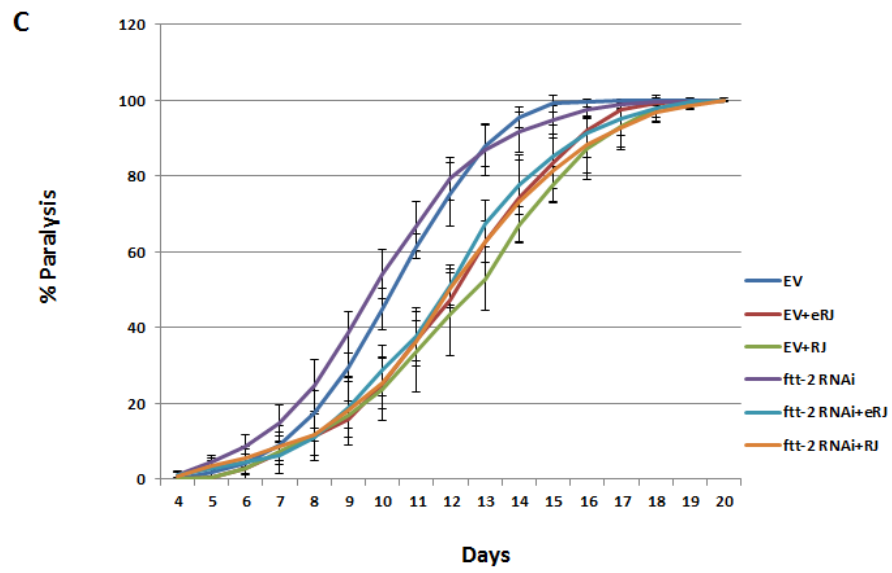


Figure 5.6 RJ/eRJ mediated protection against A β toxicity is dispensable on HCF-1 and 14-3-3, but partially dependent on SIR-2.1. (A). RJ/eRJ slowed down the progression of body paralysis in CL-2006 worms with reduced SIR-2.1 level by *sir-2.1* RNAi. (B). RJ/eRJ delayed the progression of toxicity-induced paralysis in CL-2006 worms with decreased HCF-1 level by *hcf-1* RNAi. (C). RJ/eRJ delayed the progression of toxicity-induced paralysis in CL-2006 worms with decreased 14-3-3 level by *ftt-2* RNAi. Each paralysis assay was repeated at least three independent times with similar results. “% paralysis” indicated the average paralysis among the multi-replicates and error bars represent the standard deviation. (D). RNA interference efficiently knocked down selected gene expression (*sir-2.1*, *hcf-1*, and *ftt-2*, respectively) in CL-2006 worms’ genetic epistasis assays. The expression levels of *sir-2.1*, *hcf-1*, and *ftt-2* were measured with qRT-PCR in CL-2006 worms supplemented with RJ/eRJ (2mg/mL RJ and 1mg/mL eRJ, respectively). CL-2006 worms feeding with paired RNAi control (L4440 empty vector) served as controls to check gene knock down efficiency. The data from three independent experiments were pooled to calculate the mean RNA level normalized to the internal control *act-1*. The standard errors of the mean (SEM) were shown. “*” indicated $p < 0.05$ when compared to paired RNAi bacteria controls.

Table 10. The Paralysis Data of CL-2006 Worms at 20 °C

strain	Mean±SE	Median	No. of Worms	<i>p</i> value
EV(L4440) RNAi*	10.708±0.188	11.00	253	
EV(L4440) RNAi+RJ*	13.008±0.234	13.00	261	5.68E-05
EV(L4440) RNAi+eRJ*	12.379±0.223	12.00	256	1.41E-04
<i>sir-2.1</i> RNAi*	10.520±0.151	11.00	277	
<i>sir-2.1</i> RNAi+RJ*	11.264±0.172	12.00	277	0.002
<i>sir-2.1</i> RNAi+eRJ*	11.260±0.166	12.00	285	0.002
EV(L4440) RNAi ^{†,‡}	11.099±0.133	11.00	332	
EV(L4440) RNAi+RJ ^{†,‡}	12.848±0.179	13.00	329	7.51E-04
EV(L4440) RNAi+eRJ ^{†,‡}	12.477±0.163	13.00	329	8.68E-04
<i>ftt-2</i> RNAi [†]	10.381±0.147	10.00	381	
<i>ftt-2</i> RNAi+RJ [†]	12.477±0.186	12.00	329	3.13E-04
<i>ftt-2</i> RNAi+eRJ [†]	11.922±0.160	12.00	375	4.28E-04
<i>hcf-1</i> RNAi [‡]	13.005±0.121	13.00	381	
<i>hcf-1</i> RNAi+RJ [‡]	14.736±0.146	15.00	329	0.002
<i>hcf-1</i> RNAi+eRJ [‡]	14.693±0.133	15.00	375	0.002

Notes: Paralysis and standard error were shown in days. The paralysis experiments were repeated at least three times with similar results, and the data for representative experiments are shown. The paralysis data were analyzed using the log-rank test and *p* values for each individual experiment are shown.

*Results presented in Figure 5.6 A

†Results presented in Figure 5.6 B

‡Results presented in Figure 5.6 C

5.2.4 RJ/eRJ supplementation increases protein solubility in aged *C. elegans*

Aging and persisting stresses cause cumulative damages to cells, which could destruct proteins' conformation to induce the deposition of insoluble proteins. Some nutraceuticals could combat this damage by promoting the protein solubility to maintain a functional proteostasis. Considering that RJ/eRJ slowed down the progression of paralysis in AD worms, we wondered that whether RJ/eRJ's beneficial effect is dependent on promoting solubility of proteins in aged worms. To this end, synchronized young adult N2 worms and CL-2006 worms were treated with or without RJ/eRJ for 10 days. Then the aged worms were collected for total proteins extraction. After sonication and high speed centrifugation, soluble fraction was isolated from the total proteins. SDS-PAGE and Image-J were utilized to investigate whether RJ/eRJ affect the solubility of proteins in aged *C. elegans*. Our results showed that RJ/eRJ increased 26.95% (RJ) and 26.87% (eRJ) soluble protein amount relative to non-supplemented controls in N2 worms. Similarly, RJ/eRJ also displayed an average increase of CL-2006 worms' soluble proteins amount by 27.13% (RJ) and 21.27% (eRJ) when compared with controls without RJ/eRJ supplementation (Figure 5.7 A and B). These results were statically significant, which indicated that RJ/eRJ improved functional proteostasis in aged *C. elegans*.

Our previous results indicated that DAF-16 was required in RJ/eRJ mediated lifespan extension, stress resistance, and protection against A β toxicity. It is highly possible that DAF-16 might also be required in increasing the protein solubility under RJ/eRJ supplementation. In order to confirm this hypothesis, synchronized *daf-16* (*mgDf50*) mutant worms and *daf-16* RNAi bacteria feeding CL-2006 worms were treated

with or without RJ/eRJ for 10 days. Then the aged worms were collected for total protein extraction. Sonication and centrifugation were utilized to remove the insoluble proteins, and the amount of soluble protein was measured by SDS-PAGE and Image-J software. Specifically, RJ/eRJ still increased the amount of soluble proteins by 5.44% (RJ) and 2.20% (eRJ) when compared with the non-treated controls in *daf-16* deletion mutant worms. Additionally, RJ/eRJ increased the amount of soluble proteins by 5.12% (RJ) and 3.82% (eRJ) when compared with the non-supplemented controls in CL-2006 worms fed with *daf-16* RNAi bacteria (Figure 5.8 A and B). However, the student *t* test showed that these increases were not statistically significant. To this end, these results indicated that RJ/eRJ cannot improve protein solubility of *daf-16* deletion mutant worms and *daf-16* knocked down CL-2006 worms.

