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A Role of Vitamin B2 in Reducing Amyloid-beta Toxicity in a Caenorhabditis elegans Alzheimer's Disease Model

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by

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ABSTRACT

A Role of Vitamin B_2 in Reducing Amyloid-beta Toxicity in a *Caenorhabditis elegans* Alzheimer's Disease Model

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Alzheimer's disease (AD) is associated with amyloid-beta peptide deposition and loss of mitochondrial function. Using a transgenic *C. elegans* AD worm model expressing amyloid-beta in body wall muscle, we determined that supplementation with either of the forms of vitamin B_2, flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) protected against amyloid-beta mediated paralysis. FMN and FAD were then assayed to determine effects on ATP, oxygen consumption, and reactive oxygen species (ROS) with these compounds not significantly improving any of these mitochondrial bioenergetic functions. Knockdown of the *daf-16*/FOXO transcriptional regulator or the FAD synthase enzyme completely abrogated the protective effects of FMN and FAD, while knockdown of the mitochondrial unfolded protein response factors *ubl-5* or *atfs-1* also blocked the protective effects. Therefore, vitamin B_2 supplementation could lead to the activation of conserved signaling pathways in humans to delay the onset and progression of neurodegenerative diseases such as AD.

DEDICATION

To my wife – Aishah Imam Muhideen, my son Maher Ameen, my parents – Mustapha Kayode and Rahmat Ameen and the Kwara State Government, Nigeria for my stipend allowance grant.

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease associated with progressive memory impairment, cognitive deficits, difficulty with language, and problem-solving skills (Beitnere et al. 2014; Alzheimer's Association 2017). This disease is irreversible and slowly destroys memory and thinking skills and later prevents an individual to carry-out simple daily activities (Rizzieri 2012). It is the most common cause of dementia among the older population. AD is known to be the 6th leading cause of death in the United States and about 35 million people are affected worldwide (Prince et al. 2016). Despite years of funding for research for into the pathological cause of AD and therapeutic development, it remains a significant and unresolved physiological, financial, and social burden (Chen et al. 2015). Some of the common risk factors that have been associated with AD include old age, family history, lifestyle, gender, head injury, environmental toxins, and cardiovascular diseases such as hypertension. So far, there is no cure for AD, however, emerging evidence has suggested maintaining healthy aging and living style, such as healthy eating, engaging in social and physical activities, and avoiding excess alcohol and smoking, could help to protect an individual form developing AD. Also, much research has correlated brain health to heart health, so chances of developing AD or any form of dementia increases with increased risk for heart disease (Sandeep Kumar Singh et al. 2016). However, the number of deaths from AD increased by 89 percent from 2000 to 2014 while deaths from heart disease decreased by 14 percent (Alzheimer's Association 2017).

Despite numerous biomedical research discoveries suggesting therapeutic strategies for AD, many of them have failed clinical trials. Current drugs used for treating patients with AD include cholinesterase inhibitors and the NMDA receptor antagonist memantine that only

delay symptoms of the disease without any modification of its direct cause (Misra and Medhi 2013). Therefore, the major problem for researchers and clinicians is identifying the etiology of the disease and developing therapies or drugs that could prevent its onset or delay its progression.

Molecular Pathology of Alzheimer's Disease

The main pathological signatures of AD are the extracellular deposition of insoluble amyloid-beta plaques and intracellular formation of neurofibrillary tangles in the hippocampus region and cerebral cortex of the brain (Basurto-islas et al. 2010). These two pathologies in the brain are thought to disrupt normal memory and cognitive functions of the brain. Accumulation of these plaques and tangles later leads to common symptoms associated with AD such as memory impairment, cognitive decline, personality changes and ultimately death (Sandeep Kumar Singh et al. 2016). Since, 1906 when AD was first identified, and its symptoms described by Alois Alzheimer, there have been concerted efforts by generations of researchers and clinicians to identify direct pathological causes of the disease and develop effective therapies and therapeutics (Goedert and Spillantini 2006; Santos et al. 2010). These efforts have led to the development and testing of several hypotheses using different experimental models. Also, results from these experiments have facilitated the discovery and use of current drugs used for treating AD patients.

Proposed Mechanisms for AD Development

Given the observations that multiple cellular mechanisms could be involved in the pathology of AD, there have been several hypotheses proposed for its cause. The oldest of these hypotheses is the cholinergic hypothesis, which postulates that reduced production of the acetylcholine neurotransmitter in neurons of the forebrain leads to loss of neuronal signaling and eventual loss of cognition and memory (Terry and Buccafusco 2003). However, this theory has not been widely accepted because medications developed to treat acetylcholine deficiency have not been effective. Other hypotheses proposed for AD include tau protein hyperphosphorylation and the amyloid and mitochondrial cascade hypotheses (Singh et al. 2016).

The current most widely established theory for AD is the "amyloid cascade hypothesis". This theory stipulates that AD develops due to the gradual accumulation of toxic amyloid-beta peptide (AβP) in the medial temporal lobe of the brain due to proteolytic cleavage of amyloid-beta-precursor-protein (AβPP) by β-secretase and γ-secretase. This hypothesis therefore asserts that deposition of this peptide forms insoluble plaques in the extracellular matrix of neurons thereby causing intracellular neurofibrillary tangles of tau protein, neuronal cell loss, and vascular damage (Hardy and Higgins 1992). This toxic protein accumulation has also shown to result in impaired brain cell energy metabolism, lower oxygen uptake, increased toxic reactive oxygen species (ROS) production, and neuronal cell death (Wang et al. 2015). Also, the familial form of AD, which arises due to mutations in the AβPP (amyloid-beta precursor protein) gene has been linked to amyloid-beta deposition (Owen et al. 1990). Unlike tau protein phosphorylation, which is also linked to other neurodegenerative diseases known as tauopathies, the amyloid-beta peptide deposition has only been linked to the onset of Alzheimer's disease (Mudher and Lovestone 2002).

Notwithstanding a plethora of research evidence relating amyloid-beta protein toxicity to AD, there is lack of evidence as to what triggers the proteolytic production of amyloid-beta from its precursor AβPP. A growing body of evidence now suggests that mitochondrial dysfunction caused by excessive oxidative damage by ROS production triggers increased production of the toxic amyloid-beta peptide, which later leads to a cascade of cellular physiological disruptions and eventually cell death (Thannickal and Fanburg 2000). This later assumption is known as the "mitochondrial cascade hypothesis", which proposes the cause of the most common type of AD, the sporadic or late onset AD (Swerdlow and Khan 2004).

Because of continuous shift in the understanding of the pathophysiological cause of AD, several other mechanisms, such as hormonal imbalance, neuroinflammation, calcium dysregulation, genetic defects, oxidative stress, autophagy and neuronal dysfunction, have been proposed as upstream regulators for AD progression and that amyloid-beta plaque formation and neurofibrillary tangles are downstream processes used by neuronal cells to adapt to the continuously changing cellular environment (Anand et al. 2014).

Mitochondrial Biology of AD and Neurodegenerative Diseases

The significance of mitochondrial functions for cell survival has necessitated an investigation into their role in the progression and onset of AD and other neurodegenerative diseases. Maintaining normal cellular mitochondrial distribution and functions have been reported to be challenging for aging cells, so dementia and other age-related neurological diseases are a common culprit of mitochondrial dysfunction (Santos et al. 2010). Mitochondria, the power-houses of cells, account for more than 90 percent of cellular energetic needs (Chance et al. 1979). Since neurons have limited glycolytic capacity to generate energy, they highly depend on energy derived from mitochondrial aerobic

respiration through oxidative phosphorylation (OXPHOS) for their extensive energy-dependent functions (Moreira et al. 2010). Although, the mitochondrial energy production process generates toxic reactive oxygen species such as superoxide anions and downstream hydrogen peroxide and peroxynitrite, normal cellular function requires superoxide as a second messenger for cell signaling purposes (Shigenaga et al. 1994). However, mitochondrial exposure to toxins and the aging process leads to the accumulation of abnormally high ROS levels, which triggers abnormalities in mitochondrial function and homeostasis, a hallmark of neurodegenerative diseases (Valko et al. 2007). Thus, impaired mitochondrial function, homeostasis dysregulation, and abnormal mitochondrial dynamics have been implicated in the onset and progression of AD and other neurodegenerative diseases.

AD and Impaired Mitochondrial Function

One of the major functions of the mitochondria in cells is energy production though the TCA (tricarboxylic acid) cycle and oxidative phosphorylation. Other than energy production, mitochondria also trigger cell death or apoptosis and regulate Ca^{2+} homeostasis (Moreira et al. 2006; Wang et al. 2017). A considerable number of studies with laboratory AD models, postmortem AD tissues, and AD patients have identified reduced mitochondrial respiration and ATP production and increased ROS production. Using a *C. elegans* AD model, Fong et al. 2016, reported that reduced ATP production and abnormalities in the electron transport chain (ETC) protein complexes precede global metabolic failure and pathology of AD. Also, Martire et al. 2016 have reported energetic decline in cell lines and brain tissue of mice producing human amyloid-beta peptide (Aβ1–42). They also reported that amyloid-beta peptide induced upregulation of PARP-1 (poly (ADP-ribose) polymerase),

an enzyme that catalyzes the ADP-ribosylation of proteins, leads to excessive degradation of NAD^+ (nicotinamide adenine dinucleotide), an important cofactor required for mitochondrial energy production. Also, PARP-1 hyperactivation caused reduced mitochondrial cytochrome c oxidase protein levels, oxygen consumption, and mitochondrial membrane potential.

Multiple studies have suggested that reduced brain glucose metabolism is a common hallmark of AD patients and that energy impairment precedes clinical diagnosis of AD symptoms. A study by Mosconi et al. (2011) reported glucose hypometabolism, increased amyloid-beta deposition, and reduced activity of mitochondrial cytochrome c oxidase (COX), in adult offspring of mothers with late-onset Alzheimer's disease. Based on the mitochondrial cascade hypothesis of AD proposed by Swerdlow and Khan (2004), mitochondrial dysfunction due to oxidative damage is a potential target for AD therapeutics development.

AD and Abnormal Mitochondrial Dynamics

Due to the significance of mitochondria in eukaryotic cells, abnormal modifications in mitochondrial morphology and distribution can result in significant changes in cellular functions and development of mitochondrial related diseases. Fan et al. (2010) have reported that excessive mitochondrial oxidative damage causes increased mitochondrial fragmentation or fission. A proper balance between mitochondrial fission (fragmentation) and fusion is required for mitochondrial maintenance. Proteins located on mitochondrial outer and inner membranes are responsible for regulating mitochondrial dynamics. Proteins required for mitochondrial fission include the cytosolic GTPase Drp1 (dynamin-related-protein-1), Fis1 (fission 1), and mitochondrial fission factor (Mff). Drp1 is recruited to specific sites on the mitochondrial outer membrane to initiate fission. Large GTPases such as mitofusins 1 (Mfn1)

and 2 (Mfn2) located on the mitochondrial outer membrane and optic atrophy 1 (OPA1) located in the inner mitochondrial membrane regulate mitochondrial fusion (Yoon et al. 2003; Gandre-Babbe and van der Bliek 2008; Onoue et al. 2013).

Disruption of Drp1 activity is increased due to increased mitochondrial oxidative damage causing frequent mitochondrial fission and abnormal cellular distribution of mitochondrial populations and morphology. Barsoum et al. (2006) have reported increased expression of Drp1 in cortical neuronal cultures treated with amyloid-beta peptide and nitric oxide. They also reported reduced ATP production, mitochondrial ultrastructural damage, and increased autophagy and generation of free radicals as possible consequences of the Drp1 induced excessive mitochondrial fission. Cho et al. (2009) have reported increased mitochondrial fission in the brains of AD patients due to increased formation of SNO-Drp1 intermediates (S-nitrosylation of Drp1). Cho et al. also reported that amyloid-beta peptide induced nitric oxide generation leading to increased synaptic loss and neuronal damage, which are hallmarks of AD and neurodegenerative diseases. Interestingly, using a transgenic mouse model of FAD (familial AD), Trushina et al. (2012) reported that alterations in mitochondrial dynamics and morphology precedes neurological phenotypes and amyloid-beta deposition. They also reported that altered mitochondrial energy metabolism and increased mitochondrial stress accompany the altered mitochondrial dynamics. Therefore, regulation of mitochondrial dynamics through the fission and fusion processes could also be a potential therapeutic strategy for AD.

AD and Dysregulation of Mitochondrial Proteostasis

Targeting mitochondrial proteostasis regulation is an emerging trend in the prospect for therapeutics for AD. Mitochondria possess mechanisms that regulate the expression of proteins from mtDNA, the import of proteins from the cytoplasm and the transport of ions across the inner mitochondrial membrane. These mitochondrial protein quality control processes are often referred to as mitochondrial proteostasis. Inhibition of these processes by amyloid-beta has been reported to result in inhibition of mitochondrial protein import, OXPHOS impairment, and increased numbers of misfolded mitochondrial proteins (Devi et al. 2006; Sorrentino et al. 2017). Also, evidence for the presence of amyloid-beta, functional γ-secretase, and AβPP in mitochondria is an indication that mitochondria could either be a production or storage site for amyloid-beta peptide. Amyloid-beta peptide inside the mitochondrion has been shown to compromise cytochrome c oxidase (COX) activity and increase mitochondrial ROS production. Also, the peptide induces cytochrome c dependent caspase 3 activation, and decreased ATP production (Anandatheerthavarada et al. 2003; Atamna and Frey 2004; Rhein et al. 2009). Thus, the loss of mitochondrial proteostasis could also be involved in AD.

