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Deltamethrin Induces Endoplasmic Reticulum Stress and Increases Proteotoxicity in Caenorhabditis Elegans

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DELTAMETHRIN INDUCES ENDOPLASMIC RETICULUM STRESS AND INCREASES PROTEOTOXICITY IN *CAENORHABDITIS ELEGANS*

A Thesis Presented

by

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ABSTRACT

DELTAMETHRIN INDUCES ENDOPLASMIC RETICULUM STRESS AND INCREASES PROTEOTOXICITY IN *CAENORHABDITIS ELEGANS*

FEBRUARY 2019

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Deltamethrin is a widely used type-II pyrethroid insecticide in agricultural, industrial and domestic pest control. Previous studies have shown that deltamethrin can induce ER stress response *in vivo*. However, it is still unclear whether deltamethrin can disturb protein homeostasis. To investigate how deltamethrin affects ER protein homeostasis, we used a *C. elegans* model of protein misfolding. In the current study, deltamethrin induced ER stress response in *C. elegans*. Moreover, exposure to deltamethrin increased the accumulation of unfolded and misfolded proteins in a *C. elegans* model of polyglutamine aggregation in body wall muscles. Deltamethrin also increased the toxicity of polyglutamine aggregates in *C. elegans*, as characterized by decreased locomotion with deltamethrin treatment. These data indicate that exposure to deltamethrin can induce ER stress response, increase the accumulation and proteotoxicity of unfolded and misfolded proteins. As ER stress plays a pathological role in many diseases, these findings provide evidence for the potential pathological role of deltamethrin in ER stress-related diseases.

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CHAPTER 1

INTRODUCTION

Disturbances to the regular function of the endoplasmic reticulum (ER) can lead to the accumulation of unfolded and misfolded proteins in the ER, a condition called ER stress (1, 2). ER stress has been associated with the pathology of many conditions, including aging, neurodegeneration, obesity, and diabetes (2, 3). Moreover, increasing evidence has pointed out that environmental contaminants, like pyrethroid insecticides, can contribute to diseases associated with ER stress (4, 5). Thus, identification of potential ER stress-inducing environmental contaminants may help in the prevention and treatment of diseases associated with ER stress.

Deltamethrin is a type II pyrethroid that is widely used as an insecticide in agriculture, industrial, medical and home pest control (6, 7). Pyrethroids are considered as low-toxicity replacements to organophosphorus and carbamate insecticides (6). However, recent studies have indicated that a high level of exposure to deltamethrin can result in convulsion, tremor, and ataxia in rodents (8-10). In addition, deltamethrin has been found to induce ER stress response *in vitro* and *in vivo* (4, 11). Growing evidence suggests that deltamethrin can induce ER stress and disturb protein homeostasis (4, 10, 11). Therefore, we investigate the role of deltamethrin in ER stress and protein misfolding *in vivo* for the first time.

Caenorhabditis elegans, a free-living nematode, was used in this study as a model organism to investigate the role of deltamethrin in ER stress, protein misfolding, and proteotoxicity. *C. elegans* model has many advantages over rodent models, including short lifespan, fully sequenced genome, and ease of maintenance (12). Additionally, this

nematode model has been established and used extensively for studies on ER stress and protein misfolding (13). Therefore, *C. elegans* is an appropriate model to use in the current study.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Caenorhabditis elegans is a widely used animal model that has many advantages over other *in vivo* models (12). The wildtype *C. elegans* has a short life cycle (about 2 days at 25°C), a relatively short lifespan (about 2-3 weeks), and a large brood size (more than 300 progeny) (12). In addition, experiments using *C. elegans*, which has a transparent body, only require relatively simple laboratory conditions and do not need institutional committee approval (12). For example, *C. elegans* is fed with non-pathogenic *Escherichia coli* OP50 and raised on petri dishes or in liquid media (12). Thus, research using *C. elegans* is much more time and cost-efficient than research using vertebrate models. Moreover, *C. elegans* shares the basic biology of vertebrates; 83% of its proteome is homologous to that of humans, and about two thirds of its genes are related to human diseases (14). In addition, *C. elegans* has a completely sequenced genome and more than 3,000 mutants are available, and RNA interference (RNAi), microinjection, as well as genetic crosses, are amenable in this worm model (14). These reasons make this animal model a powerful toolbox in many life science research areas (15).

2.2 Basic biology of *C. elegans*

C. elegans is a free-living nematode about 1-1.5 mm long in its adult stage (14). It has a life cycle of about 2 days; this includes an embryonic stage, four larval stages (L1-L4), and the adult stage (12). Its eggs have an impermeable shell that isolates them from the outside environment (12). Hatched eggs turn into L1-stage worms, which proceed with

adequate food into the L2-L4 stages in the developmental process. The cuticle of the worms is reestablished after each larval stage (12). When the nematode encounters harsh environments, including an absence of food and the presence of unfavorable chemicals, it arrests development and enters a dormant state named a dauer larvae stage (16, 17). Nematodes in the dauer stage will continue to grow into L4 stage worms with sufficient food and favorable environmental conditions (16).

C. elegans has a high degree of cell differentiation and specialization (16). The worm has five systems; the epidermis, and muscular, digestive, nervous, and reproductive systems (16). The presence of well-defined tissues and organs makes the worm useful for research in biological processes involved in multicellular organisms (18).