Taken together, RJ/eRJ improved protein solubility in aged worms and thus delayed the development of AD. Noticeably, DAF-16 displayed a very important role in this process to maintain a healthy proteostasis.

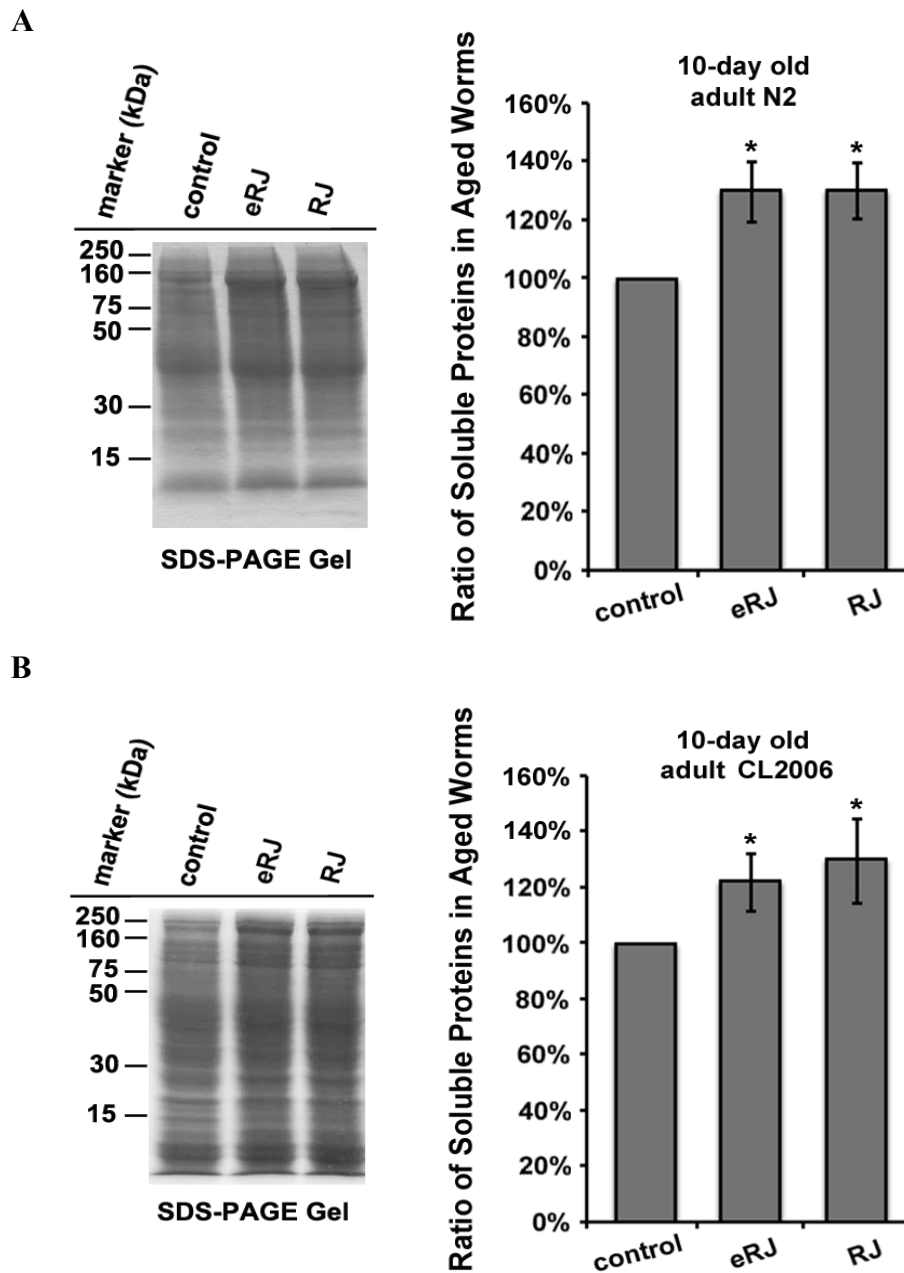
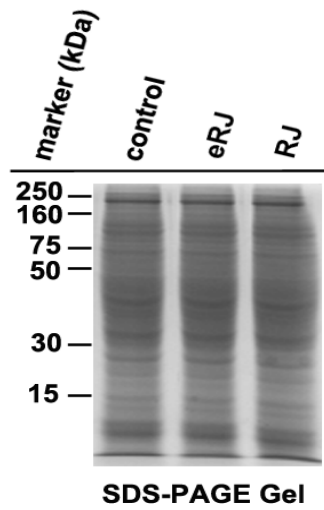


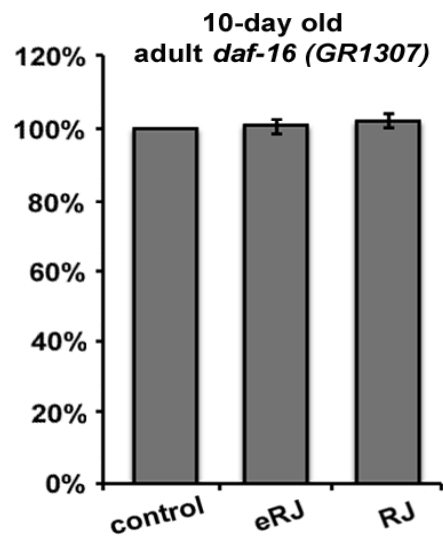
Figure 5.7 RJ/eRJ improves proteostasis in aged *C. elegans*. (A). RJ/eRJ treatment increased protein solubility in 10-day old N2 worms. (B). RJ/eRJ supplementation increased protein solubility in 10-day old CL-2006 worms. Soluble proteins on the SDS-PAGE gel were quantified by using Image-J software. Data were

expressed as mean intensity from three independent experiments. The standard errors of the mean (SEM) were shown. p value was calculated using Student's t -test. “*” indicated $p < 0.05$ when compared to controls.