Mitochondrial Unfolded Protein Response Pathway

One important mechanism that is conserved in living cells from single celled organisms to higher animals is the activation of conserved stress response pathways when exposed to stressors such as toxins, starvation, misfolded proteins, or inhibition of normal cellular function. However, the activation of some of these mechanisms has been reported to decline with aging. Vital cell organelles such as endoplasmic reticulum and mitochondria have evolved mechanisms that act as defense systems during cellular stress to protect the cell

from damage. Specifically, mitochondria have evolved a mechanism to regulate their folded protein content (and proteostasis) during mitochondrial stress conditions such as during times of limited mitochondrial protein import and accumulation of misfolded and unassembled mitochondrial proteins (Jovaisaite et al. 2014). The most common defense mechanism involved in this task is called the mitochondrial unfolded protein response (UPR^{mt}). It evolved to protect mitochondria from overwhelmed chaperones and to boost the activities of proteasomal enzymes and mitochondrial proteases that degrade misfolded proteins. The normal mitochondrial chaperone systems found in the matrix space (mtHSP70 and HSP60/HSP10) and intermembrane compartments (protein quality control (PQC) proteases) are overwhelmed during disease conditions associated with misfolded proteins. As a result, the mitochondria sense the increased unfolded protein burden and induce the UPR^{mt} to improve protein quality control mechanisms through activation of ATFs (activating transcription factors). Several of these factors have been identified in humans such ATF4 and ATF5. However, this mechanism has been best studied in *C. elegans* models and is known to require a master transcriptional regulator in this species called ATFS-1 (Activating transcription factor associated with stress). ATFS-1 functions in conjunction with UBL-5 (ubiquitin-like protein-5) and DEV-1 (a homeodomain-containing transcription factor) (Jovaisaite et al. 2014; Pickles et al. 2018).

Under normal conditions, ATFS-1, which has both a nuclear localization sequence (NLS) and a mitochondrial targeting sequence, is imported into the mitochondria and degraded by the LonP protease (Melber and Haynes 2018; Pickles et al. 2018). However, during misfolded protein stress in mitochondria, ATFS-1 is translocated into the nucleus together with DEV-1 to upregulate the transcription of a network of genes such as chaperones, proteases and proteins required for mitochondrial import, metabolism and

biogenesis. In this way mitochondria become protected and can once again resume optimal function.

Activation of the mitochondrial unfolded protein response pathway has been reported to decrease with aging suggesting this inability may participate in the onset and development of many age-related diseases associated with misfolded proteins. For instance, Ruan et al. 2017, reported that defective cytosolic heat shock protein, Hsp70s, in yeast cells leads to an increased entry of misfolded proteins into mitochondria. They also reported that blocking mitochondrial protein import caused a decrease in proteasomal degradation with an increase in mitochondrial stress. More so, in a study conducted by Sorrentino et al. 2017 to study the role of mitochondrial proteostasis in worms, mice, and cell lines containing high levels of amyloid-beta peptide ($A\beta_{1-42}$), there was reduced mitochondrial respiration and an upregulation of mitochondrial stress response pathways, mainly the mitochondrial unfolded protein response pathway and mitophagy. Activation of these mitochondrial stress response pathways was shown to improve health and lifespan of the *C. elegans* AD model studied and also reduce amyloid-beta peptide deposition. Therefore, results from these and other studies are indicators of the role of mitochondria in the onset and progression of AD and many other neurodegenerative diseases caused by misfolded proteins.

Alzheimer's Disease Drug Discovery Research

Alzheimer's disease drug discovery research over the last three decades has only yielded two types of drugs currently approved by the FDA for the treatment and management of AD patients. These two types, acetylcholinesterase inhibitors and NMDA (N-methyl-D-aspartate) receptor antagonists, only ameliorate the disease symptoms but have little to no effect on the pathological process (Anand et al. 2014). Attempts by researchers to develop

effective therapies and therapeutics for AD have led to findings revealing more information about the level of cellular complexities involved in the pathological progression of the disease. Promising studies have found several natural compounds and genetic approaches to modifying some of the currently known pathological causes of AD. Some of the strategies used in Alzheimer's disease drug discovery research include modulation of neurotransmission, tau pathology inhibition, amyloid-beta based therapies, modulation of intracellular signaling cascades, oxidative stress reduction, mitochondrial based therapies, regulation of cellular calcium homeostasis, neuroinflammation based therapies, and other emerging therapeutic strategies (Citron 2010; Grill and Cummings 2010; Anand et al. 2014).

Due to the complexities involved in AD pathological processes and the need for studying therapeutic effects of multiple compounds, researchers have developed several laboratory models based on amyloid-beta and tau production for high-throughput drug screening of potential pharmacological and natural drug candidates for AD. Some of the most commonly used laboratory models for studying AD pathology and therapeutics development include transgenic mice, worms, flies, and mammalian cell lines expressing amyloid-beta peptide ($\beta_{1\text{-}42}$), mutant AβPP, or mutant tau protein (Sandeep Kumar Singh et al. 2016).

Amyloid-beta Based Strategies

Based on the understanding of the role of amyloid-beta peptide in AD pathology, researchers have developed strategies to reduce amyloid-beta peptide production, modify its cellular transport, prevent oligomerization, promote cellular clearance, or delay proteotoxicty. One of the top strategies is an immunization-based approach (Anand et al. 2014). However, amyloid-beta peptide likely plays some minor role in normal cellular physiological functions such as in neurite outgrowth, synaptogenesis, cell adhesion, calcium metabolism, axonal

protein trafficking, and others. Amyloid-beta peptide is produced through the β-secretase and γ-secretase (not α-secretase) cleavage of AβPP and the intracellular and extracellular oligomerization of the peptide leads to the formation of Aβ fibrils and plaques. So, inhibiting β-secretase or γ-secretase activity has been a potential strategy to prevent the production of toxic Aβ-peptide species (Zheng and Koo 2006). But this strategy has largely failed in clinical trials.

Treatments targeting amyloid-beta cellular transport are also being developed. The apolipoprotein E (ApoE) and RAGE (Receptor for advanced glycation end products) receptor-mediated transport of Aβ-peptide into and out of the brain are targets for AD drug development (Anand et al. 2014). The ability of amyloid-beta peptide to oligomerize and aggregate to form amyloid fibrils in the hippocampus and frontal brain region has been shown to be a key factor in promoting its neurotoxicity. Drugs and plant extracts such as tramiprosate (homotaurine), scyllo-inositol, the hormone melatonin, caffeine, NAD^+ precursors, *Gingko biloba*, and others have been shown to possess anti-oligomerization properties by interacting with the monomeric Aβ species and preventing their interaction to form fibrils (Wu et al. 2006; Olcese et al. 2009; Dostal et al. 2010; Sorrentino et al. 2017). In addition, genetic manipulations and immunotherapies are also being developed to delay Aβ proteotoxicty and enhance Aβ clearance (Singh et al. 2016).

Mitochondrial Targeted Strategies

Mitochondrial dysfunction has also been shown to be involved in AD pathology through the generation of excessive ROS leading to cellular oxidative damage and neuronal cell death (Anand et al. 2014). Therapeutics targeted to mitochondria attempt to reduce mitochondrial ROS production and improve activity and availability of enzymes and proteins

involved in mitochondrial energy production pathways such as the citric acid cycle, ETC, and OXPHOS. Also, nutritional therapy for regulating mitochondrial dynamics and homeostasis have been developed. Dietary supplementation of CoQ, a coenzyme required for electron shuttling in the mitochondrial ETC, or MitoQ, a mitochondrial targeted derivative, have been shown to possess neuroprotective effects by suppressing ROS production and improving mitochondrial function (Wadsworth et al. 2008). Methylene blue has also been shown to have neuroprotective effects to improve mitochondrial function by serving as an alternative electron carrier of the ETC (Wen et al. 2011) to decrease ROS production. Other drugs targeting mitochondrial ROS production and ETC have been proposed such as N-acetyl-L-carnitine and R-α-lipoic acid, vitamins C and E, and Szeto-Schiller peptide-31 (SS-31) (Anand et al. 2014).

Mitochondrial based therapies have also been developed to modify mitochondrial dynamics (fission and fusion) and improve mitochondrial proteostasis. However, several of these drugs have failed clinical trials and have been shown to affect normal cellular functions and trigger inflammatory reactions in patients (Anand et al. 2014). Therefore, there is a need to develop mitochondrial targeted treatments that solely target damaged mitochondria and have minimal negative side effects on healthy mitochondria.

Transcriptional Regulator DAF-16/FOXO

DAF-16 (Abnormal Dauer formation 16) is the *C. elegans* homolog of mammalian FOXO transcription factors, which are induced in different cellular stress conditions. The ability to activate DAF-16 declines with aging. FOXO transcription factors are involved in cell cycle arrest, tumor suppression, protection against oxidative stress, and promotion of longevity. They also upregulate genes involved in the innate immune response, DNA repair, insulin sensitivity, and activation of gluconeogenesis. Four FOXO genes are present in humans (FOXO1, FOXO3, FOXO4, and FOXO6), while only one is present in *C. elegans* and *Drosophila melanogaster*, but multiple proteins are synthesized from the one gene in these organisms. One of the key roles of FOXO proteins is to recruit chromatin modifying enzymes to DNA to regulate target gene expression (Tia et al. 2018).

DAF-16/FOXO transcriptional activity is regulated by different proteins in response to extracellular signals. The activity of the DAF-16/FOXO transcription factor can be regulated through phosphorylation by Akt/ PKB, JNK (c-Jun N-terminal kinase), MST1 (mammalian ste20- like kinase 1), ERKs (extracellular signals regulated kinases), p38 MAPK (mitogen-activated protein kinase), and AMPK (AAK-2 in *C. elegans*). Phosphorylation by Akt in the cytoplasm prevents nuclear localization in response to insulin signaling, while phosphorylation by the other kinases stimulates translocation form the cytoplasm to the nucleus to modulate gene expression (Brunet et al. 2004). Some of the genes induced by FOXO proteins include SOD2 and catalase (for ROS detoxification), PTEN (tumor suppressor), P21CIP1 and P27KIP1 (cell cycle arrest), and PEPCK and G6Pase (during starvation). Also, DAF-16/FOXO transcriptional activity is modulated by acetylation (by HATs (Histone acetyltransferases)) , methylation (by PRMT1 and PRMT2 (protein arginine N-methyltransferases 1 and 2)), and ubiquitin-dependent proteolysis (by SKP2 (S-phase

kinase-associated protein 2)) (Yamagata et al. 2008; Tia et al. 2018). All four human FOXO proteins are expressed in the brain.

FOXO transcriptional regulators have been shown to be activated through pharmacological agents and genetic manipulations in *C. elegans* AD models. For instance, Chondrogianni et al. (2014) reported that DAF-16 is required for enhanced proteasomal degradation and reduced Aβ proteotoxicity in a *C. elegans* AD model overexpressing the β5 proteasome subunit. Several other studies have also reported DAF-16 to be required for the protective effect of added compounds against amyloid-beta toxicity. Studies by Zhi et al. (2017) and Wang et al. (2016) are examples of cases where compounds such as Dianxianning (DXN) (a traditional Chinese formula) and royal jelly alleviate Aβ toxicity and improve AD symptoms through DAF-16 activation. Thus, FOXO protein activation could be a target for improving health and lifespan of AD patients.

A *Caenorhabditis elegans* AD Model

As the cost of maintaining a research laboratory is on the rise, biotechnology companies and biomedical researchers have sought to develop quick and inexpensive laboratory models with little maintenance cost, efficient handling, short reproduction time, high level of gene and pathway conservation with vertebrates, and high amenability to genetic manipulation. The worm *C. elegans* has now been extensively used as a laboratory model to study developmental biology, embryology, disease mechanisms, and high throughput drug screening for neurodegenerative disease (Teschendorf and Link 2009).

C. elegans is a free-living, transparent, and non-parasitic nematode with a short life cycle of 3 days from egg to adult at 25°C (Brenner 1974). The mean lifespan of a worm population is between two to three weeks and thus allows for a timely study of its biology

and therapeutic effects of drugs candidates for neurodegenerative diseases. In 1998 the genome sequence of *C. elegans* was published. It shows 38 percent gene conservation with humans (*C. elegans* Sequencing Consortium 1998). Although, *C. elegans* do not naturally produce amyloid-beta peptide or show other AD-like memory phenotypes, orthologs of genes mutated in familial AD such as AβPP, tau, and presenilin are present. Also, since *C. elegans* neuronal connectivity has been established, worms have been very useful in studying the pathology and drug interventions for AD (Alexander et al. 2014). One of the most prominent genetic manipulations of *C. elegans* is RNAi interference (RNAi)-based gene knockdown. This method has allowed for unbiased insights into *C. elegans* physiology (Teschendorf and Link 2009).

C. elegans AD models have been developed based on amyloid-beta peptide and tau pathology, the major correlates for AD progression. The most popular *C. elegans* AD model expresses amyloid-beta peptide ($\beta_{1\text{-}42}$) in the muscle leading to the formation of amyloid-immunoreactive inclusions and fibrils and paralysis (Lublin and Link 2014). Also, a tauopathy worm model expressing either wild-type or mutated human tau protein has been developed. Marked phenotypic consequences of Aβ or tau expression has allowed for easy tracking and direct observation of Aβ and tau proteotoxicity (Singh et al. 2016).

One of the major limitations of using *C. elegans* as a neurodegeneration model is the lack of evidence that the observed pathology in the worm is relevant for human disease. Also, amyloid-beta needs to be overexpressed in worms at higher levels to induce the same level of toxicity observed in human brains or mammalian cell lines (Teschendorf and Link 2009). Other experimental limitations of *C. elegans* disease models is the absence of the immune system and the relative ineffectiveness of the RNAi technique in neurons, although this second limitation can be overcome by genetic manipulation (Firnhaber and Hammarlund 2013). Also, some challenges of using *C. elegans* for drug screening is the inability of some

compounds to penetrate the thick worm cuticle and false positive or negative results from compounds that are metabolized by the bacteria that the worms are co-cultured with to consume as a food source. However, these can mostly be overcome by using worm strains engineered to have a more permeable cuticle (Partridge et al. 2008) or increasing drug dosage or time of supplementation (Alexander et al. 2014).

