12. Nutraceutical modulation of the aging process and age-related diseases – What the worm has taught us

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1. Abstract. Increased subsets of elderly persons in the total populations of many countries have suffered from age-related diseases in the past few decades. Diseases that at one point in time seemed rare are amplified in aged individuals. Sedentary lifestyles and unhealthy diet choices have also exacerbated these conditions. It is well known that a healthy diet and moderate exercise improves the overall quality of our physical being and improves mental functions. Recent reports have associated instances of Alzheimer’s disease (AD) among obese individuals, and suggest that abnormalities in insulin signaling in the brain maybe the underlying cause. The link between insulin signaling and aging has been illustrated in the invertebrate models Drosophila melanogaster and the nematode Caenorhabditis elegans. In C. elegans, there are several mutations involving the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway that will either accelerate or delay the aging process. There are also several models that express the human amyloid beta peptide, one of the pathohistological hallmarks associated with AD. These models have been indispensable for nutraceutical studies. Studies in our laboratory have revealed that in C.elegans, nutraceuticals such as
the *Ginkgo biloba* extract EGB 761 and a green tea constituent epigallocatechin gallate (EGCG) increase the worm’s life span and stress resistances. EGB 761 has also been shown to delay age-associated tissue degeneration and alter serotonin (5-HT) sensitivity in *C. elegans*. Evidence suggests that there is substantial crosstalk between the insulin and 5-HT signaling pathways. This chapter will discuss studies that utilize *C. elegans* as a system to elucidate the relationship between nutraceuticals, the aging process and their effects on amyloid beta toxicity. The chapter will also illustrate what has been learned from *C. elegans* is likely to be applied to mammalian systems.

2. Introduction

Many countries around the globe are experiencing an increase in their elderly populations, which has elevated interest in the scientific community, not only in the normal aging process, but in age-related diseases as well. Although the maximum life span has significantly increased in most developed nations in the past several decades, due to sanitation requirements and vaccine development, there is still much work to be done on increasing the health span of individuals. The old argument that debates the quality of life versus quantity still persists, but a diagnosis that continues to persevere throughout is the benefits of healthy lifestyle choices. It is well known that a healthy lifestyle, which includes a balanced diet and moderate exercise, improves the overall quality of your physical well being. However, recent scientific evidence has demonstrated that these choices/modifications can also increase cognition and other mental functions[1]. There is a clear linear relationship between unhealthy lifestyles, obesity and diabetes mellitus. Research is now correlating dysfunctions in insulin signaling to certain types of dementia, including Alzheimer’s disease (AD) [2-4]. Although more than a century has pasted since the first diagnosed case of AD, we are still far from finding a cure for this neurodegenerative goliath. As the elderly populations steadily rise in many developed nations, the prevalence of AD also continues to rise and is projected to reach proportions that will strain health care systems throughout the globe. Though the etiology of this disease is still unknown, scientific evidence has reported that the instances of dementia are increased in obese individuals [5]. Genetic studies performed in the nematode *Caenorhabditis elegans* have given important insight into gene regulation of life span and health span. These studies have demonstrated that life span in the worm can be regulated by several different mechanisms, including, caloric restriction, reduced insulin/ (insulin-like growth factor) IGF-1 signaling and serotonin (5-HT) receptor modulation [6-9], which control life span either by accelerating or delaying the aging process. Many natural compounds, such as the *ginkgo biloba* extract EGB 761 and several of its active components, epigallocatechin gallate (EGCG) and α-lipoic acid all affect the life span and/or health span of the worms [10-13] which in turn, could delay or even prevent the development of age-related neurological disorders.
3. Obesity and AD