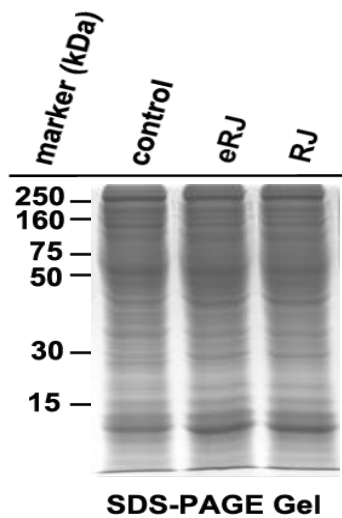
A



Ratio of Soluble Proteins in Aged Worms



B



Ratio of Soluble Proteins in Aged Worms

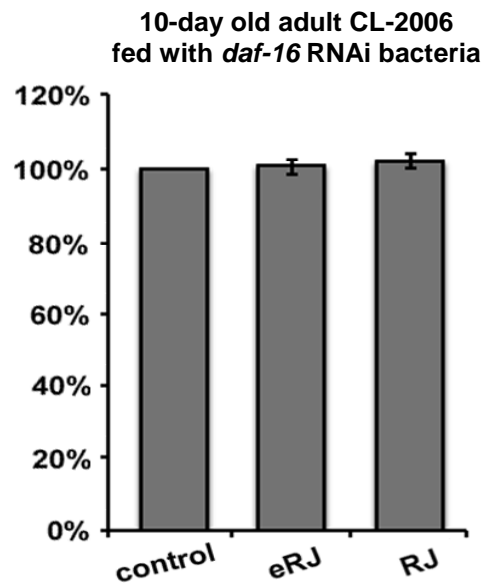


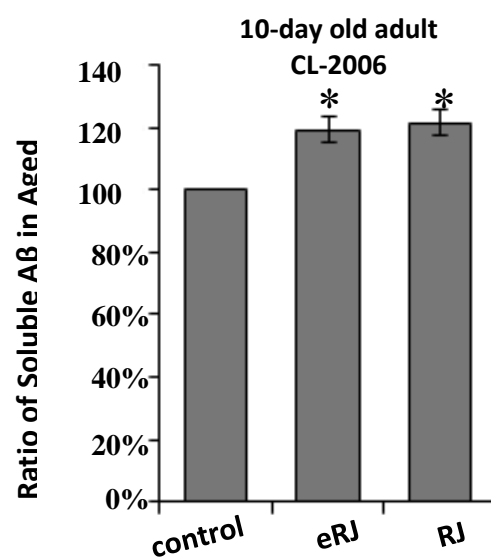
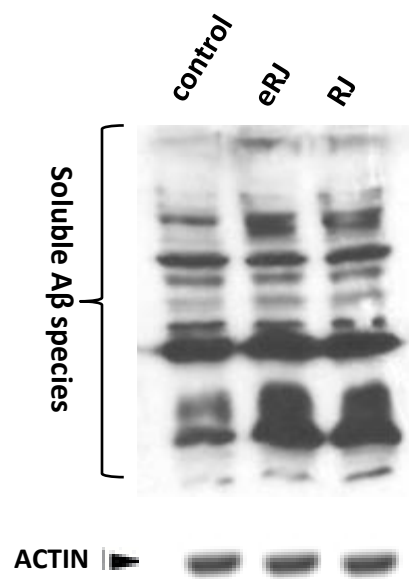
Figure 5.8 RJ/eRJ improves proteostasis in aged *C. elegans* dependent on DAF-

16. (A). RJ/eRJ treatment increased protein solubility in 10-day old *daf-16* (*mgDf50*) mutant worms. (B). RJ/eRJ supplementation increases protein solubility in 10-day old CL-2006 worms fed with *daf-16* RNAi bacteria. Soluble proteins on the SDS-PAGE gel were quantified by using Image-J software. Data were expressed as mean intensity from three independent experiments. The standard errors of the mean (SEM) were shown. *p* value was calculated using Student's *t*-test. “*” indicated *p* < 0.05 when compared to controls.

5.2.5 RJ/eRJ supplementation significantly increases solubility of A β species in aged worms

It is well known that healthy aging is often associated with functional proteostasis to reduce the proteotoxicity in cells. Given that RJ/eRJ could mitigate A β toxicity in AD worms, we wondered whether RJ/eRJ might promote the solubility of A β species in aged worms. To address this question, synchronized young adult CL-2006 worms were supplemented with or without RJ/eRJ for 10 days. Then the 10-day aged worms were collected for total proteins extraction. Sonication and centrifugation were utilized to divide proteins into soluble fraction and insoluble fraction. Afterwards, the amount of soluble A β species and insoluble A β species were measured by western blot and Image-J software. Our results showed that RJ/eRJ increased solubility of A β species in aged AD worms. Specifically, comparing with non-treated controls, RJ/eRJ supplementation increased the amount of soluble A β species 21.71% (RJ) and 19.31% (eRJ), whereas the amount of insoluble A β species was decreased by 27.63% (RJ) and 28.61% (eRJ), respectively (Figure 5.9 A and B). Considering that RJ/eRJ dramatically slowed down the progression of paralysis in AD worms, this result indicated that RJ/eRJ might promote the solubility of A β species in a toxicity reduction manner.

A



B

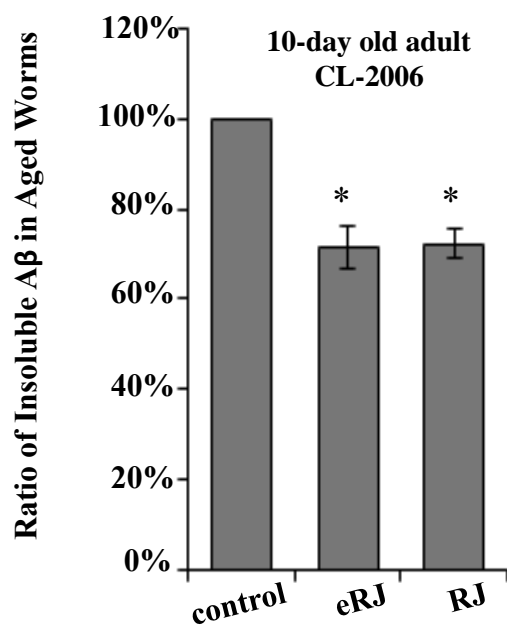
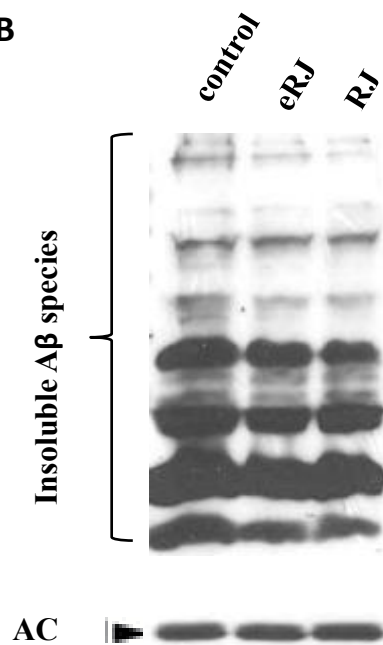


Figure 5.9 RJ/eRJ improves solubility of A β species in aged CL-2006 worms.

(A). RJ/eRJ supplementation increased soluble A β species in 10-day old CL-2006 worms.

(B). RJ/eRJ supplementation decreased insoluble A β species in 10-day old CL-2006 worms. The amount of soluble and insoluble proteins was quantified by using western blot and Image-J software. DATA were expressed as mean intensity from three independent experiments. The standard errors of the mean (SEM) were shown. *p* value was calculated using Student's *t*-test. “*” indicated *p* <0.05 when compared to controls.