Neurological Importance of B Vitamins

B vitamins are a class of water-soluble vitamins that play key roles in brain energy metabolism. B vitamins serve as precursors for coenzymes involved in cellular metabolism, most prominently in mitochondrial energy production. For instance, vitamin B_2 (riboflavin) is a precursor for FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide), while vitamin B_3 (niacin) is a precursor for NAD (nicotinamide adenine dinucleotide), all three of which serve as electron carriers for mitochondrial energy production. Other types of B vitamins such as B_1 (thiamine), B_5 (pantothenic acid), B_6 (pyridoxine), B_7 (biotin), B_9 (folate), and B_{12} (cobalamin) play key roles as cofactors and molecules mediating multiple brain functions (Kennedy and Haskell 2011).

B vitamins cannot be synthesized in the human body, except for niacin (vitamin B_3) which can be synthesized to a limited extent from the essential amino acid tryptophan, and must be regularly obtained from the diet (Sechi et al. 2016). Natural sources of vitamin B_1 include brown rice, fruits, vegetables, and seafoods, while vitamin B_2 is naturally present in dark green vegetables, meat (liver), dairy products, and whole grains. However, epidemiological studies have shown that B vitamin deficiency is on the rise in both developed and developing countries, especially among the older population, vegetarians, patients with heart failure, and postoperative bariatric surgery. Vitamin B_1 and B_2 deficiency is more

prevalent among the elderly (Sechi et al. 2016). Due to interactions between B vitamin functions, specifically the role of active forms of riboflavin (FMN and FAD) as an enzyme cofactor involved in the synthesis of other classes of B vitamins, B_2 deficiency has been shown to limit folate, pyridoxine, and cobalamin availability (McCormick 1989; Sechi et al. 2016).

Vitamin B Deficiency and Neurodegenerative Diseases

Due to lack of robust tissue storage of B vitamins, cells depend on a regular supply of B vitamins for vital functions and to prevent disease-symptoms associated with their deficiency. The physiological importance of B vitamins, such as for energy production, amino acid metabolism, synthesis of proteins and neurotransmitters, and synthesis of cholesterol and nucleotides bases, have made their cellular deficiencies a leading cause of many neurological and cardiovascular diseases. Specifically, active forms of vitamin B_2 and B_6 have roles as antioxidants in cellular processes (Powers 2003).

Several lines of evidence have suggested vitamin B supplementation as a strategy to improve brain function and reduce incidence of neurological diseases. For instance, vitamin B_1 (thiamine) deficiency has been associated with neurological problems such as cognitive deficits and encephalopathy. Thiamine-dependent enzymes perform critical roles in brain glucose metabolism. As a result, reduced levels of these enzymes have been reported in brains of AD patients. In contrast, excess levels of thiamine have been shown to diminish AD-like pathologies (Gibson et al. 2016).

Vitamin B_2 Synthesis and Metabolism

Vitamin B_2 (also known as riboflavin) is not synthesized by humans and must be obtained from the diet. Vitamin B_2 is present abundantly in a wide variety of animal and plant foods such as in legumes, meat ,and dairy products, etc. (Udhayabanu et al. 2017). The chemical structure of riboflavin consists of an isoalloxazine ring and a ribityl side chain. Riboflavin obtained from the diet is enzymatically converted to FMN by riboflavin kinase by the addition of a phosphate group to its ribityl side chain. FMN may then be converted to FAD by the FAD synthase enzyme by the addition of an adenine monophosphate group (AMP). FAD can be converted back to riboflavin by FAD pyrophosphatase (This process is called the Rf-FAD cycle.). Riboflavin has a half-life of approximately one hour and is mainly stored in the liver, spleen, kidney and cardiac muscle in the form of FAD. Thus, its deficiency could impair essential functions of these vital organs (Saedisomeolia and Ashoori 2018). FMN and FAD are the main active forms of vitamin B_2 and perform active roles as coenzymes of enzymes and proteins called flavoproteins, mostly involved in mitochondrial metabolic processes.

Mutations in riboflavin transporters and riboflavin-dependent enzymes can limit absorption, transport, or conversion of riboflavin to its different active forms leading to disease. Specifically, mutation in FAD synthase (FLAD1) causes FAD deficiency in patients diagnosed with multiple acyl-CoA dehydrogenase deficiency (MADD) (Auranen et al. 2017a). Also, mutations in riboflavin plasma membrane and mitochondrial transporters have been shown to contribute to the development of MADD, with riboflavin supplementation decreasing disease symptoms and improving mitochondrial flavoprotein activities. Other symptoms of riboflavin deficiency have been reported in rat studies to include demyelination in the cerebrum and cerebellum and delay and impairment in brain development and maturation (Udhayabanu et al. 2017).

Riboflavin also plays a key role as an antioxidant in the glutathione redox cycle. FAD is involved in the activation of the glutathione reductase (GR) enzyme through its reduction by reduced nicotinamide adenine dinucleotide phosphate (NADPH). This crucial step is required for the continuous activity of the glutathione redox cycle (Saedisomeolia and Ashoori 2018). As a result, cellular riboflavin status is commonly obtained through measurement of GR activity. Riboflavin deficiency could therefore potentially play a role in the development and treatment of neurodegenerative diseases where oxidative stress is increased.

Due to a dearth of effective and readily available disease modifying therapeutics for AD treatment and due to the failure of multiple clinical trials of already proposed drugs, there is a need to develop new strategies that could address the complex multifactorial causes of AD onset and progression. Because of this necessity, this study was conceived to study the role of vitamin B_2 in delaying amyloid-beta induced proteotoxicity using both mitochondrial and amyloid-beta cascade hypotheses as rationale. Therefore, our initial hypothesis was that vitamin B_2 supplementation would improve mitochondrial bioenergetic function, which would in turn improve amyloid-beta clearance to delay amyloid-beta induced proteotoxicty in a *C. elegans* amyloid beta-expressing AD model.

CHAPTER 2
MATERIALS AND METHODS

Caenorhabditis elegans Strain Maintenance

C. elegans AD model strain CL4176 *(smg-1(cc546)+ [myo-3p::A-Beta (1-42)::let-851 3'UTR) + rol-6(su1006)])* expressing $A\beta_{1-42}$ peptide specifically in body wall muscle at 25°C, but not 15°C, through the use of a temperature-inducible expression system and wild-type N2 strain were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota). The CL4176 worm strain was maintained at the permissive temperature of 15°C on Nematode Growth Media (NGM) agar 10 cm plates and fed with the OP50-1 (or HT115(DE3) for RNAi experiments) *Escherichia coli* bacterial strain, while wild type N2 worms were cultured similarly as a control.

Chemicals

Nicotinic acid (RPI), nicotinamide adenine dinucleotide (NAD^+) (Alfa Aesar), nicotinamide adenine dinucleotide phosphate ($NADP^+$) disodium salt (Alfa Aesar), flavin adenine dinucleotide (FAD) disodium salt (TCI), riboflavin-5'-phosphate (FMN) (Alfa Aesar), Carbenicillin disodium salt (RPI corporation), IPTG (Fisher scientific), (adenosine-5-triphosphate (ATP) disodium salt hydrate (MP Biomedicals) and Nystatin (RPI corporation).

Drugs Preparation and Concentrations

The water-soluble vitamins B_2 and B_3 were dissolved in either double deionized water or M9 buffer. The active forms of vitamin B_2 (riboflavin), FMN and FAD, were used at varying concentrations until a protective concentration was obtained. FMN was used at final concentrations of 0.07 mM, 0.37 mM, 0.74 mM and 1.48 mM. FAD was used at final

concentrations of 0.2 µM, 0.37 µM, 0.74 µM, 2.2 µM, 3.7 µM, and 37 µM. Vitamin B_3 (niacin) was used in both the precursor (nicotinic acid) and active forms, NAD and NADP. Nicotinic acid was used at final concentrations of 0.07 mM, 0.37 mM, 0.74 mM, and 1.11 mM. NAD was used at final concentrations of 1.48 µM, 1.85 µM, 2.22 µM, 3.70 µM, 7.40 µM, and 14.80 µM. Lastly, NADP was used at final concentrations of 0.37 µM, 1.48 µM, 2.96 µM, and 7.40 µM.

Amyloid-beta Peptide Mediated Paralysis Assays

To test for a protective effects against amyloid-beta peptide induced paralysis, worms were assayed to determine any delay in paralysis after administration of different concentrations of vitamin B_2 or B_3 using methods described by Dostal and Link 2010. Briefly, age synchronized worm populations were obtained by picking 30 gravid adult worms onto several freshly prepared drug-free (no vitamins added) NGM agar plates using a dissecting stereo microscope with a worm picker. Worms were allowed to lay eggs for 2-3 hours at 15°C and gravid adult worms were picked off. Eggs were then allowed to hatch and grow on NGM (0.01 mg/ml streptomycin (an antibiotic) and (0.01 mg/ml nystatin or 0.01 mg/ml fluconazole (antifungals)) plates at 15°C for 7 days. On the 7th day, 10-12 gravid adult worms were picked onto freshly prepared NGM plates containing appropriate drug (B vitamin) concentrations or controls. After 3-4 hours of egg laying on the drug-containing or control NGM plates, gravid adult worms were picked off while eggs were allowed to hatch and grow at 15°C for 48 hours. After 48 hours of growth at 15°C, all plates were upshifted to a 25°C incubator for 30 hours. After 30 hours of incubation and growth at 25°C, worms were assayed for paralysis at 2 hours intervals for 20-30 hours or until all worms on the plates had paralyzed. Paralyzed worms were detected by prodding worms with a worm picker (platinum wire) under a dissecting microscope. Paralyzed worms cannot translocate across the plate,

will cease to move after 2-3 proddings, or cannot form bacterial halos around their moving heads without whole body movement. At least 65 worms were used in triplicate for each treatment group.

Obtaining Worm Samples for ATP, Oxygen Consumption, and Reactive Oxygen Species (ROS) Assays

Confluent gravid adult worms growing on 3-5 drug-free NGM plates at 15°C for 7 days were bleach synchronized to obtain eggs of approximately equal age. Equal numbers of hatched eggs were grown on 12-well plates containing liquid S-media, 0.01 mg/ml fluconazole, and the appropriate drug concentration together with 1 x 10^{10} CFU OP50-1 *E. coli* per ml. Worms were grown at 15°C with shaking for 48 hours. Plates were then upshifted to 25°C to induce Aβ-peptide expression with shaking for 36 hours. After 36 hours of incubation at 25°C, the 800-1000 worms in each well were washed thoroughly with ice-cold 0.1 M NaCl solution in a 15 ml centrifuge tube 4 times at 2500 rpm for 2 minutes to remove bacteria. 1 ml of worm pellets were then transferred to 1.5 ml centrifuge tubes and spun down at 5,000 rpm for 5 minutes. 500 µl of sample suspension was then divided into 3 taking 350 µl worms for the oxygen consumption assay, 50 µl of worms for the ROS assay, and 100 µl of worms for the ATP and BCA protein quantification assays. 100 µl of worm samples for ATP and BCA assays were snap frozen in liquid nitrogen and stored at -80°C for later use.

ATP assays

Worms treated with the protective FMN (0.74 mM) and FAD (0.74 µM) concentrations were assayed for changes in ATP level using the CellTiter-Glo (Promega, Madison, WI) firefly luciferase system as described by Edwards et al. 2013. As described above, 100 µL of worm samples were pelleted, snap frozen to disrupt membranes and release

ATP, and stored at -80°C. For analysis, the samples were thawed on ice and divided into equal halves. 50 µl of the worm sample was used for BCA protein quantification assay while 50 µl was used for the ATP assay. 50 µl of each sample was added into a well of a 96-well plate with 50 µl of CellTiter-Glo reagent. The plate was then shaken for 2 minutes at room temperature and incubated for 10 minutes at room temperature in a Biotek Synergy 2 microplate reader after which the luminescence of the samples was measured. ATP concentrations in the samples were calculated using a standard curve of known ATP concentrations.

Oxygen Consumption Assays

Worms were assayed for basal oxygen consumption using methods described by Edwards et al. 2013 using a Clark oxygen electrode (MT200A chamber, Strathkelvin Instruments). 350 µl of worm sample containing approximately 700 worms was added to the electrode chamber and respiration was monitored for 5 minutes for each sample. Basal worm oxygen consumption was normalized to protein concentration.

ROS Assays

Worms were assayed for ROS levels using the method described by Delic et al. (2017). Briefly, 50 µl of live worms containing approximately 100 worms were added into each well of a 96-well plate with 50 µl of the dye 5'-dichlorodihydrofluorescein diacetates ($DCFH_2$-DA). The plate was shaken in the dark for 2 minutes and then incubated in the dark for 30 minutes to allow for the dye penetration into the worms. The plate was then read for ROS-induced DCF fluorescence using a Biotek Synergy 2 microplate reader for 4 hours at 10 minutes intervals. Worm ROS levels were calculated using the slope of the fluorescence curve and normalized to protein concentration.

BCA Protein Quantification Assay for ATP, Oxygen Consumption, and Reactive Oxygen Species (ROS) Assays

Measurements from ATP, oxygen consumption, and ROS assays were standardized to protein content. Protein quantification was performed using the method described by Edwards et al. (2013) with slight modification. Flash frozen worm samples were thawed on ice and vacuum dried to remove excess washing buffer using a speed vacuum SC110A (Savant) for 1 hr. Worm pellets were then dissolved in 20 µl of 1 M NaOH solution heated at 70°C for 25 minutes. Worm samples were cooled on ice and vortexed. 200 µl of deionized water was added to each sample to give a concentration of 0.1 M NaOH. Samples were centrifuged at 14, 000 rpm for 5 minutes. 25 µl of each sample was used for protein quantification using a BCA assay kit (Pierce) according to the manufacturer's instruction.