Since many conditions that are associated with obesity are also associated with aging, research in recent years has focused on understanding the role of insulin signaling in cognitive process and how dysfunctions in insulin metabolism contribute to age-related pathologies, in particular AD [2, 14]. These relationships have become even more important in the present society where conditions that directly involve insulin regulation, such as obesity and diabetes, have dramatically increased [15-18]. A recent clinical study shows that intranasal administration of insulin modulates Aβ and facilitates memory without affecting fasting plasma glucose or insulin levels in early AD patients [19]. Evidence has also demonstrated that in aging individuals, insulin receptors in the brain become less sensitive to insulin [20, 21], an event which possibly reduces synaptic plasticity in the brain [4]. There is evidence that this reduction in insulin sensitivity, which is due to aging and exacerbated by diabetes and obesity, decreases the clearance of Aβ from the brain [4, 22-24]. Also, insulin and amyloid beta protein are in direct competition for the insulin degrading enzyme (IDE) [22, 23] in the brain. This activity can affect the hippocampus, a brain region that is well known for its role in cognition. Another study reported that obesity-related leptin had the ability to modify Aβ levels both in vitro and in vivo by reducing beta secretase levels [25]. This evidence has made many researchers postulate that Aβ deposition would be increased because of excessive levels of insulin. Many studies have demonstrated that manipulation of insulin can affect cognitive process and Aβ levels that are detected in cerebrospinal fluid [26, 27]. Although there is a fair amount of evidence that supports the role of increased insulin levels in AD, this only indicates a correlative relationship. The evidence does not implicate excessive insulin in the causation of the disease; rather this condition may compound and amplify the known pathologies.

4. A worm model for studying aging, insulin & serotonin (5-HT) signaling

Since the introduction of C.elegans into the laboratory setting [28], the area of worm research has exploded. The majority of the research has focused on genetic studies that have lead to many insights into the complex and ill-understood aging process. The discovery that metabolic health can regulate the worm’s life span highlights the importance of healthy lifestyle choices [7, 8, 29]. Years ago it was reported that a high-calorie diet shortened the life span of the worm, but animals that had their calories restricted lived much
longer [30, 31]. Also, mutant worms that have their normal feeding behavior genetically disrupted live 20-40% longer [8] than their wild type counterparts. These studies, yielded similar results when they were later preformed in the fruit fly drosophila and mice [32-34]. There are at least 38 insulin-like peptides that are encoded by the C. elegans genome that act as either agonist or antagonist to DAF-2, which is the only insulin receptor found in the animals [6, 35-37]. The use of mutant worms elucidated the relationship between the insulin/IGF-1 and 5-HT pathways and their regulation of life span and health span in the worm. The worm’s body fat content is controlled by metabolic activity and feeding behaviors; both of which have afforded researchers the opportunity to use this model in the identification of genes that control feeding/satiety, fat metabolism and nutritional uptake [31].

The neuromodulator 5-HT controls a variety of behaviors in invertebrates and vertebrates alike. Several behaviors in C. elegans are affected by 5-HT which include: locomotion, pharyngeal pumping, egg-laying and feeding [38-42]. Also, 5-HT receptors have recently been found to antagonistically modulate life span in the worms, probably through the insulin/IGF-1 pathway and independent of caloric restriction [9]. This work was confirmed by another laboratory that also used the worm model, although this group antagonized the receptors in a pharmaceutical approach, instead of utilizing mutant worms [43].

Worms that are deficient in the serotonergic biosynthetic enzyme tryptophan hydroxylase (tph-1) are defective in behaviors and metabolism that is normally coupled to the ingestion of food, such as decreased mating and egg retention [44-46]. These worms also accumulate larger stores of fat than their wild type counterparts, although their feeding rates are reduced [45]. Despite the fact that exogenously applied 5-HT increases the feeding rates, it potently decreases fat accumulation in the worm [42]. When 5-HT is applied exogenously, it modulates the worms behaviors in a similar fashion as changing the levels of food [45] i.e., decrease in locomotion and increase in egg-laying [38, 39]. Prior evidence has demonstrated that both the tph-1 and the daf-2 mutants have a tendency to developmentally arrest at the alternate dauer larvae stage and accumulate excess fat, and deletion mutations of daf-16 suppresses these phenotypes [45, 47, 48]. Research has also shown that the DAF-2 insulin/IGF-1 receptor which regulates DAF-16, the FOXO transcription factor, is targeted by 5-HT to affect stress responses [48]. In mammals, 5-HT influences the synthesis, release, and sensitivity of insulin as well as many other hormones that regulate the appetite and metabolic homeostasis. Evidence has shown that dysregulation in metabolism is partially due to the decrease in insulin-like neuroendocrine signals and that the activity of the serotonergic system in metabolic regulation and obesity is similar in the worm and mammalian models [45, 48].
5. Modeling amyloid-beta toxicity in the worms