5.3 Discussion

In chapter three and four, our results showed that RJ/eRJ supplementation had beneficial effects of pro-longevity and stress resistance in *C. elegans*. A growing body of evidence suggests that some anti-aging nutraceuticals could delay the development of neurodegenerative diseases[123, 146, 195]. For example, cranberry protected *C. elegans* from thermal stress and slowed down the development of AD; Resveratrol, an antioxidant component from grape seed, not only had pro-longevity function but also delayed the development of Alzheimer's diseases and Parkinson's disease[147]. Therefore, it is interesting to further investigate whether RJ/eRJ also has the capacity to slow down the development of Alzheimer's disease.

CL-2006 mutant strain, an AD *C. elegans* model, which transgenically expresses human A β ₃₋₄₂ in the worms' body muscle cells, has been considered as a well-established model to investigate the link between AD and A β pathology[192]. During the aging process, A β species were aggregated and accumulated inside and outside the muscle and neuron cells, which causes the worms' body paralysis via deposition of cytotoxic plaques. To date, paralysis is a direct indicator to reflect A β toxicity in AD worms.

In our study, our results showed that RJ/eRJ supplementation reduced the total amount of A β species and thereby delayed the progression of paralysis in AD worms. Additionally, RJ/eRJ supplemented CL-2006 worms' lifespan. It is worth noticing that RJ treatment better protected AD worms from A β toxicity when compared with eRJ treatment. Our paralysis assay results showed that RJ/eRJ treatment increased the mean time of paralysis by 16.10% (RJ) and 10.92% (eRJ) when compared with the mean

paralysis time in non-supplemented controls in CL-2006 worms feeding with OP50 E. coli (figure 5.2 A and Table 7). Moreover, RJ/eRJ supplementation extended the mean paralysis time by 21.48% (RJ) and 15.61% (eRJ) when compared with the mean paralysis time in non-supplemented controls in CL-2006 worms feeding with RNAi control (L4440 empty vector) bacteria. In addition to these two sets of data, another RNAi control (PAD12 empty vector) bacteria treatment also showed the same trend that RJ extended nearly 1 day longer of mean paralysis when compared with the mean time of paralysis in eRJ treatment. In agreement with these paralysis assay results, our dot blot assay showed that RJ treatment reduced 21.49% A β species, while eRJ treatment only reduces 14.51% A β species.

As we mentioned in chapter three, the difference between the two types of RJ powders is that RJ contains more large proteins than eRJ powder. It is possible that these large proteins might promote immune response to eliminate toxic A β species. In agreement with these paralysis assay results, our dot blot assay showed that RT treatment reduced the A β species by 21.49%, while eRJ treatment only reduced the A β species by 14.51%, comparing with the amount of A β species in non-supplemented controls. However, further experiments are needed to be done to better elucidate the different beneficial effects between RJ and eRJ powders.

In our study, CL-2006 worms were treated with RJ/eRJ after L4/young adult stage rather than egg stage. The reason was that RJ/eRJ affected the hatching rate and development of CL-2006 worms. This negative effect was only observed in CL-2006 strain rather than other types of worms in the previous study. Based on our observation,

when CL-2006 worms were treated with RJ/eRJ from the egg stage, their hatching rate and worms' size were reduced when compared with that of non-treated CL-2006 worms. To note, these negative effects were even worse in RJ treatment than eRJ treatment. It is possible that RJ/eRJ might contain some proteins to reduce CL-2006 worms' hatching rate and affect their development to a certain degree. Moreover, the large proteins in RJ powder might dramatically exacerbate these negative effects.

Generally, although there were some differences between RJ/eRJ's protein component and beneficial effects, both RJ and eRJ could slow down the progression of A β toxicity induced paralysis. Considering that CL-2006 worms' A β species were human derived, it is highly possible that RJ/eRJ supplementation might have potential capacity to mitigate A β toxicity in humans. However, clinical research is needed to further confirm this hypothesis.

As shown in this chapter, RJ/eRJ supplementation possessed the ability to alleviate A β toxicity in AD worms. However, the molecular mechanism underlying this beneficial effect is unknown. Moll and colleagues reported that reduction of Insulin Signaling (IIS) Pathway could prevent neurodegeneration in worms and mammals. They demonstrated that the reduction of IIS pathway could activate its three important transcriptional factors. DAF-16, HSF-1, and SKN-1, to inhibit age-mediated proteotoxicity and thereby maintain a healthy aging[144]. It is possible that IIS pathway and its three important transcriptional factors might also play a key role in RJ/eRJ mediated protection against A β toxicity. To address this concern, RNA interference (RNAi) was utilized to inhibit the gene expression of two important components (*daf-2*

and *age-1*) and three transcriptional factors (*daf-16*, *skn-1*, and *hsf-1*), respectively, to conduct genetic epistasis assays. Our findings indicated that IIS and DAF-16, rather than HSF-1 and SKN-1 were required in this RJ/eRJ mediated beneficial anti-AD effect (Figure 5.3 and 5.5). Moreover, quantitative PCR experiments were carried out to test whether DAF-16's trans-activity is influenced by RJ/eRJ supplementation. As expected, the increased level of *sod-3* and *mtl-1* confirmed that RJ/eRJ supplementation elevated the transactivity of DAF-16. Additionally, other DAF-16 target genes, *Y71H2AR.2*, *hsp-12.6*, and *hsp-16.2*, were also up-regulated in RJ/eRJ treated CL-2006 worms (Figure 5.4). Taken together, the genetic analysis and quantitative PCR results indicated that RJ/eRJ alleviated A β toxicity via influencing IIS pathway to elevate the transactivity of DAF-16.

SKN-1 and HSF-1 are also important factors to protect organisms from proteotoxicity by maintaining a healthy proteostasis. For example, HSF-1 regulates numerous heat shock proteins (HSPs) to facilitate the refolding of misfolded proteins in neurodegenerative diseases[37]. Similarly, SKN-1 also plays an important role in controlling protein quality and regulating detoxification of proteome[40, 196]. It is well known that a functional proteostasis could reduce the proteotoxicity and thereby alleviate the progression of paralysis. However, our genetic results showed that RJ/eRJ supplementation still dramatically extended the mean time of paralysis dramatically when compared with non-treated controls in *hsf-1* RNAi bacterial treatment and *skn-1* RNAi bacterial treatment (Figure 5.5) when compared the data among these two treatments and paired RNAi control (L4440 empty vector) bacteria, the increasing rates of mean

paralysis time were nearly the same (Table 9). This result further confirmed that SKN-1 and HSF-1 were dispensable in RJ/eRJ mediated protection against A β toxicity.

In addition to IIS pathway, the JNK pathway is also reported as an important regulator in neurodegenerative disorders. Specifically, Chen's lab demonstrated that JNK pathway was significantly up-regulated in AD mammalian models[197]. Based on our knowledge, JNK-1, an essential regulator in the JNK pathway, could modulate DAF-16, HSF-1, and SKN-1 in parallel to IIS pathway. Intriguingly, our results showed that RJ/eRJ treatment extended the same rates of mean paralysis in CL-2006 worms feeding with *jnk-1* RNAi bacteria and paired RNAi control (L4440 empty vector) bacteria (Figure 5.5 and Table 9). These results indicated that JNK pathway was not required in RJ/eRJ mediated protection against A β toxicity.