RNAi Feeding Experiments

E. coli daf-16, ubl-5, and flad-1 RNAi clones were obtained from the Ahringer *C. elegans* RNAi library (Source BioScience Life Sciences). The *atfs-1 E. coli* RNAi clone from the Vidal RNAi library was purchased from Dharmacon. Using methods described by Edwards et al. (2015) and the Ahringer group who synthesized the *E. coli* RNAi library (Kamath et al. 2000), clones were grown in LB liquid media with 50 µM ampicillin at 37°C with shaking at 220 rpm for 18 hours. Bacteria cultures were pelleted and resuspended in sterile double deionized water at a concentration of 100 mg/ml and stored at 4°C until further use. NGM plates for RNAi experiments contained appropriate vehicle controls or concentrations of FMN or FAD at 0.74 mM and 0.74 µM respectively. Also, the plates contained final concentrations of 25 µg/ml carbenicillin and where appropriate 1 mM IPTG (to induce dsRNA production). Synchronized worm populations feeding on the appropriate *E.*

coli RNAi clone (*daf-16, ubl-5, flad-1, or atfs-1*) were then grown on these plates and assayed for Aβ-mediated paralysis.

Statistical Analysis

Statistical analysis for the paralysis curves was performed with Kaplan-Meier survival analysis and the Log-Rank test using Sigma plot version 11.0 software. Each replicate experiment of the paralysis assays was done in triplicates. Statistical analysis for bioenergetic assays was performed using One-way ANOVA repeated measures with GraphPad Prism version 7.04. Bioenergetic assays experiments were repeated four times in triplicates. All data were analyzed to check for outliers.

CHAPTER 3

RESULTS

Protective Effects of B Vitamins on Amyloid-beta Mediated Paralysis

This study investigated the protective effects of B vitamins, specifically vitamins B_2 and B_3, on the toxicity of amyloid-beta peptide using a *C. elegans* AD model. In this model, temperature upshift induces amyloid-beta expression in body wall muscle leading to a paralysis in some worms beginning at 30 hours following upshift with greater than 90% of worms paralyzing by 40 hours after upshift. Paralysis assays using different concentrations of active forms and precursors of these two B vitamins were performed.

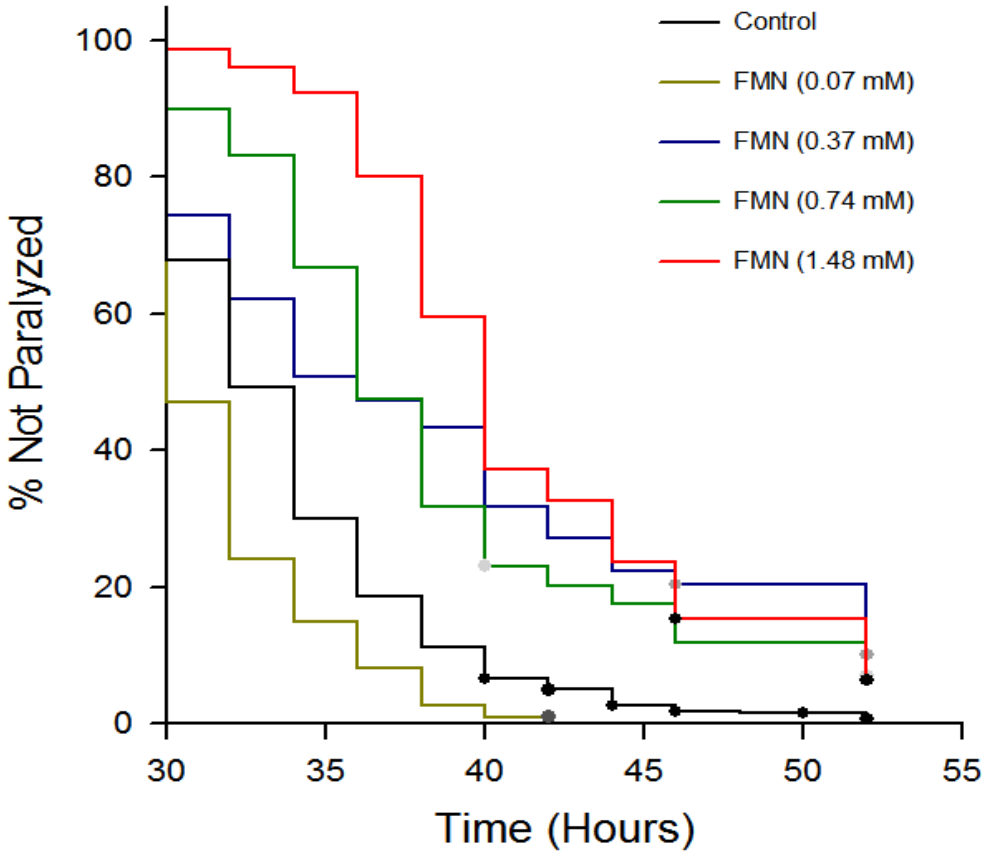

Figure 1 Effect of FMN Treatment on Amyloid-beta Mediated Paralysis

The active forms of B_2 (FMN and FAD) protected the worms against amyloid-beta toxicity depending upon the concentration added. FMN addition significantly delayed paralysis at the three highest concentrations tested with increased mean worm survival ranging from 13 % to 22 % (Figure 1, Table 1) when compared with the control group, while the lowest concentration slightly increased the rate of paralysis. Only one of the two FAD concentrations tested (0.74 µM) significantly protected against amyloid-beta toxicity and this concentration delayed mean paralysis time by 13 % (Figure 2, Table 1). The 3.7 µM concentration had no significant effect on the mean time to paralysis.

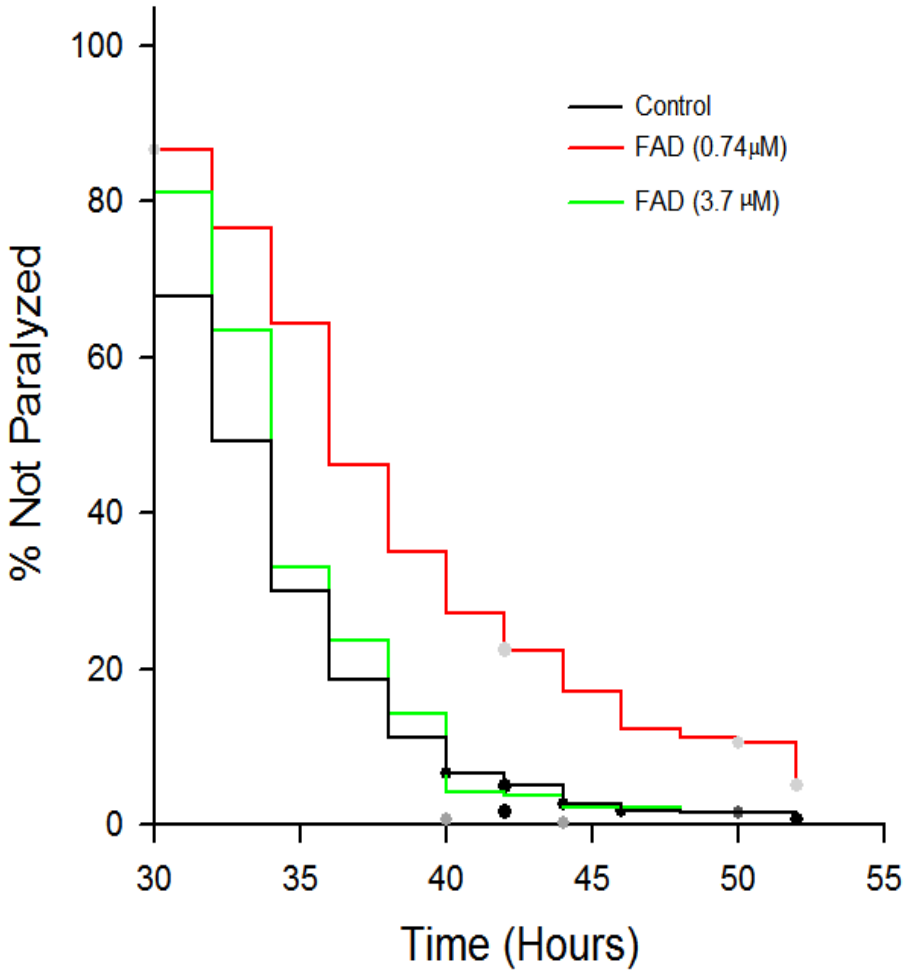

Figure 2 Effect of FAD Treatment on Amyloid-beta Mediated Paralysis

Nicotinic acid (a vitamin B_3 precursor) supplementation moderately protected the worms against amyloid-beta toxicity at one of the three concentrations tested (0.74 mM) (Figure 3) and at this concentration increased mean time to paralysis by 10 % (Table 1) compared to the control group. Two other lower NA concentrations used decreased mean time to paralysis by 13 % and 14 %.

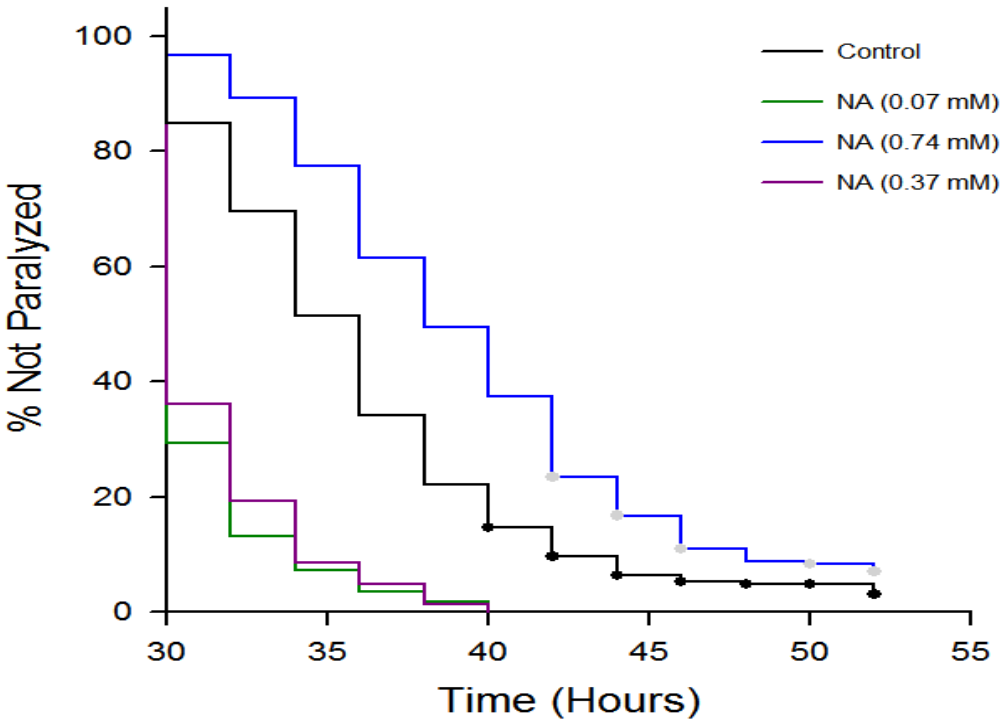

Figure 3 Effect of Nicotinic Acid (NA) Treatment on Amyloid-beta Mediated Paralysis.

Active forms of vitamin B_3 (NAD and NADP) were also assayed to determine if they could provide protection against amyloid-beta-mediated toxicity. NAD addition delayed mean time to paralysis by 15 % at a concentration of 1.85 µM and by 3 % at a concentration of 7.40 µM (Figure 4), while two concentrations added did not lead to protection with one concentration even facilitating paralysis. NADP addition failed to protect against amyloid-beta toxicity at either of the two limited concentrations tested with one concentration decreasing time to paralysis (Figure 5, Table 1).