Different animal models have been constructed over the years to try to mimic the pathogenesis of AD. Although none have been totally successful, there are several ways humans disease states can be partially mimicked in the worm. The first model is by 1) knocking out (mutants) or knocking down (RNA interference) a worm gene that is homologous to the human gene that is known to be involved in a certain disease 2) targeting a biological process in the worm that reproduces certain aspects, whether cellular or molecular, that are involved in that particular disease mechanism and 3) expressing a human gene in the worm that can induce a disease-related phenotype [31, 49]. The transgenic worm strains that carry human Aβ (1-42) were all constructed in the laboratory of Dr. Chris Link [50-53]. These worms were made by injection of the minigene construct, unc-54/Aβ-(1-42) in their gonads, in conjunction with the dominant marker rol-6 gene [53, 54]. The transgenic progeny obtained exhibit a non-sinusoidal, roller movement phenotype due to the rol-6 marker. So, expression of the Aβ gene causes the animals to rotate along their longitudinal axis [50, 54], and later there is a progressive paralysis [53, 55]. This paralysis is attributed to the accumulation of intracellular Aβ deposits in the C. elegans muscles. From this construct, two strains CL2006 and CL4176, along with their respective vectors were identified. CL2006 is cultivated at 20°C, the same as wild type animals, throughout the duration of their life span, and Aβ-(1-42) is continuously expressed. CL4176, on the other hand, is maintained at the lower permissive temperature of 16°C, without the expression of Aβ [50, 52]. Aβ is expressed in this strain, when the worms are upshifted to the non-permissive temperature of 23°C [52]. This mechanism of temperature inducibility was explained by Link et al [52] and is caused by mutations in the mRNA surveillance (smg) system of the worm. In CL4176, smg-1 is inactivated, and this allows for mRNA translation of the human Aβ-(1-42) transgene at the non-permissive temperature. CL2355 is the strain that express human Aβ in the neurons. Although it exhibits wild type movement because there is no insertion of the rol-6 marker, there have been other phenotypes characterized for this strain [56].

The advantages of using C. elegans for drug combination studies are: 1) highly conserved biochemical pathways, cell signaling and stress response between worms and human; 2) well established mutants linking biological pathways with pathological phenotypes; 3) the small size, ease of maintenance, genetic amenability and rapid generation time makes evaluation of numerous animals on microtiter plates feasible. Several examples illustrated the power of C. elegans in screening for new drugs [57-59].
genetic target of drugs such as fluoxetine was identified in worm mutants [60]. As stated by Dr. WR Schafer [61]: “Drugs have historically been effective tools for investigating how worm neurons work; worm’s neurons may prove equally effective for investigating how drugs work”.

Intriguingly, data obtained from three AD-relevant models; Aβ cell culture, Aβ worm behaviors and hippocampal slices exposed to non-fibrillar oligomers called Aβ-derived diffusible ligands or ADDLs, support each other for nutraceutical studies. For example, among the EGb 761 constituents tested, ginkgolide J (GJ) enhanced phosphorylation of cyclic AMP response element-binding protein (CREB) in Aβ expressing cells [62], reduced Aβ-induced paralysis in CL4176 Aβ-muscle expressing worms [56] and restored ADDL-evoked LTP defect [63].