In chapter three and four, our research revealed that besides DAF-16, co-factors SIR-2.1, HCF-1, and 14-3-3 also play a very important role in RJ/eRJ mediated pro-longevity and stress resistance. Based on these results, we questioned whether these three DAF-16 co-factors are involved in RJ/eRJ mediated anti-AD function. Our results showed that SIR-2.1, rather than HCF-1 and 14-3-3, were partially required in RJ/eRJ mediated protection against A β toxicity. Unlike DAF-16, SIR-2.1 was partially involved in RJ/eRJ mediated anti-AD effect, because our genetic data showed that RJ/eRJ still extended the mean time of paralysis by 7.07% (RJ) and 7.03% (eRJ) in CL-2006 worms feeding with *sir-2.1* RNAi bacteria (Table 10), but this increasing rate was only half of the increasing rate in worms fed with CL-2006 RNAi control bacteria.

Numerous studies have revealed that SIRT1/SIR-2.1 plays a key role in prevention of neurodegenerative diseases. Araki reported that SIRT1 repressed axonal degeneration to decrease neuronal death rate in AD and Parkinson's disease in murine model[198]. Besides neuronal protection, Chen lab discovered that overexpression of SIRT1 could inhibit NF- κ B signaling via decreasing the acetylation of RelA/P65, which in turn protected mice against A β toxicity in AD development[199]. In agreement with their mammalian studies, our results showed that SIR-2.1 was partially involved in RJ/eRJ mediated protection against A β toxicity. Given that SIR-2.1 is an important DAF-16 co-factor to fine-tunes DAF-16's transactivity in RJ/eRJ mediated pro-longevity and stress resistance effects, it is conceivable that SIR-2.1 might modulate DAF-16's transactivity to slow down the progression of CL-2006 worms' paralysis under RJ/eRJ supplementation. Further experiment is needed to better understand the function of SIR-2.1 in neurodegenerative diseases. Taken together, although three DAF-16 factors-SIR-2.1, HCF-1, and 14-3-3-were involved in pro-longevity and stress resistance under RJ/eRJ supplementation, only SIR-2.1 was further required in RJ/eRJ mediated protection against A β toxicity in the AD worm model.

Considering that RJ/eRJ supplementation mediated anti-AD effect acted through IIS pathway and DAF-16, we further questioned how IIS/DAF-16 functions in this process. Several studies demonstrated that the imbalance of proteostasis is often linked to neurodegenerative diseases. For instance, Ben-Zvi further demonstrated that an age-related protein homeostasis disorders may lead to impaired protein solubility and increased cytotoxicity[190]. Reis-Rodrigues and colleagues further claimed that

accumulation of insoluble proteins with diverse biological functions may be shared as a general feature of normal aging and late onset neurodegenerative diseases[200]. The collective research of these age-related studies showed that better protein solubility directly reflects the healthier proteostasis and lower proteotoxicity. Interestingly, our results showed that RJ/eRJ significantly increased protein solubility in aged wild type worms and AD worms, which indicated that RJ/eRJ treated worms acquired the capacity to maintain a healthy proteostasis. Our previous results showed that DAF-16 was required in RJ/eRJ mediated protection against A β toxicity, which implied that RJ/eRJ might also require DAF-16 to retain a healthy proteostasis. In accordance with this hypothesis, our further solubility assay showed that the protein solubility of aged *daf-16* deletion mutant worms was barely influenced by RJ/eRJ supplementation. Taken together, our results indicated that RJ/eRJ improved the healthy proteostasis to mitigate A β toxicity in aged worms. This beneficial effect was dependent on DAF-16.

It is worth noticing that RJ/eRJ supplementation remarkably increased the solubility of high molecular weight (HMW) proteins (protein size over 70 KD) by more than 2 fold when compared with the increasing rates of solubility for low molecular weight (LMW) proteins (protein size smaller than 70 KD) (Figure 5.10 A and B). However, in *daf-16* (*mgDf50*) and *daf-16* RNAi feeding AD worms, this difference of increasing ratios in HMW soluble proteins and LMW soluble proteins was abolished, indicating that DAF-16 played a very important role in maintaining the configuration of large soluble proteins (Figure 5.10 C and D). As of yet, there is no conclusive evidence

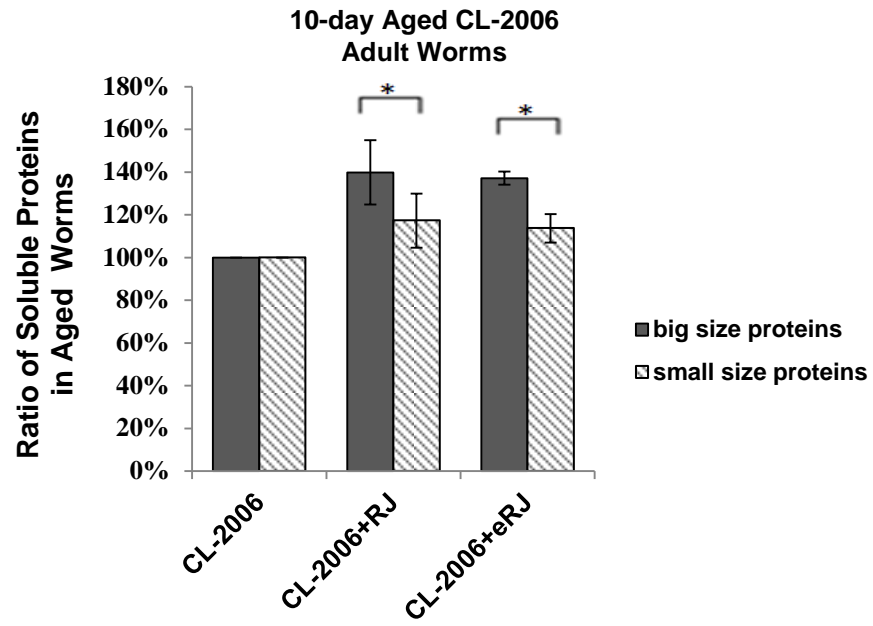
proving the relationship between the well-maintained conformation of LMW and healthy aging, but the further research would confirm this relationship.

To date, more and more research lab focus on investigating the link between A β conformation and its toxicity. Several clinical studies revealed that the amount of A β species detected in AD patients was not always higher than healthy people, which indicated that the amount of A β species is not a direct indicator to determine AD[201]. Furthermore, numerous studies have shown that not all of A β species are toxic to induce AD pathology, and the toxicity of A β species were dependent on their conformation[202]. Specifically, some scientists discovered that LMW A β / A β oligomers which prone to seed A β aggregation could accelerate the development of neurodegenerative diseases and proteinopathy[203]. Although the detailed molecular mechanism of proteotoxicity underlying neurodegenerative diseases is still unclear, numerous studies demonstrated that IIS pathway and its three critical transcriptional factors play an important role to regulate protein aggregation and proteotoxicity. In order to decipher the molecular mechanism of A β induced cytotoxicity, Cohen research lab reported that DAF-16 can promote protein aggregation to protect cells from the accumulation of soluble and toxic protein species[204].