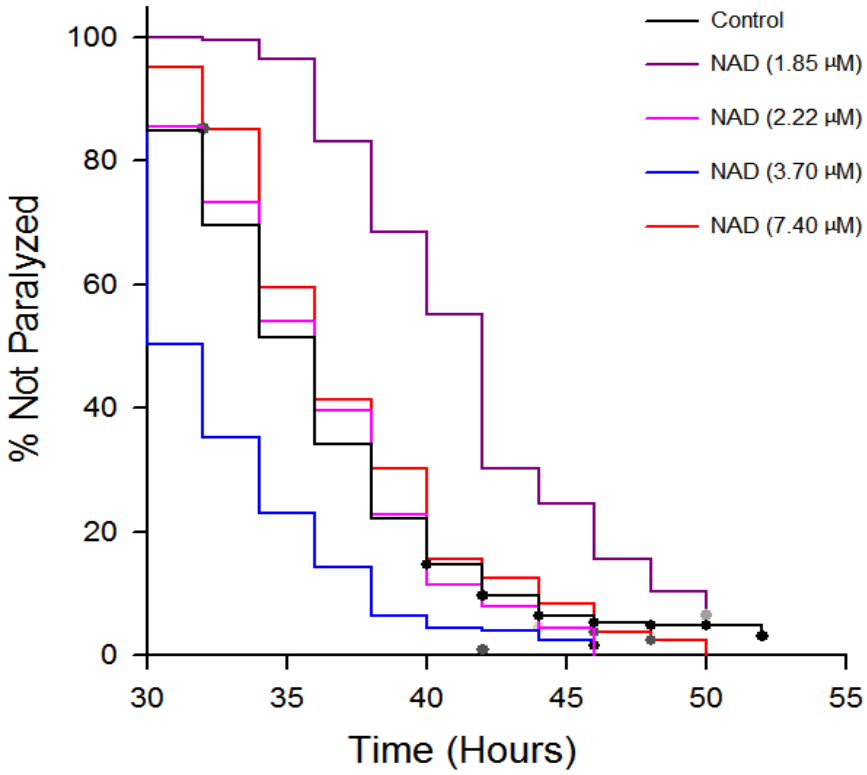

Figure 4 Effect of NAD Treatment on Amyloid-beta Mediated Paralysis

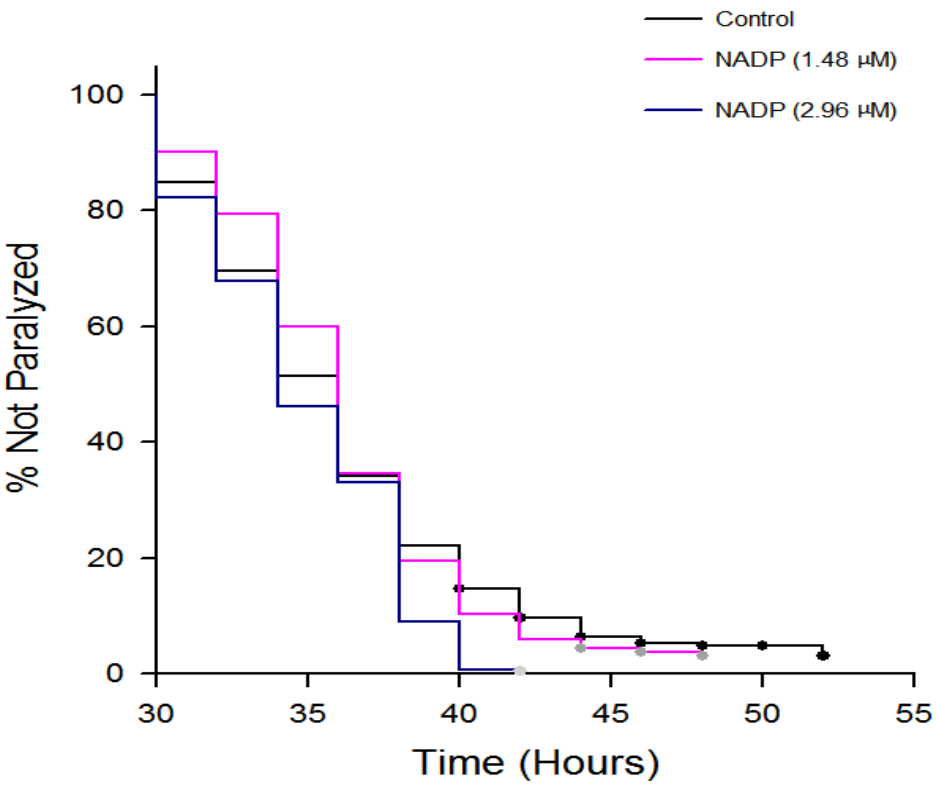

Figure 5 Effect of NADP Treatment on Amyloid-beta Mediated Paralysis.

Table 1 The Effects of B Vitamins on Aβ-peptide Mediated Paralysis in a *C. elegans* AD Model

Treatment	Concentration	% change in mean paralysis time	p-value	# of worms	Replicates
FMN (flavin mononucleotide)	0.07 mM	-6	<0.001	342	2
	0.37 mM	+13	<0.001	148	3
	0.74 mM	+13	<0.001	325	3
	1.48 mM	+22	<0.001	156	2
FAD (flavin adenine dinucleotide)	0.74 µM	+13	<0.001	385	5
	3.7 µM	+2	0.055	191	2
NA (nicotinic acid)	0.07 mM	-14	<0.001	271	2
	0.37 mM	-13	<0.001	163	2
	0.74 mM	+10	<0.001	1060	8
NAD (nicotinamide adenine dinucleotide)	1.85 µM	+15	<0.001	232	1
	2.22 µM	0	0.915	202	3
	3.7 µM	-9	<0.001	205	2
	7.40 µM	+3	0.003	595	6
NADP (nicotinamide adenine dinucleotide phosphate)	1.48 µM	0	0.854	446	4
	2.96 µM	-4	<0.001	403	2

Effects of Vitamin B_2 on Mitochondrial Bioenergetic Function and Cellular Energy Metabolism

Since vitamin B_2 is an essential cofactor for several mitochondrial enzymatic reactions, the concentrations of FMN (0.74 mM) and FAD (0.74 μM) that protected against amyloid-beta toxicity by 13% were assayed for changes in measures of mitochondrial bioenergetic function and cellular energy metabolism including ATP level, oxygen consumption, and reactive oxygen species (ROS) production. Figure 6 shows ATP levels of untreated, FMN treated, or FAD treated Aβ-expressing worms (strain CL4176) and untreated wild-type N2 worms. FMN or FAD treatment did not show a significant effect on ATP levels.

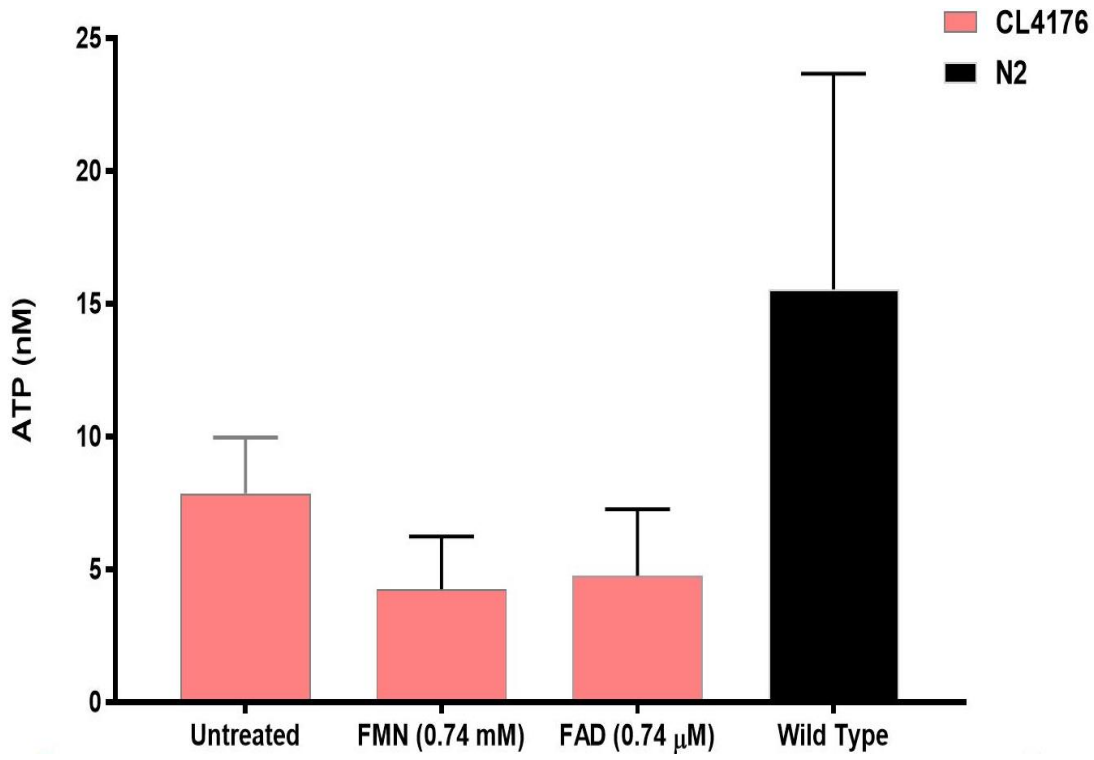

Figure 6 Effect of FMN and FAD Treatment on ATP Level
The data indicate the mean and standard error of the mean (SEM) of four independent triplicate trials. The p values compared to untreated amyloid-beta expressing worms are 0.40, 0.59, and 0.64 for FMN treated, FAD treated, and untreated wild type N2 worms respectively.

The basal oxygen consumption rate of FMN or FAD treated, and untreated CL4176 worms was also measured as well as that of wild type N2 worms (Figure 7). FMN or FAD treatment or Aβ expression did not significantly alter the rate of oxygen consumption.

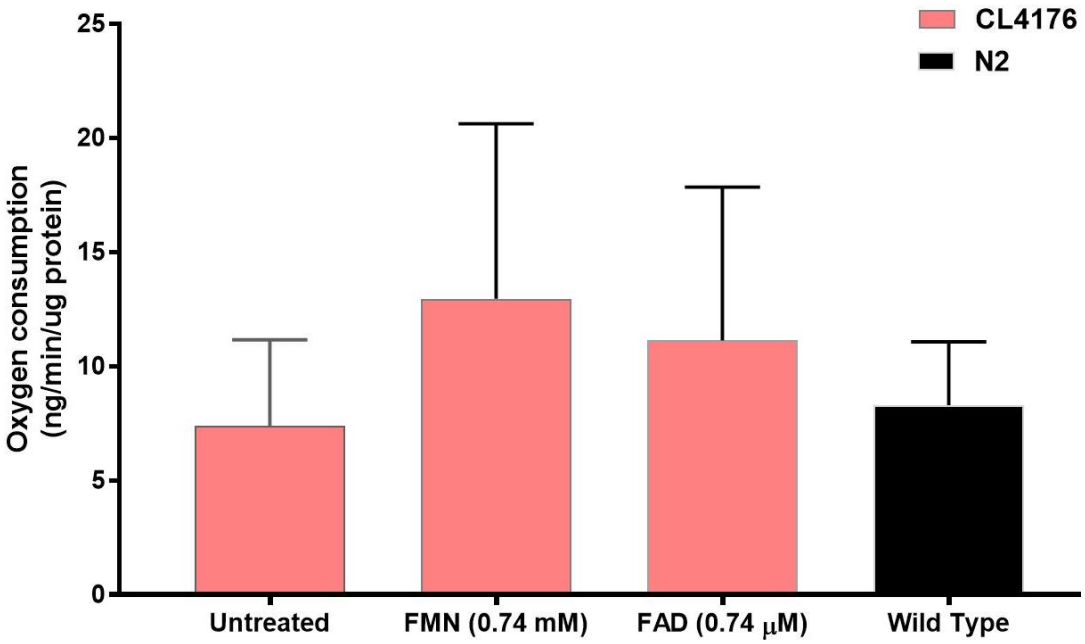

Figure 7 Effect of FMN and FAD Treatment on the Basal Oxygen Consumption Rate

The data indicate the mean of four independent triplicate trials with error bars representing the SEM. The p values compared to untreated amyloid-beta expressing worms are 0.63, 0.72, and 0.99 for FMN treated, FAD treated, and untreated wild type N2 worms respectively.

The effect of FMN or FAD supplementation on reactive oxygen species (ROS) production in the amyloid-beta expressing CL4176 worms was also determined and compared to untreated wild-type N2 worms (Figure 8). Although there were trends for increased ROS levels in CL4176 worms and decreased ROS levels following FMN or FAD treatment these values were not significantly different than the control. For consistency among assays, ROS levels were measured from the same cohort of worms used for the ATP and oxygen consumption measurements.

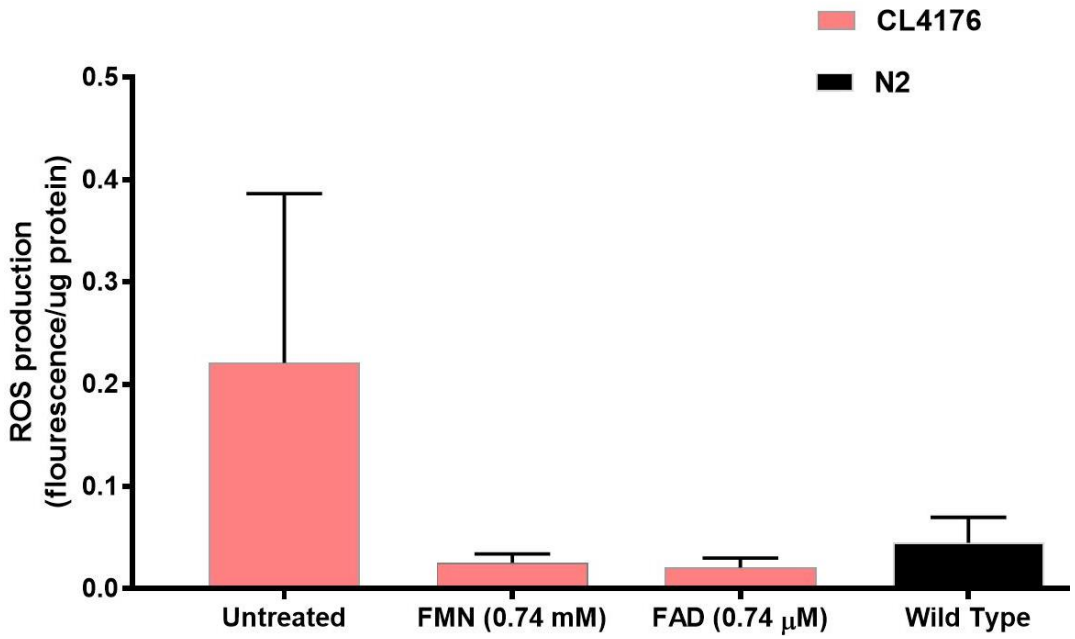

Figure 8 Effect of FMN and FAD Treatment on Reactive Oxygen Species (ROS) Production
The means and SEMs shown are from four repeated triplicate trials. The p values compared to untreated amyloid-beta expressing worms are 0.32, 0.33, and 0.30 for FMN treated, FAD treated, and untreated wild type N2 worms respectively.

Effects of DAF-16/FOXO or FAD Synthase Knockdown on Vitamin B_2 Protection

To identify molecular mechanisms through which vitamin B_2 functions to protect from amyloid-beta peptide toxicity, the stress response transcriptional regulator DAF-16/FOXO was knocked down by RNA interference (RNAi) and the worms were assayed for FMN or FAD-mediated protection from amyloid-beta mediated paralysis. All RNAi knockdown experiments were repeated three times in triplicate with each point on the graphs representing the mean of all worms for all groups. For RNAi knockdown experiments worms were fed the HT115(DE3) strain of *E. coli* used for making the whole genome knockdown library instead of the standard OP50-1 strain. This change caused an increase in the mean paralysis time of control worms from approximately 35 hours to 45 hours. Concentrations of FMN or FAD that increased the mean paralysis time by 13% when using OP50-1 bacteria were used.