6. Insulin/ IGF-1 like signaling, heat shock and modulation of proteotoxicity

Substantial experimental evidence has demonstrated the regulatory effects of insulin signaling and heat shock factors (HSF) in the aging process [64, 65]. Heat shock factor 1 (HSF-1) has been reported to have roles in the developmental process, stress responses and the circadian rhythm cycle [66-68]. Reports have shown that when the expression of HSF-1 was increased, the worms’ life span was extended 40% and this extension required daf-16 [64]. Recent studies are now focusing on these factors and their relationship to protein misfolding or aggregation: proteotoxicity. DAF-16 and HSF-1 transcriptomes both result in the expression of a variety of chaperons, leading researchers to hypothesize that protein folding plays a key role in life span and aggregate-associated proteotoxicity [65, 69]. In a paper published by Cohen et al, the relationship between the insulin signaling pathway and Aβ was examined. It reported that Aβ worms that were grown on daf-2 RNAi showed a significant extension in life span and delay in paralysis in comparison to their vector control groups [69]. This study summarized that protein toxicity is highly dependent on the aging process and that daf-16 and hsf-1 are both required for the reduced insulin signaling mediated alleviation of Aβ (1-42) in the body wall muscles of the worm [69]. Dietary restriction functions through a pathway that is genetically distinct from the insulin/IGF-1 like signaling pathway. Bacterial food deprivation or BD was found to suppress age-associated paralysis in the Aβ (1-42) worms through an hsf-1 dependent mechanism [70]. A study conducted in our laboratory demonstrated that a protective heat shock dramatically delayed paralysis in the Aβ worm strain (Figure 4). More importantly, we found that the heat shock is not
associated with Aβ transgene expression, but with a reduction of Aβ oligomeric protein [71]. Although direct regulation of HSF-1 by the insulin/IGF-1 receptor has not been established and many molecular interactions have not been elucidated, the indirect evidence from these intermingling factors, suggest that altering one may modulate the other [65, 69, 72]. With the obesity epidemic reaching epic proportions, particularly in the United States [16], the public is now looking to nutraceutical interventions as potential treatments for weight management.

7. *C. Elegans* as a model organism for screening of nutraceuticals

The current literature has expounded on the virtues of the roundworm *C. elegans* in pharmacological studies. With its tiny size, adult hermaphrodites are ~1mm in length, to its ease and low cost of cultivation, extremely short generation time and life span; it is not surprising that this model organism has branched beyond the primarily genetic studies. The latest trends in *C. elegans* research are leaning toward the drug discovery arena. Since about half of the worm’s genome is homologous to the human genome, this makes targeting specific genes to study and treat fairly easy. Pharmacological modulation of targets that have been correlated with obesity, depression and Alzheimer’s disease (AD) are all being tested in the worm. Altering the activity of a specific gene can be a fundamental readout of the genes function [31]. This approach has aided us in our understanding of the general aging process, but it will probably be less effective in a multi-factorial disease such as AD; in particular the sporadic and most common form, which unlike the familial version of this disorder, has no solid genetic links.

8. Epigallocatechin-3-gallate (EGCG)

Green tea has seen a boost in its popularity in the Western Hemisphere over the past few years because of its beneficial effects on health. Catechins, the polyphenolic plant metabolites that are common in the drink, and EGCG, which is the most abundant and active component, has become mainstream. Now EGCG can be found in a variety of commercial products ranging from fortified supplements to facial creams. EGCG has several pharmacological and biological properties. These include free radical scavenging activity, antioxidant activity, iron-chelating capabilities and attenuation of lipid peroxidation, due to various forms of radicals [73]. Researchers have reported that EGCG can rescue PC12 cells and protect rat hippocampal cells
against amyloid β-induced neurotoxicity in a dose dependent manner, [74, 75] and attenuate Aβ-induced toxicity in cultured hippocampal neurons [76]. EGCG has been demonstrated to have potent antioxidative properties in vitro and is also displays biphasic action, e.g., it acts as a prooxidant or antioxidant depending on the cellular environment and concentration [77, 78]. Although our laboratory did not find an increase on life span at the concentrations used in this particular study; we did find that EGCG delayed the age-related slowing of sinusoidal movement (Figure 1A) [11], which probably delays severe muscle degeneration termed sarcopenia.