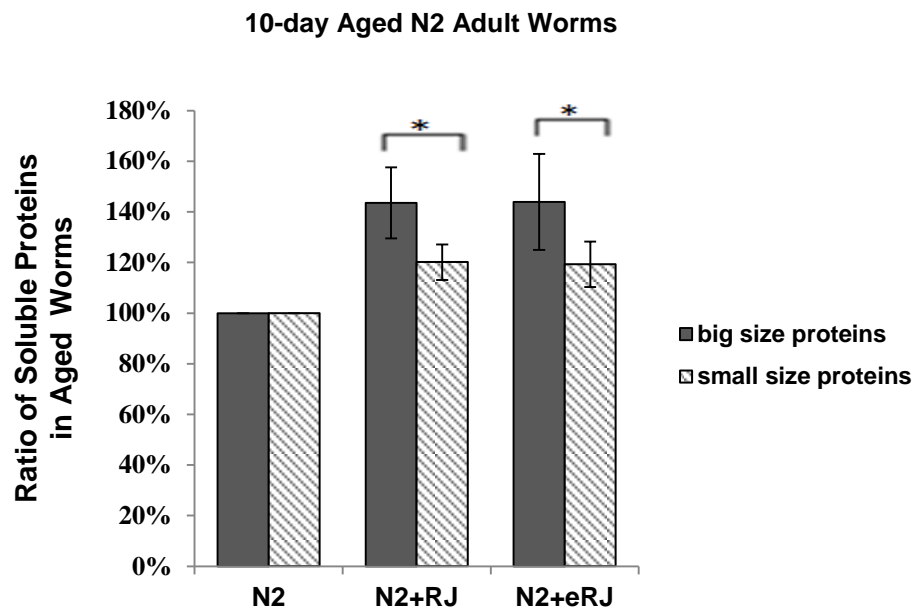
In our research, the solubility results implied that DAF-16 was regulated by RJ/eRJ supplementation to increase more than 2 times of soluble HMW proteins than LMW proteins in aged worms. There are two possible reasons to explain this phenomenon. First, in agreement with Cohen's discovery, DAF-16 could promote soluble LMW proteins aggregation to form more soluble HMW proteins in aging process.

Another possibility is that DAF-16 protected HMW proteins from degradation by retaining their soluble status conformation and stability. Based on our results, it is hard to tell whether DAF-16 functions to promote protein aggregation or prevent the degradation of HMW proteins. Therefore, further research was needed to be carried out to investigate DAF-16's function in proteostasis. Taken together, our findings suggested that RJ/eRJ supplementation improved the healthy proteostasis by maintaining the functional protein conformation in aged worms.

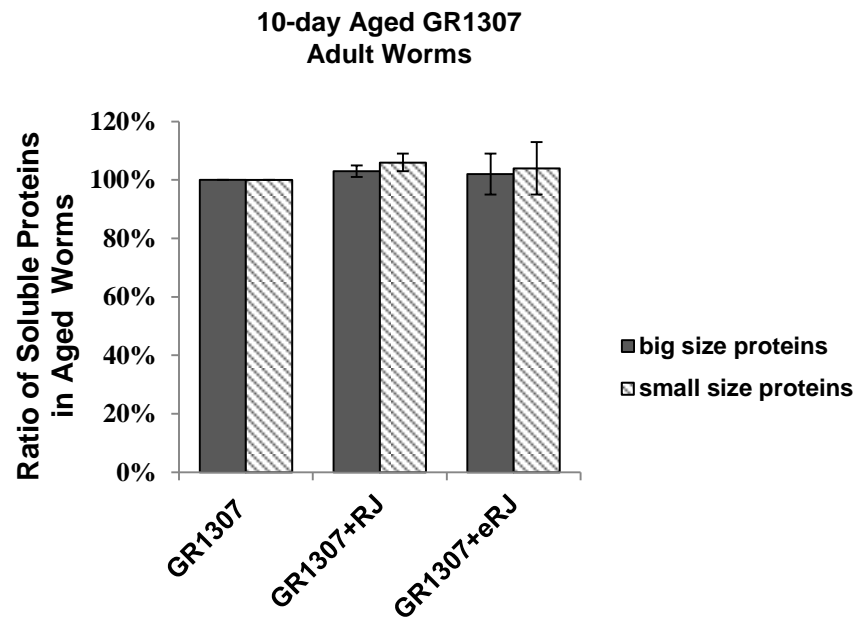
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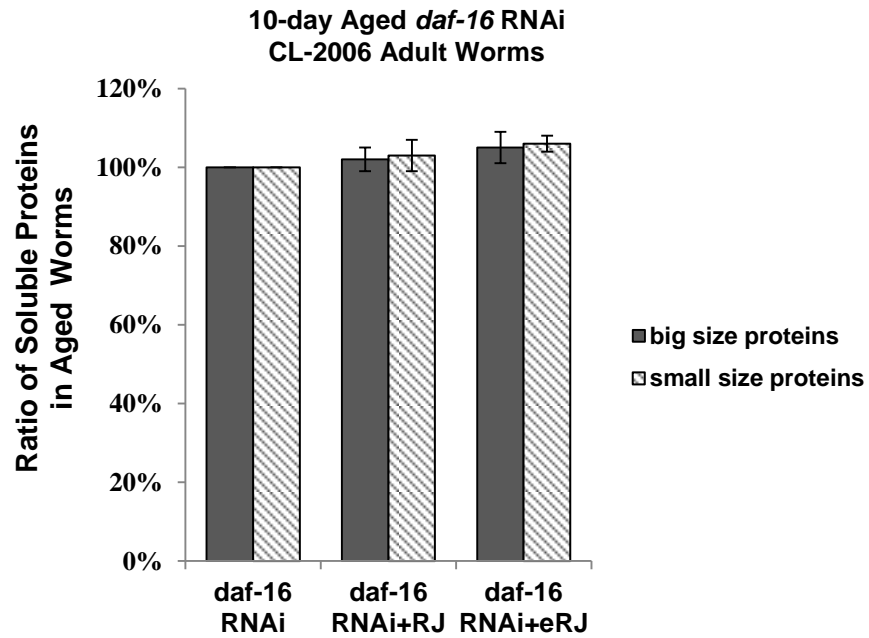


Figure 5.10 RJ/eRJ supplementation remarkably increases the solubility of high molecular weight (HMW) proteins (protein size over 70 KD) more than low molecular weight (LMW) proteins in a DAF-16 dependent manner. (A). RJ/eRJ treatment increased more HMW protein solubility than LMW protein solubility in 10-day old N2 worms. (B). RJ/eRJ treatment increased more HMW protein solubility than LMW protein solubility in 10-day old CL-2006 worms. (C). RJ/eRJ treatment increased same ratio of HMW protein solubility and LMW protein solubility in 10-day old *daf-16 (mgDf50)* worms. (D). RJ/eRJ treatment increases same ratio of HMW protein solubility and LMW protein solubility in 10-day old CL-2006 worms feeding with *daf-16* RNAi bacteria. Soluble proteins on the SDS-PAGE gel are quantified by using Image-J software. Data were expressed as mean intensity from three independent experiments. The standard errors of the mean (SEM) were shown. *p* value was calculated using Student's *t*-test. “*” indicated *p* < 0.05 when compared to controls.