Figure 9 shows results of FMN treatment of worms feeding on *daf-16* RNAi knockdown *E. coli* and assayed for amyloid-beta peptide mediated paralysis. Untreated control groups were also fed *daf-16* RNAi knockdown bacteria or empty vector (ev) bacteria as controls. Knockdown of *daf-16* did not significantly alter the rate of paralysis in untreated worms. FMN treatment of empty vector fed worms increased mean paralysis time by 14 % compared with the empty vector control fed worms, while FMN treatment of *daf-16* knockdown worms decreased mean paralysis time by 7 % (Table 2) when compared to the empty vector control fed worms (Table 2). Therefore DAF-16 is required for the protective effects of FMN treatment on amyloid-beta toxicity.

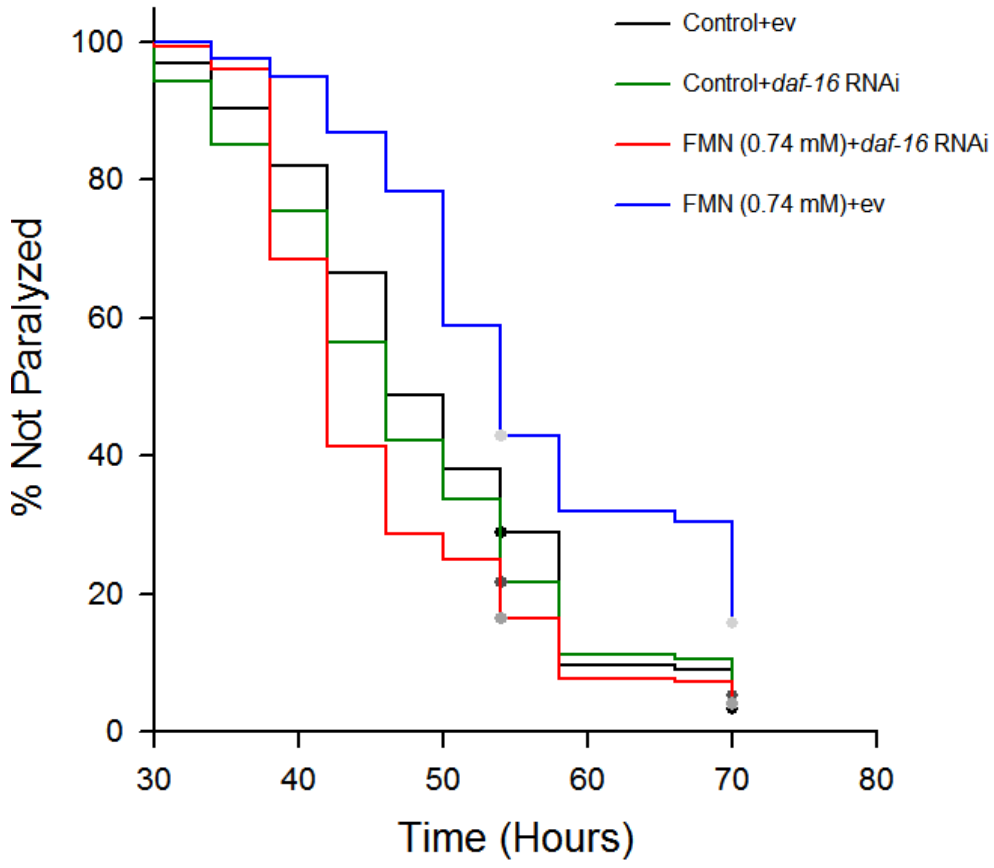

Figure 9 DAF-16 is Required for the Protective Effect of FMN on Amyloid-beta Mediated Paralysis

Figure 10 shows the effects of FAD supplementation on amyloid-beta mediated paralysis in the presence of *daf-16* knockdown. FAD treated empty vector fed worms showed a 21 % increase in mean paralysis time, while FAD treatment of *daf-16* knockdown worms did not significantly alter their rate of paralysis (Table 2). Therefore, DAF-16 is required for FAD mediated protection from amyloid-beta toxicity.

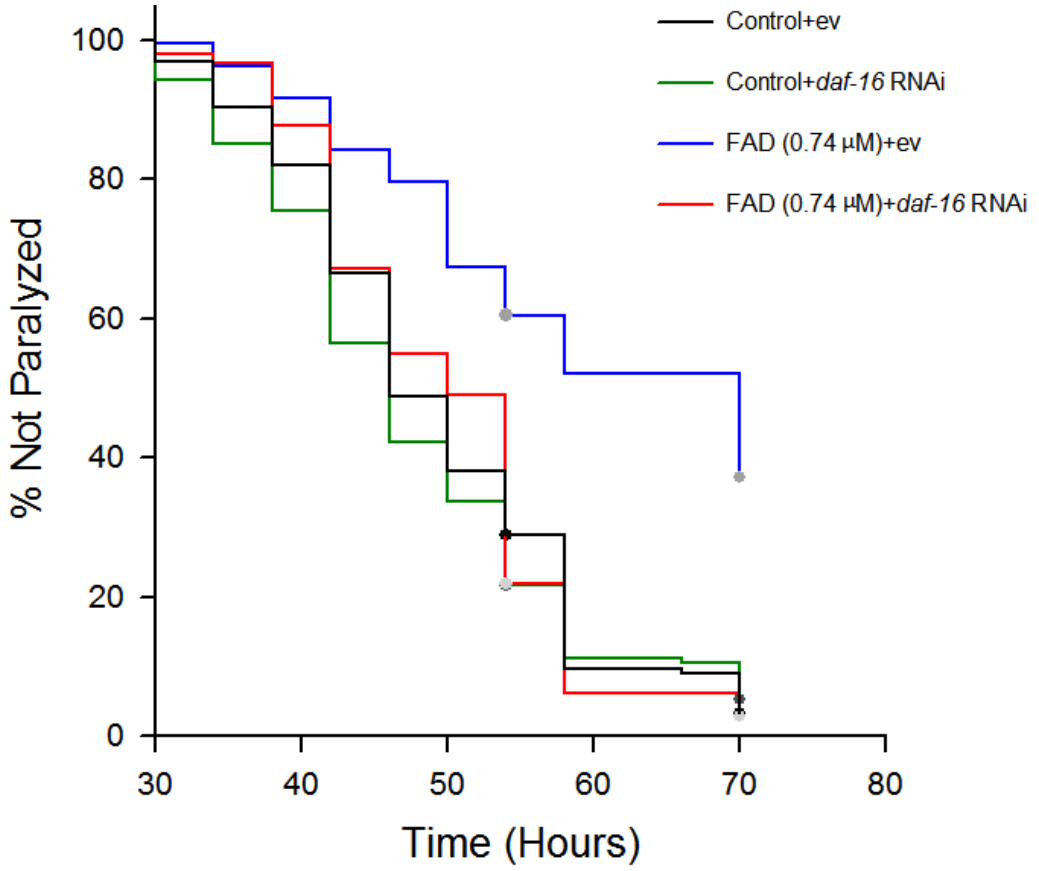

Figure 10 DAF-16 is Required for the Protective Effects of FAD on Amyloid-beta Mediated Paralysis

The *C. elegans flad-1* gene is homologous to the human FAD synthase gene whose protein product converts FMN to FAD. In attempt to determine the most protective active form of vitamin B_2, worms were subjected to *flad-1* RNAi feeding and treated with either FMN or FAD. As shown in Figure 11, knockdown of *flad-1* in untreated worms decreased mean paralysis time by 12 % indicating that decreased FAD levels likely sensitize the worms to amyloid-beta toxicity. FMN treatment of empty vector fed worms increased paralysis time by 14 %, while FMN treatment of *flad-1* RNAi knockdown did not alter the mean time to paralysis compared to untreated *flad-1* knockdown worms (Table 2). Therefore, FLAD-1 is required for FMN-mediated protection from amyloid-beta toxicity.

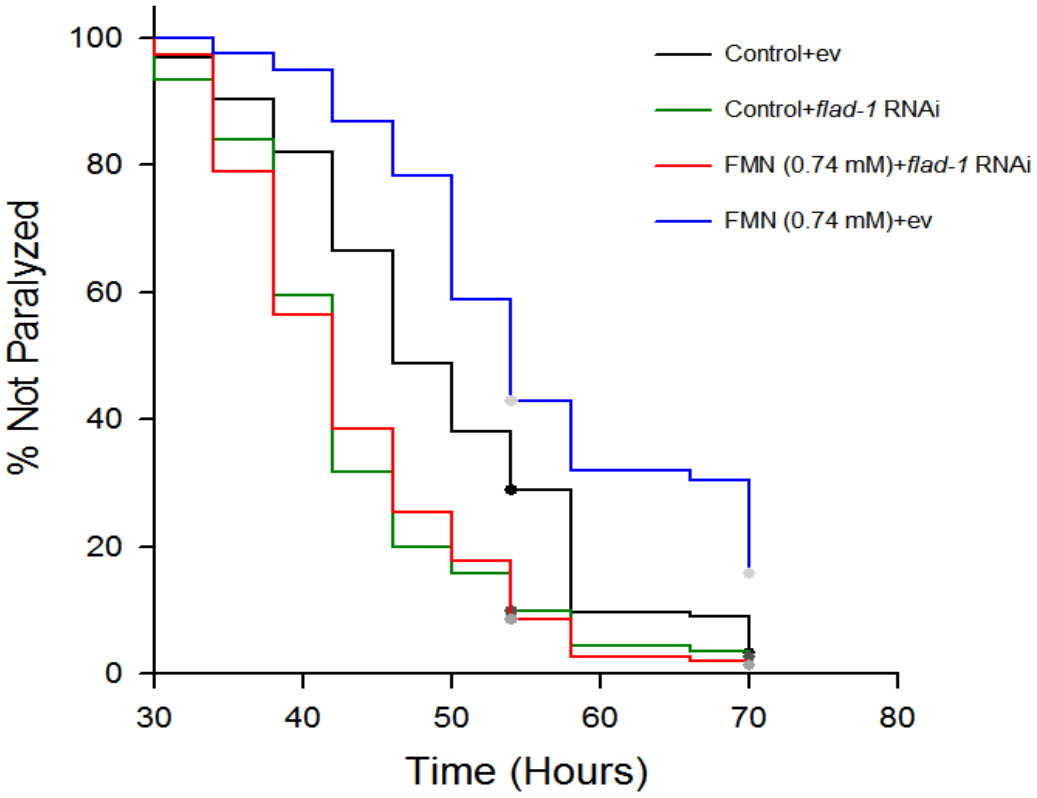

Figure 11 FLAD-1 is Required for FMN Mediated Protection Against Amyloid-beta Toxicity

Similarly, *flad-1* RNAi knockdown experiments were performed with FAD supplementation as shown in Figure 12. FAD supplementation of empty vector fed worms increased mean paralysis time by 21 %. FAD supplemented, *flad-1* knockdown worms had a mean paralysis time roughly equal to untreated *flad-1* knockdown worms. Surprisingly, FLAD-1 appears to be equally important for the protective effects of FAD supplementation as it is for FMN supplementation.

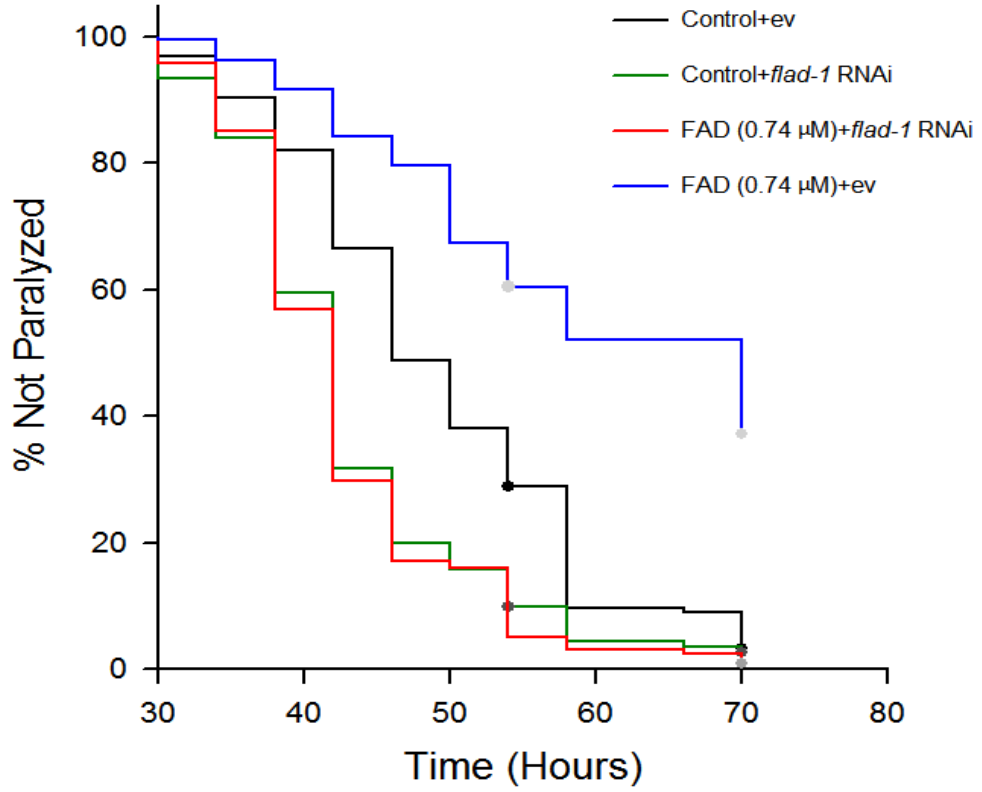

Figure 12 FLAD-1 is Required for FAD Mediated Protection Against Amyloid-beta Toxicity

Table 2 Vitamin B_2 Requires DAF-16 and FAD Synthase for Protection Against Aβ-peptide Toxicity in a *C. elegans* AD Model

RNAi treatment	Treatment	% change from control mean paralysis time	% change from empty vector, control mean paralysis time	p-value	# of worms	Replicates
empty vector (ev)	Control		0		384	3
	0.74 mM FMN	+14		<0.001	336	3
	0.74 µM FAD	+21		<0.001	291	3
daf-16	Control		-3	0.200	175	3
	0.74 mM FMN	-4		0.064	279	3
	0.74 µ FAD	+4		0.206	156	3
flad-1	Control		-12	<0.001	255	3
	0.74 mM FMN	0		0.908	338	3
	0.74 µM FAD	-1		0.325	336	3

Effect of Vitamin B_2 Supplementation on Worms Where Mitochondrial Unfolded Protein Response (UPR^{mt}) Pathway Genes are Knocked Down

FMN or FAD supplementation was further tested for the ability to delay amyloid-beta mediated paralysis in worms where mitochondrial unfolded protein response pathway genes *ubl-5* or *atfs-1* were knocked down. Knockdown of *ubl-5* or *atfs-1* did not alter the rate of paralysis in untreated worms. FMN was slightly more effective in these experiments than in previous experiments increasing the time to paralysis by 27 % in empty vector fed worms. Worms treated with FMN and *ubl-5* RNAi bacteria feeding did not have a significantly different mean time to paralysis than untreated *ubl-5* RNAi worms or untreated, empty vector control worms (Table 3 and Figure 13). Therefore, UBL-5 is required for the protective effects of FMN on amyloid-beta mediated toxicity.