9. α-lipoic acid

Thioctic acid, more commonly known as α-lipoic acid (LA), has been shown to increase insulin sensitivity when administered orally in patients diagnosed with type-2 diabetes [79]. LA is synthesized in a host of organisms that range from bacteria to humans and is a natural cofactor in the pyruvate dehydrogenase complex, where it will bind acyl groups and transfers them from one complex to another [80]. LA also exhibits networking capabilities, because of its capacity to regenerate endogenous antioxidants [80]. LA has been reported to extend the life span of the fruit fly Drosophila [81] and C.elegans [11], in a daf-16 dependent manner. Our laboratory found that the chemotaxis index, which decreases in an age-dependent manner in the worms, was enhanced in older animals that were fed with LA. Also, combined treatment with EGCG showed a markedly positive additive effect (Figure 1B) [11]. Although LA and EGCG both exhibit favorable effects on age-dependent declines of the worms’ behavior, it is obvious that their mechanism of action is different.

10. Ginkgo biloba extract EGB 761 & ginkgolide J

The ginkgo biloba leaf extract EGB 761 has been at the forefront of many studies involving complementary and alternative medicine [12, 82-85]. Our laboratory reported many pharmacological effects of EGB 761 in the C.elegans model. It increased the worm’s median life span, by 8%, with the flavonoid fraction exhibiting a much higher median life span increase of 25% [10]. EGB 761 also dramatically increased the worm’s resistance to the oxidative stressor jugalone [10] and directly attenuated elevated levels of reactive oxygen species in the Aβ worms [86]. We concluded through these findings that EGB 761 extended the worm’s life span through alleviation of oxidative stress, which is probably the most prominent aging theory and has been around since the middle of the twentieth century [87, 88]. This conclusion, although valid, may
Figure 1. (A) Locomotive assay in wild type *C. elegans* fed EGCG or LA at day 12 or day 16 of age. Worms were scored as A, if they moved spontaneously and without the aid of touch stimulus. Worms were scored B, if a touch stimulus was needed in order for the worms to move. Worms were scored as C, if they only moved their head or tail and it required a touch stimulus. (B) Effects of EGCG, LA, or their combination, on the chemotaxis index (CI) in adult day 5 *C. elegans*. For the chemotaxis assays, 1 µl attractive odorant diacetyl were spotted near one edge of the plate plus 1 µl of sodium azide (1 M) to paralyze the worms which arrived at the odorant. After 1 h, worms that reached the odorant were quantitated. The Chemotaxis Index (CI) was defined as (the number of worms at the attractant − the number of worms at the control spot) / the total number of worms. * p<0.05, ** p<0.01, *** p<0.001 [11].
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not be the complete story. EGb 761 has been shown to reverse the age-related loss of 5-HT\textsubscript{1A} receptors [83]. Studies conducted in our laboratory demonstrated that worms that express the human A\beta transgene in the neurons (CL2355) show dysfunctions in serotonergic transmission [56]. This was demonstrated by their defect in two characteristic 5-HT controlled behaviors: the chemotaxis behavior, which activates input from several sensory, and interneurons and translates them into a motor output [89] [90] and in their hypersensitivity to 5-HT. When exogenous 5-HT is applied to wild type animals, it causes a decrease in their locomotor behavior [60, 91]. However, the animals that express A\beta in the neurons (CL2355) were defective in these responses [56]. Both of these behavioral defects were reversed in worms that were fed with EGb 761 (Figure 2A and B). Although no clear mechanism of action has been defined for the effects of EGb 761 on serotonin transmission in the worm, there are several scenarios that could be possible: 1) Response to 5-HT could be affected by the transgene expression, 2) Acetylcholine (ACh), which negatively regulates 5-HT in the worms, could be decreased or 3) there could be direct or indirect blockage of 5-HT reuptake by A\beta expression [56].