It is well known that a healthy proteostasis indicates a balance of protein synthesis, folding, aggregation, and degradation. However, imbalance among these processes engenders deposition and aggregation of aberrant proteins, causing toxicity and damage to induce age-related disorders. In order to fight against this negative impact, cells are equipped with protective machinery to maintain a protein homeostasis in normal condition. The misfolded and unfolded proteins are usually refolded by HSPs or targeted by ubiquitin for proteasomal or lysosomal degradation[205]. However, this mechanism is overwhelmed in aging cells or under persisting harsh stresses conditions. As a result, the misfolded and unfolded proteins are aggregated and accumulated inside and outside the cells instead of being refolded or destroyed, and thus induces the neurodegenerative disorders. It is well known that HSPs function as a master regulator to maintain this important machinery in all conditions[187]. Several studies demonstrated that even overexpression of one HSP could dramatically delay the neurodegenerative diseases, which underscored HSPs' critical function in retaining the healthy proteostasis.

In agreement with this view, RJ/eRJ supplementation also positively regulated the levels of several protein turnover genes in AD worms, such as *mtl-1*, *hsp-12.6*, *hsp-16.2*, and *Y71H2AR.2*. *mtl-1*, an important downstream genes of DAF-16, plays an important role in metal detoxification and maintaining the protein homeostasis in stress conditions[206]. HSP-12.6 and HSP-16.2 are two essential heat shock proteins, which function as ubiquitous molecular chaperones to prevent the aggregation of misfolded and unfolded proteins in response to proteotoxic stressors[196, 207]. Additionally, Link's lab discovered that overexpression of HSP-16.2 could suppress the A β toxicity in AD worms,

which might be selected as a target to design anti-AD drugs. The last gene dramatically influenced by RJ/eRJ treatment was *Y71H2AR.2*, which encodes a papain family cysteine protease to modulate proteolysis[208]. The up-regulated of this gene might indicate a well-maintained detoxification function to destruct the aberrant proteins. Besides protein folding and degradation, we also investigate whether RJ/eRJ supplementation could affect CL-2006 worms' protein synthesis. Intriguingly, *ZK218.8* and *T05G5.10*, which encode translation initiation factor 2C and 5A respectively, were not affected by RJ/eRJ supplementation in our quantitative PCR experiment[209]. Collectively, the elevated expression level of these DAF-16 targeted protein turnover genes indicated that RJ/eRJ improve the healthy proteostatic status by strengthening the function of protein folding and degradation in AD worms.

Besides these genes, several protein turnover related genes' level were also measured in our quantitative PCR experiment. *sip-1* (stress induced protein 1) and *hsp70* are also reported to control protein quality, guide protein folding, and maintain proteins' biological function[118, 210]. *aip-1* (arsenite inducible protein) and *F10D7.5* are important regulator to induce the degradation of toxic proteins under stress or disease conditions[209, 211]. To note, it is reported that *aip-1* played an important role to regulate 26S proteasome in response to protein aggregation in AD. In contrast, no study demonstrated *F10D7.5*'s function in AD related research, but this gene encodes one of the E3 ubiquitin ligases in *C. elegans*, which could bind the target protein and E2 enzyme together to signal ubiquitin targeted protein degradation or modulate its function. Unfortunately, these genes were not affected by RJ/eRJ supplementation in AD worms. It

is well known that AIP-1, SIP-1, and HSP-70 were mainly regulated by HSF-1, and HSF-1 was not required in RJ/eRJ mediated protection against A β toxicity. Thus this result further confirmed our genetic data and supported our proposed genetic model. Additionally, this result indicated that DAF-16's activity is fine-tuned by co-factors and pathways. Further experiments are needed to best understand the precise post-translational modification of DAF-16 under RJ/eRJ supplementation, which will provide novel ideas to develop effective anti-AD drugs.

Given that RJ/eRJ supplementation could promote the functional proteostasis to attenuate proteotoxicity in aging cells, next we tested whether RJ/eRJ mediated A β detoxification acted through affecting the solubility of A β species. Our results showed that RJ/eRJ supplemented worms' soluble A β species were significantly higher than non-treated CL-2006 worms, while the insoluble A β species in RJ/eRJ treated worms were dramatically lower when compared with non-treated controls (Figure 5.9 A and B). In consideration of RJ/eRJ possessed the ability to alleviate A β toxicity in AD worms, our finding suggested that RJ/eRJ mediated A β species detoxification might act through changing the conformation of A β proteins to alleviate its toxicity in AD worms.

Tyedmers and colleagues reported that the forming aggregates' structure varied from most disordered aggregates to prefibrillar species and highly ordered amyloid fibrils in AD patients' cells. Comparing with highly ordered aggregates, the unstructured and disordered aggregates are dramatically inclined to be degraded and thereby be considered as less toxic aggregates. Noticeably, chaperones play an important role in this aggregates formation process[131, 212]. Given that RJ/eRJ changed the solubility of A β species in

aged worms and up-regulated the levels of several chaperone proteins, it is possible that RJ/eRJ could promote the formation of disordered A β aggregates instead of highly ordered insoluble A β aggregates and hence improves the detoxification of A β species in AD worms.

Further experiment is needed to be carried out to best understand how A β conformation is changed and detoxified by DAF-16. Numerous studies revealed that the A $\beta_{3(\text{PE})-42}$ was one type of toxic A β species, which formed more than 50% of A β plaques in cells and seeded A β aggregation to accelerate the development of AD. Accordingly, glutaminyl cyclase (QC) has been proposed to catalyze A β_{3-42} to A $\beta_{3(\text{PE})-42}$ in clinical study to exacerbate neuronal cytotoxicity. It is possible that RJ/eRJ might possess capacity to affect A β species conformation by influencing QC[213]. Currently, there is no publication talking about the link between A β conformation and QC in *C. elegans* model, and further research will investigate this relationship.

Overall, our finding not only demonstrated that the molecular mechanisms of RJ/eRJ mediated alleviation of A β toxicity in *C. elegans* but also underscored RJ/eRJ's potential function to lead a healthy aging. In considering that IIS/DAF-16 is highly conserved in species ranging from *C. elegans* to human and RJ/eRJ mediated healthy proteostasis is dependent on the regulation of IIS/DAF-16, it is highly possible that RJ/eRJ might also be beneficial for human consumption to prevent or slow down the onset neurodegenerative diseases.

CHAPTER SIX CHAPTER SIX: FUTURE DIRECTION

In previous chapters, our findings indicated that Royal Jelly (RJ/eRJ) supplementation extended *C. elegans* lifespan, increased their stress tolerance, and protected against A β toxicity in AD worms. Intriguingly, this anti-aging effect acted through increasing the transcriptional activity of DAF-16 and, in turn, improving the healthy proteostasis in *C. elegans*. To this end, our studies not only revealed the molecular mechanism underlying RJ/eRJ mediated beneficial effects but also underscored the essential function of Insulin Signaling Pathway (IIS) and DAF-16 under aging and disease conditions.