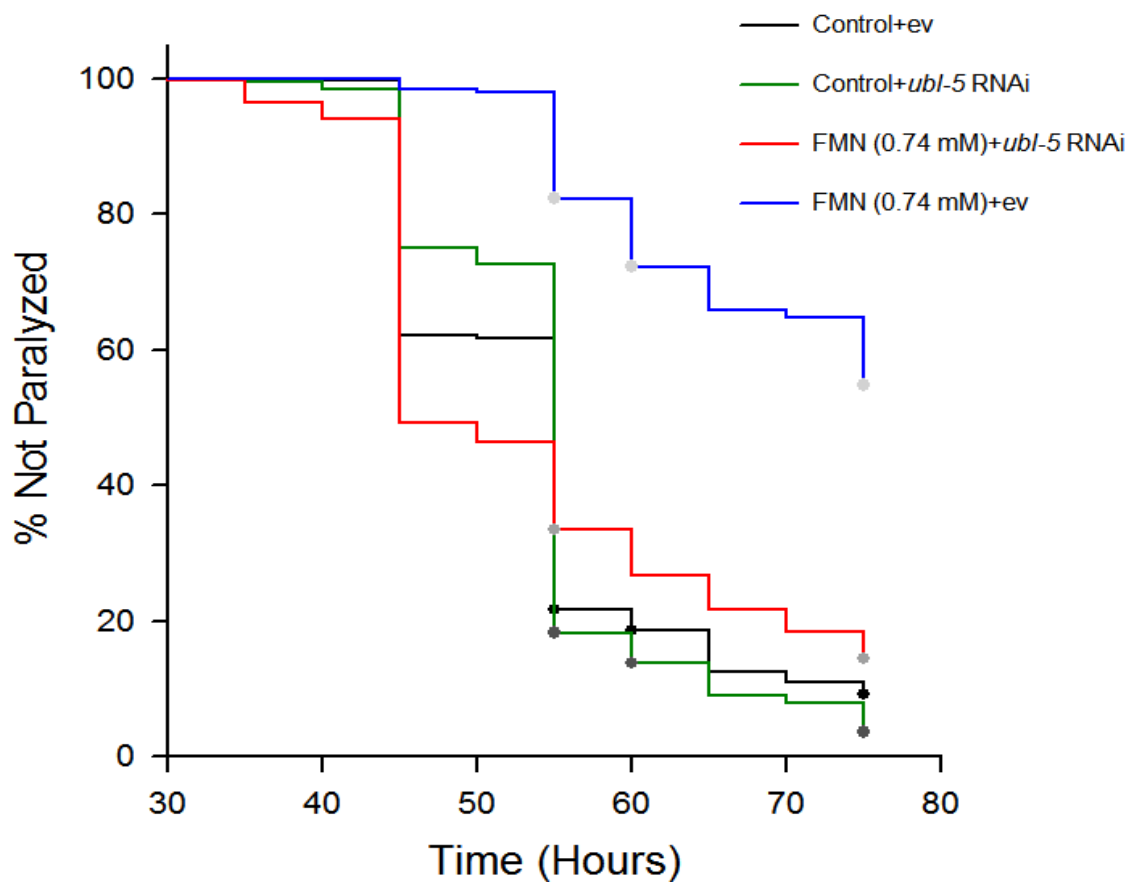

Figure 13 The Mitochondrial Unfolded Protein Response Factor UBL-5 is Required for FMN Mediated Protection Against Amyloid-beta Toxicity

Similar experiments were performed with FAD supplementation as shown in Figure 14 and Table 3. In these experiments FAD supplementation to empty vector fed worms was slightly less protective than in previous experiments increasing mean time to paralysis by only 7%. However, *ubl-5* knockdown of FAD treated worms completely prevented the FAD-mediated increase in mean time to paralysis. Therefore, UBL-5 is required for FAD-mediated protection from amyloid-beta toxicity.

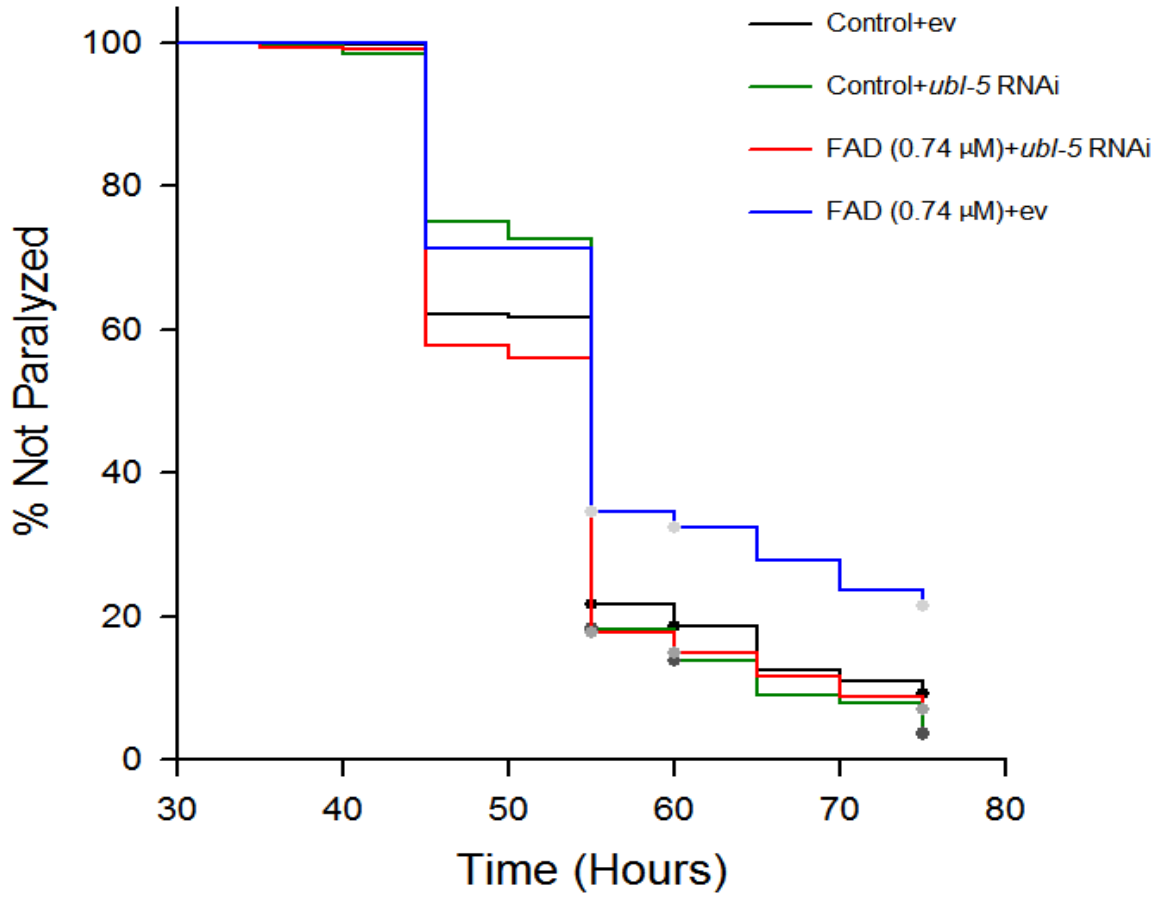

Figure 14 The Mitochondrial Unfolded Protein Response Factor UBL-5 is Required for FAD Mediated Protection Against Amyloid-beta Toxicity

To verify the effects of *ubl-5* knockdown, a transcriptional regulator of the UPR^{mt}, *atfs-1*, was knocked down. FMN treatment increased mean time to paralysis by 27% in the empty vector treated worms. FMN treatment of *atfs-1* knockdown worms led to a 11 % increase in time to paralysis (Figure 15 and Table 3). This indicates either a partial requirement of *atfs-1* for FMN-mediated protection or possibly incomplete knockdown of *atfs-1* by the RNAi clone used. Either way the data support a role for the UPR^{mt} in the protective effects of FMN on amyloid-beta mediated toxicity.

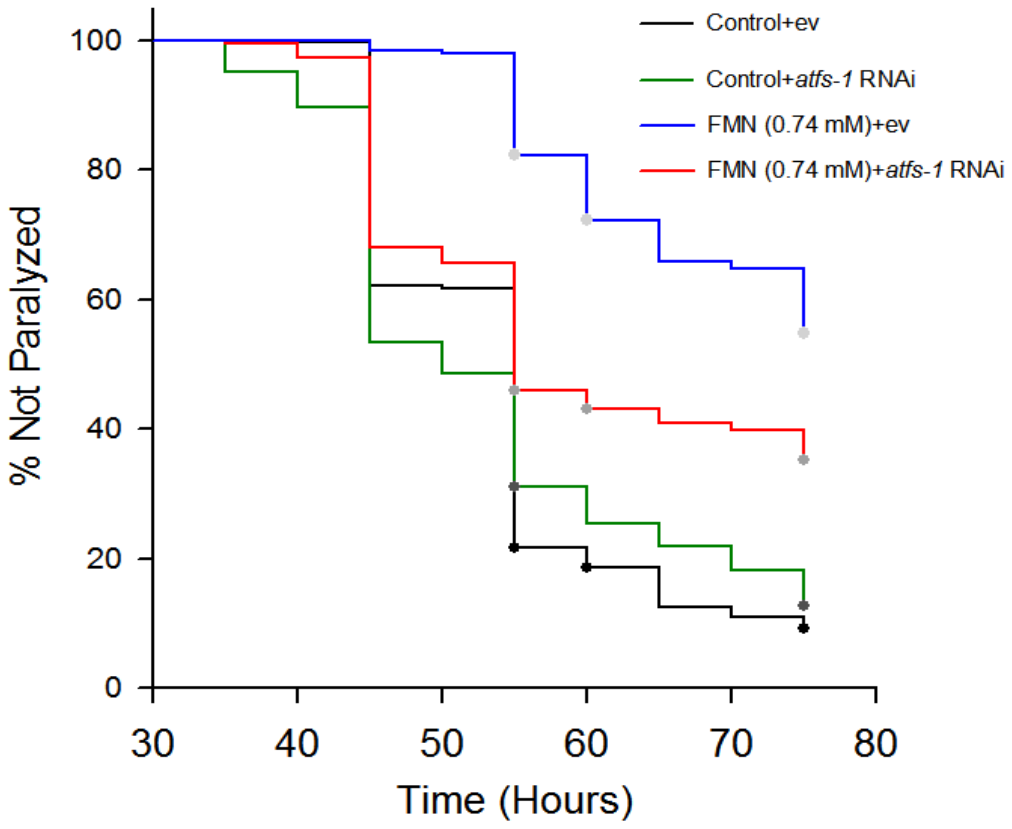

Figure 15 The Mitochondrial Unfolded Protein Response Factor ATFS-1 is Partially Required for FMN Mediated Protection Against Amyloid-beta Toxicity

Similar experiments were also performed with *atfs-1* knockdown and FAD supplementation. Once again FAD supplementation was slightly less effective in these experiments only increasing the mean time to paralysis by 7% in the empty vector fed worms. However, *atfs-1* knockdown prevented the increase in mean time to paralysis in the FAD treated worms as shown in Figure 16 and Table 3. Therefore, UPR^{mt} is required for the FAD mediated protection from amyloid-beta toxicity.

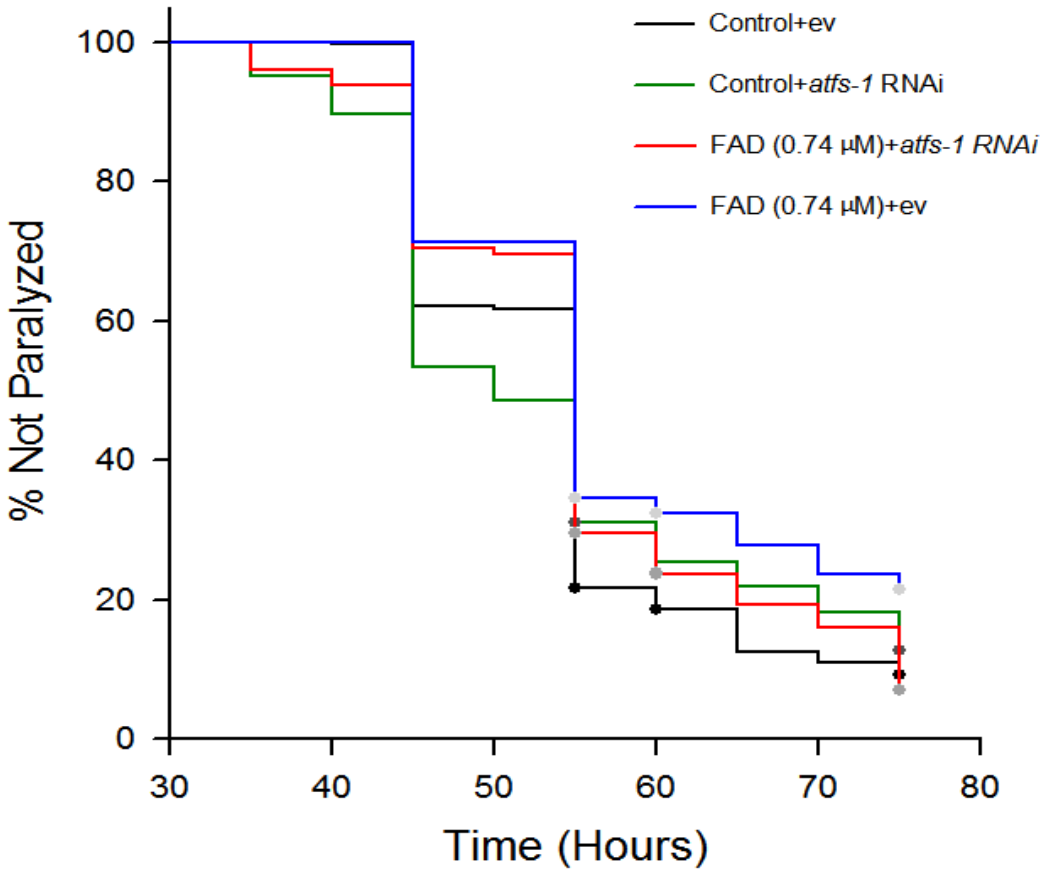

Figure 16 The Mitochondrial Unfolded Protein Response Factor ATFS-1 is Partially Required for FAD Mediated Protection Against Amyloid-beta Toxicity

Table 3 The Mitochondrial Unfolded Protein Response Factors UBL-5 and ATFS-1 are Required for FMN and FAD Mediated Protection Against Aβ-peptide Toxicity in a *C. elegans* AD Model.