Several of the single components of EGb 761 are pharmacologically active on their own. EGb 761 suppresses A\beta deposition and its single component Ginkgolide J (GJ) alleviates paralysis induced by A\beta expression in the transgenic CL4176 worm strain (Figure 3B) [56]. RNA interference of the serotonin receptor (SER-1), which has been shown to increase thermotolerance, UV –related stress and life span in the worms [9, 92] was knocked down in A\beta worms that paralyze at non-permissive temperature. These worms were also fed Ginkgolide J, to determine if GJ functions in the serotonergic pathway (unpublished data). Although GJ and SER-1 each delayed paralysis separately, there was no combinatorial effect (Figure 3B). The unique biological properties of the ginkgolides are attributed to their unique “cage skeleton” structure. Other compounds such as Congo red and/or curcumin [93], may share structural similarities with the ginkgolides, which may explain why all exhibit affinity for amyloidogenic conformations, Minuet differences in the chemical structure of the ginkgolides can have varying effects their biological activity. Ginkgolide J is able to inhibit the deleterious effects of A\beta on LTP impairment of CA1 hippocampal slices [63, 94].

Although some may question the validity of using \textit{C.elegans} as a model system because a direct line cannot be drawn from different phenotypes to human pathological behaviors, several basic biochemical processes are conserved between these species. We do not expect \textit{C.elegans} to answer the complexity of disease states of higher organisms, but we do expect them to give us insight into basic gene functions that can provide clues for novel therapeutic interventions.
Figure 2. (A) Chemotaxis behavior in neuronal Aβ strain, CL2355, was reduced compared with the transgenic control strain CL2122 (no Aβ strain). Feeding with EGB 761 alleviated this descent in the transgenic strain, but not in the control strains (n=4; *p<0.05). (B) Serotonin hypersensitivity in CL2355 is normalized by EGB 761 and its constituents. Strain CL2355 were fed with EGB 761 (100µg/ml), vehicle (Ctrl; 100µg/ml), ginkgolides (GA, GB, GC, GJ) (10µg/ml each), BB (10µg/ml), quercetin (QC) (10µg/ml), kaempferol (KA; 10µg/ml) L-ascorbic acid (VC) (100µg/ml), and CR (139µg/ml). The worms were collected at 36 h after temperature upshift to 23°C. 5-HT sensitivity assay was conducted in 96-well plate containing 200µl of 1mM 5-HT solution. Percentage of active worms is calculated as follows: number of the active worms (after placed into serotonin solution for 5 min and still kept moving for 5 s)/total worms. (n=3; **p<0.01) [56].
Figure 3. Effect of EGB 761, its constituent GJ and ser-1 (RNAi) on Aβ-induced paralysis in muscle Aβ strain CL4176 (A) Representative images of Aβ-induced paralysis in the transgenic CL4176 strain. CL1175, vector control (no Aβ) untreated (Control; CL1175, open circles), and in the transgenic CL4176 strain (muscle Aβ strain) fed with (EGB 761; closed circles) or without EGB 761 (Control; CL4176, open squares) at 36 h after temperature upshift. (B) Time course of paralysis assays in CL4176 with serotonin receptor (SER-1) knocked down by RNA interference; ser-1(RNAi) (purple circle) fed with Ginkgolide J (GJ) (green squares) or a combination of ser-1(RNAi) and GJ (blue squares). Worms were grown for 38 h at 16°C followed by upshifting the temperature to 23°C to induce the transgene expression. The paralysis was scored at 60 min intervals.
Nutraceuticals may exhibit disease modifying effects by abrogating the deleterious effects induced by amyloid beta toxicity and neurofibrillary tangles (NFTs). The green tea component EGCG was shown to promote the non-amyloidogenic pathway by increasing \( \alpha \)-secretase cleavage of the amyloid precursor protein, APP [95] and reducing potentially toxic sarkosyl-soluble phospho-tau isoforms [96]. \( \alpha \)-lipoic acid was found to improve learning and memory retention in a transgenic mouse model of cerebral amyloidosis associated with AD [97]. Utilizing nutraceuticals to target insulin signaling and the serotonergic system could possibly provide novel therapeutic strategies to ameliorate dysfunctions which are associated with both the aging process and the development of AD.

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