It is well known that DAF-16/FOXO, a highly conserved forkhead transcriptional factor from *C. elegans* to mammals, is tightly regulated by post-translational modifications (PTMs) and protein-protein interaction with its co-factors[214]. Noticeably, DAF-16/FOXO plays a very important role in cell proliferation, energy metabolism, and stress response. Additionally, DAF-16/FOXO supports the survival of organisms under low glucose and fasting conditions, and protects cells from stress-induced cellular damages. However, the dysregulation of DAF-16/FOXO results in age-related disorders, such as cancer, diabetes and neurodegenerative diseases[35]. Based on DAF-16/FOXO's important anti-aging functions, it is worth studying how the regulatory specificity of DAF-16/FOXO is determined in cells under normal and aging conditions.

Numerous studies demonstrated that FOXO transcriptional factors are regulated by certain external factors, including nutrients, environmental stressors, and cytokines.

These external stimuli could induce the post translational modifications of FOXO to alter its structural and functional properties, which in turn regulate the subcellular location and transactivity of FOXOs. To date, phosphorylation, acetylation and ubiquitylation are best-studied PTMs of FOXOs.

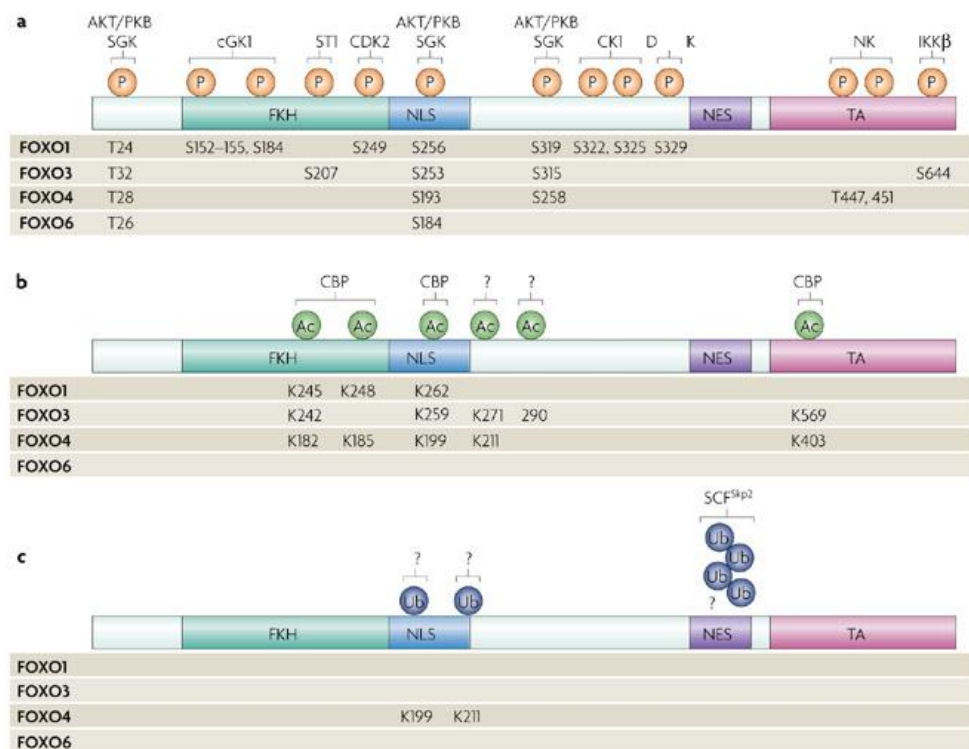
It has been proposed that FOXO phosphorylation is often induced by signaling pathways and cellular stresses. For instance, under the activation of IIS pathway, AKT/PKB phosphorylates FOXO and, in turn, sequesters FOXO in the cytosol rather than assists their nucleus translocation. In contrast, under oxidative stress, the c-Jun N-terminal kinase (JNK) pathway phosphorylates FOXO and thereby increases its transactivity to protect cells from oxidative damage. Taken together, the different sites of FOXO phosphorylation result in opposite functions in cells, either activation or inactivation of FOXO, which highlights the link between precise PTMs and specific functions[45].

In addition to phosphorylation, another frequently observed PTM of FOXO is acetylation. Under the oxidative stress, FOXO can bind to several histone acetyltransferase (HATs) , which might reduce the DNA binding capacity of FOXO and thereby decrease its transactivity. However, this negative effect can result in reduced HDACs (histone deacetylases). It is reported that in mammalian models, FOXOs are acetylated by HATs, such as P300/CBP and CBP-associated factor (P/CAF) and deacetylated by SIRT1[215].

In addition to phosphorylation and acetylation, ubiquitylation also modulates FOXO's activity. Several studies have described that polyubiquitylation leads to the proteasomal degradation of FOXOs, whereas monoubiquitylation strongly increases the transcriptional activity of FOXOs. In addition to these three PTMs, methylation and glycosylation also regulate FOXO's activity. In our research, RJ/eRJ significantly up-regulated the activity of DAF-16, but further experiments are needed to be done to investigate which types of DAF-16's PTMs are triggered in RJ/eRJ supplementation under normal and disease conditions[216].

Furthermore, as well as PTMs, several co-factors (binding partners) also modulate FOXO's activity and function. For instance, co-factor 14-3-3 could bind and sequester DAF-16 in the cytosol under the activation of IIS pathway, which represses the DAF-16's nucleus translocation. However, under heat shock stress, 14-3-3 functions as a bridging protein to promote the physiological interaction of DAF-16 and SIR-2.1, which in turn increases the transactivity of DAF-16 in the *C. elegans* model. Another important DAF-16 co-factor is HCF-1, which also physiologically interacts with DAF-16 and represses its activity. Specifically, in our research we found that SIR-2.1, 14-3-3 and HCF-1 were required to modulate DAF-16's activity under RJ/eRJ supplementation. Moreover, besides of these three co-factors, other DAF-16 binding partners might be also involved in RJ/eRJ mediated anti-aging effect, such as BAR-1, SMK-1 and RLE-1[159]. Further experiments are needed to be carried out to better understand the detailed molecular mechanism underlying this intricate protein-protein interaction[48, 49, 65].

Overall, RJ/eRJ supplementation provides us a good nutrigenetic model to further investigate the precise mechanism of how DAF-16/FOXO is regulated under aging and disease conditions.



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Figure 6.1 Summary of post-translational modifications on the various FoxO isoforms. (Horst *et al.*, 2007)

In order to approach our goal, several experiments are needed to conduct in the future. Firstly, we wondered what PTMs of DAF-16 are triggered under RJ/eRJ supplementation. The wildtype N2 and AD worms will be supplemented with RJ/eRJ for several days, and then the purified DAF-16 proteins will be analyzed by MS-Spec methods to analyze their PTM pattern. After comparing the differences between RJ/eRJ treated worms and non-treated controls, we will determine the types of PTMs on DAF-16 triggered by RJ/eRJ supplementation. Noticeably, this unique pattern on DAF-16 could be utilized as an indicator to screen efficient anti-AD drugs. As well as we know the RJ/eRJ mediated specific PTMs of DAF-16, we will further investigate the protein-protein interaction between DAF-16 and its binding partners, which will help us to better understand how the regulatory specificity of DAF-16 is determined in healthy and aging cells.

In conclusion, our findings revealed the molecular mechanism of RJ/eRJ's anti-aging effect, and underscored the link between an improving proteostasis and a healthy aging. Moreover, we highlight that IIS/DAF-16 plays a key role in pro-longevity, stress resistance and age-related disease prevention.

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