RNAi	Treatment	% change from control mean paralysis time	% change from empty vector, control mean paralysis time	p-value	# of worms	Replicates
empty vector (ev)	Control		0		410	3
	0.74 mM FMN	+27		<0.001	244	3
	0.74 µM FAD	+7		<0.001	203	3
ubl-5	Control		+1	0.979	532	3
	0.74 mM FMN	-1		0.701	340	3
	0.74 µM FAD	-3		0.032	350	3
atfs-1	Control		0	0.734	396	3
	0.74 mM FMN	+11		<0.001	392	3
	0.74 µM FAD	+3		0.336	309	3

CHAPTER 4

DISCUSSION

B vitamin deficiency has been linked to the development and onset of several diseases such as cancers, heart disease, and neurodegenerative diseases (Saedisomeolia and Ashoori 2018). This class of vitamins is water soluble and is most abundantly found in plant and animal human food sources such as legumes, milk, organ meats, sea foods, etc. B vitamins are also essential nutrients that must be consumed as part of the human diet (although vitamin B_3, niacin, can be synthesized at a low level in the body from the amino acid tryptophan) (Sechi et al. 2016). Due to evidence linking B vitamin deficiencies, specifically B_2 deficiency, to the development of AD and other neurological diseases (Udhayabanu et al. 2017), this study was initiated to investigate a possible protective role of vitamin B_2 supplementation on amyloid-beta toxicity, a pathological hallmark of AD, in a *C. elegans* AD model.

A Protective Role of Vitamins B_2 and B_3 Against Amyloid-beta Mediated Paralysis

Data from our experiments suggest a protective function of the active forms of vitamin B_2 (FMN and FAD) against amyloid-beta toxicity. FMN or FAD supplementation resulted in a delay in amyloid-beta mediated paralysis by 22 % and 13 % respectively at optimal concentrations (Figure 1-2, Table 1). Although, there has been no study testing for a protective role of FMN or FAD in any *C. elegans* neurodegenerative disease models, several human clinical trials and molecular studies using high dose riboflavin supplementation have reported improved symptoms and improvement in biomarkers of disease such as improved cognitive function (Bell et al. 1992), improved muscular function (Anand et al. 2012; Foley et al. 2014), or increased flavoenzyme activities such as glutathione reductase antioxidant activity (riboflavin-dependent) (Hoey et al. 2009).

Given the importance of the active forms of vitamin B_3, NAD(H) and NADP(H), in hundreds of cellular redox and biosynthetic reactions, including their role as cofactors for metabolic and antioxidant enzymes, it was not unexpected that supplementation with an NAD precursor, nicotinic acid (0.74 mM), at an optimal concentration delayed amyloid-beta mediated paralysis (Figure 3, Table 1). This result is consistent with studies reported by Sorrentino et al. (2017) using an alternative NAD precursor, nicotinamide riboside, which was protective when supplemented to the GMC101 *C. elegans* strain (an alternative AD worm model constitutively expressing amyloid-beta in body wall muscle). This is also consistent with our finding that an optimal supplemented concentration of NAD (1.85 µM) protected against amyloid-beta mediated paralysis (Figure 4, Table 1). However, NADP supplementation failed to protect against amyloid-beta mediated paralysis even at a higher concentration of 7.4 µM (Figure 5, Table 1). This could be due to either a low absorption rate of NADP by the worms (due to the negative charge of the phosphate group) or due to us not finding the optimal dose (Chen et al. 2015), as NADP(H) levels are roughly 10 fold lower than NAD(H) levels in the cell. NADPH serves as a cofactor for the reduction of glutathione reductase in the glutathione redox cycle. NADPH is 20-100 fold more abundant than its oxidized form NADP and controls the rate limiting step of the glutathione cycle (Saedisomeolia and Ashoori 2018). Thus, the failure of NADP (oxidized) supplementation to protect against amyloid-beta toxicity could be due to its excessive accumulation and alteration of the physiological NADP/NADPH ratio. NADPH is oxidized to NADP in solution fairly rapidly, so NADPH supplementation to the worms would almost undoubtedly lead to similar results as NADP supplementation.

Vitamin B_2 Supplementation and Mitochondrial Bioenergetic Function

Our data shows no significant difference in ATP levels, oxygen consumption, or reactive oxygen species (ROS) production between FMN or FAD treated CL4176 worms and untreated controls. There was also no significant difference in the mitochondrial bioenergetic function between treated or untreated CL4176 AD worms and wild-type N2 worms. In the AD worm model we used, amyloid-beta is only expressed in body wall muscle. Although amyloid-beta is known to inhibit complex IV of the electron transport chain, this is likely not measurable in this worm strain where over 80% of the cells do not express amyloid-beta. Both FMN and FAD serve as important coenzymes for several essential mitochondrial enzymes functioning in energy production or apoptosis. The simplest explanation for the lack of effect of FMN or FAD on mitochondrial bioenergetics function is that FMN and FAD are endogenously present at high enough levels that exogenous supplementation does not further increase mitochondrial enzyme activities. Results from supplementation with these two compounds (Figures 6-8) on mitochondrial bioenergetic function in this *C. elegans* model should not be extrapolated to suggest that vitamin B_2 does not play a protective role on mitochondrial bioenergetics in other human diseases.

Studies conducted by Karakoyun et al. (2017) reported riboflavin supplementation to reduce reactive oxygen species generation in acetic-acid induced colonic injured rats. Also, Peluchetti et al. (1991) reported riboflavin supplementation to increase the maximal oxygen consumption rate of a muscle biopsy in a small clinical trial of four patients with multiple acyl-coenzyme A dehydrogenase deficiency (MADD). Riboflavin supplementation also improved mitochondrial function or disease state in disease models resulting from a deficiency in mitochondrial enzymes such as those required for the assembly of mitochondrial complex I in a complex I-mutant *C. elegans* strain or in cells deficient in complex IV activity (Grad and Lemire 2006). Another possible reason for no significant

effect of FMN or FAD supplementation on mitochondrial bioenergetic function might be due to the low supplementation dose when compared to other studies where riboflavin was supplemented at much higher doses. For example, high dose supplementation showed significant delay in disease progression in clinical studies of Brown-Vialetto-Van Laere syndrome (a disease associated with respiratory insufficiency in children) (Anand et al. 2012).

Vitamin B_2 May Activate Transcriptional Regulators of Stress Response Pathways

We further investigated a role of the stress response transcriptional regulator DAF-16 in the protective effects of FMN and FAD to delay amyloid-beta toxicity. RNAi knockdown of *daf-16* significantly blocked the protective effect of FMN or FAD supplementation on amyloid-beta mediated paralysis (Figure 9 & 10, Table 2). This data suggests that FMN and FAD protect through activation of DAF-16. This discovery may provide a possible answer to a question posed by many biomedical researchers and clinicians for the main molecular mechanism through which riboflavin supplementation is protective. Since humans have 4 homologs of DAF-16 (FOXO1, FOXO3, FOXO4, and FOXO6), which can induce protective gene expression programs such as stimulation of antioxidant genes and autophagy genes, induction of one or more of these FOXO genes may be responsible for the protective effects reported in published animal and clinical riboflavin supplementation studies, such as those reported by Shukitt-Hale (2012) and Saedisomeolia and Ashoori (2018). Our results on the dependency of DAF-16 for protection by FMN or FAD are different from the results of the study reported by Sorrentino et al. (2017) where nicotinamide riboside (NR) supplementation protected GMC101 AD worms. NR supplementation significantly delayed amyloid-beta

mediated paralysis like we found for FMN or FAD supplementation. However, the protective effects of NR did not rely upon DAF-16 activation.

Knockdown of *flad-1* (an ortholog of human FLAD1) completely prevented the protective effects of added FMN or FAD on amyloid-beta toxicity (Figure 11 & 12, Table 2). This suggests a key role for FLAD-1 in maintaining a balanced FMN/FAD ratio for the protective effects observed. In line with our findings of the significance of FLAD-1 function during FMN or FAD supplementation, Auranen et al. (2017) reported that riboflavin supplementation ameliorated disease symptoms in patients suffering from partial loss of function of the FLAD1 enzyme resulting in multiple acyl-CoA dehydrogenase deficiency (MADD). Given the fact that deficiency of normal endogenous FLAD1 activity levels lead to abnormal flavin metabolism and cause disease and that *flad-1* knockdown sensitizes worms to amyloid-beta toxicity, our findings suggest an important role of increased levels of FAD in activating DAF-16 and UPR^{mt} to protect against amyloid-beta toxicity. In addition, since *flad-1* knockdown prevents the protective effects of FAD supplementation, added FAD may get catabolized to riboflavin and then resynthesized to FMN and FAD to exert its protective effects.

Vitamin B_2 Activation of the Mitochondrial Unfolded Protein Response Pathways

In addition to activation of the DAF-16 stress response pathway by FMN or FAD supplementation, the essential factors of the UPR^{mt} *ubl-5* (ubiquitin-like protein, UBL5) and *atfs-1* (Activating transcription factor associated with stress) were also required for the protective effects. UBL-5 is required for normal mitochondrial morphology, assembly of mitochondrial multi-protein complexes, and induction of the UPR^{mt} (Benedetti et al. 2006). In contrast to the results presented here where no effect was shown on CL4176 worms, *ubl-5*

or *atfs-1* knockdown facilitated paralysis in untreated GMC101 amyloid-beta expressing worms.

ATFS-1 shares sequence homology with several proteins in the ATF transcription factor family in mammals. Mammalian ATF4 or ATF5 are activated during mitochondrial dysfunction and translocate to the nucleus to activate networks of mitochondrial stress response genes required for the UPR^{mt} and mitochondrial proteostasis (Melber and Haynes 2018). Thus, results from *ubl-5* and *atfs-1* RNAi knockdown experiments suggest a role for high levels of vitamin B_2 in upregulating genes required for mitochondrial protein refolding (Grad and Lemire 2006) and protein import. The UPR^{mt} was shown to be activated in GMC101 worms by the mitochondrial dysfunction caused by amyloid-beta peptide deposition (Sorrentino et al. 2017), while it may not have been activated in the untreated CL4176 strain of worms that we used, as we did not find that knockdown of UPR^{mt} factors hastened the rate of paralysis.

Conclusions

This study has established that supplementation with the appropriate concentrations of the active forms of vitamin B_2 (FMN or FAD) can protect against amyloid-beta peptide toxicity through activation of the DAF-16/FOXO stress response pathway and the mitochondrial unfolded protein response (UPR^{mt}) pathway. Although, our initial hypothesis was that vitamin B_2 supplementation would improve mitochondrial bioenergetic function, results from our findings have suggested otherwise. Thus, it can be concluded from this study that vitamin B_2 supplementation might serve a limited role in improving cellular metabolic function such as improving mitochondrial bioenergetic function, but instead protect through inducing the transcription of genes associated with cellular stress response pathways.

Future Studies and Study Limitations

To further confirm the protective roles of FMN or FAD supplementation through the activation of conserved stress response signaling pathways, the following future experiments could be conducted in *C. elegans*:

- Perform RNAi knockdown of the riboflavin kinase gene (R10H10.6) to confirm if inhibition of endogenous FMN synthesis can prevent the protective effects of added FMN or FAD.
- Treat SOD-3::GFP expressing DAF-16 reporter worms with FMN or FAD to check for the transcriptional activation of the SOD-3 promoter by the DAF-16/FOXO transcription regulator.
- Perform lifespan assays of FMN or FAD supplementation and check for genes required for lifespan extension.
- Treat HSP-6::GFP or HSP-60::GFP expressing UPRmt reporter worms with either FMN or FAD to check for upregulation of GFP expression through mitochondrial unfolded protein response activation.

The study has the following limitations. The *C. elegans* AD model used for this study only expresses the amyloid-beta peptide, $A\beta_{1-42}$, and not full-length AβPP since *C. elegans* apparently lack the β and γ secretases involved in the processing of AβPP to toxic amyloid-beta peptide. Amyloid-beta is expressed in body wall muscle instead of neurons as only a much delayed, mild phenotype was observed when the peptide was expressed in neurons. Also, the peptide must be overexpressed in the worms at a higher level to induce the same level of toxicity observed as when expressing it in mammalian brains or cell lines. Therefore, similar experiments with FMN or FAD treatment should be performed in mammalian models of amyloid-beta toxicity to confirm the findings presented here.

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