The epidermis of the worm is composed of epidermal cells that secrete the cuticle, an exoskeleton that helps in the locomotion, protection, and growth of the worm (12). The cuticle of *C. elegans*, which consists of collagens, proteins, glycoproteins, and lipids, is reestablished five times during its development (12). Movement is controlled by muscles that are attached to the epidermis (16). The relaxation and contraction of these muscles are necessary for the sinusoidal movement of the animal (16). For example, one of the *unc* (uncoordinated) genes, *unc-54*, encodes proteins that are needed for the typical sinusoidal movement of the worms, and alterations to *unc-54* can cause a paralytic phenotype (16).

The digestive system of *C. elegans* is primarily composed of the pharynx, intestine, rectum, and anus (16). When the worm eats, food passes through the mouth opening first and then gets grinded at the pharynx (16). Next, the ground food reaches the intestine, where it is further digested, and where nutrients are absorbed, utilized, or stored

(12). After the food has been digested in the intestine, remaining waste passes the rectum and anus and gets excreted through a rectal valve; the defecation cycle is about 50 seconds (12).

The nervous system of *C. elegans* contains 302 neurons for an adult hermaphrodite (12). *C. elegans* has three classes of neurons; chemosensory, mechanosensory, and thermosensory neurons (19). The worm model shares many proteins with mammalian neurons involved in the formation, trafficking, and releasing of synaptic vesicles (19). Because *C. elegans* uses many of the same neurotrasmitters as vertebrates - including dopamine, serotonin, gamma-amino butyric acid, and acetylcholine - it has been used as a model for many neurodegenerative diseases (16).

The reproductive tissue of the adult hermaphrodite *C. elegans* is comprised of the somatic gonad that houses the germline and egg-laying apparatuses (12). Over 99% of the worms are self-fertilizing hermaphrodites, which helps to preserve homozygous clones (16).

2.3 ER Stress

Proteins are synthesized by the ribosomes attached to the rough ER. They are modified and folded by chaperones and enzymes in the ER, and then transported out of the ER-lumen (20). As the ER is a highly dynamic organelle, many parameters in the ER microenvironment can affect the elements of protein folding, including chaperones, foldases, calcium levels, the redox milieu, the phospholipid composition of the ER membrane, etc. (20). Once protein folding is disturbed or inadequate, unfolded and misfolded proteins can accumulate in the ER lumen and the cytoplasm, a condition named ER stress (1, 2). The ER has a ‘quality control’ system to degrade unfolded and

misfolded proteins: the ER-associated degradation (ERAD) pathway delivers damaged protein to the proteasome, and the autophagy pathway transports unfolded and misfolded proteins to the lysosome (21). However, overaccumulation of unfolded and misfolded proteins leads to activation of the unfolded protein response (UPR^{ER}), which has both protective and apoptotic components (22). It has been reported that the UPR^{ER} has a pathological role in many conditions, including aging, neurodegenerative diseases, obesity, and type 2 diabetes (2). Thus, understanding the role of UPR^{ER} may provide important insights into the pathology and new treatment of these and other diseases.

2.4 Unfolded protein response in ER (UPR^{ER})

The UPR^{ER} is a signaling pathway that is conserved from yeasts to mammals, which allows researchers to study this pathway in a variety of models, including *C. elegans* (23). The UPR^{ER} stress response system can monitor the accumulation of unfolded and misfolded proteins and correct protein folding and processing (20). The UPR^{ER} system tries to maintain normal cell function by reducing protein-folding workload, strengthening protein-folding capacity, as well as removing and degrading unfolded and misfolded proteins (23). However, when ER stress cannot properly handle unfolded and misfolded proteins, the apoptotic pathway can be triggered by the UPR^{ER} via UPR^{ER} signaling pathways (20).

2.5 The UPR^{ER} Signaling Pathways

The UPR^{ER} system includes three branches, each represents by one of three transmembrane proteins: the inositol-requiring kinase-1 (IRE-1), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF-6)

(23). All these branches have a luminal domain that can sense the amount of unfolded and misfolded proteins and initiate responses to manage ER stress (21).

2.5.1 IRE-1 Pathway

The IRE-1 branch of the UPR^{ER} is conserved from yeasts to humans (5). Although the exact mechanism of IRE-1 activation is not completely understood, one study suggests that unfolded and misfolded proteins, as well as binding immunoglobulin protein (BiP), participate in modulation of IRE-1 (24). Without unfolded or misfolded proteins, BiP binds to IRE-1 to inhibit its activity (25). In the presence of unfolded and misfolded proteins, BiP binds to them and activates IRE-1 (25). Activation of IRE-1 results in the dimerization and autophosphorylation of the protein (26). The activated IRE-1 acts as an endoribonuclease that cleaves a 26-base fragment from X-box binding protein-1 (XBP-1) mRNA, and the spliced mRNA is translated into spliced X-box binding protein-1 (spliced XBP-1) (26). In mammals, spliced XBP-1s regulate components in protein degradation, protein folding, and ER membrane biogenesis, and this regulation can alleviate ER stress as well as increase ER capacity (26). In *C. elegans*, spliced XBP-1s can upregulate heat shock protein 4 (HSP-4, the homolog of mammalian BiP), as well as ER-associated Degradation (ERAD) components and other chaperones (27-31).

2.5.2 PERK Pathway

PERK is an ER-resident transmembrane protein (32). PERK is activated by dissociation of BiP from its luminal domain (25). The activated PERK can phosphorylate eukaryotic translational initiation factor 2α (eIF2α) and downregulate global protein synthesis to alleviate ER workload (32). Moreover, the activation of eIF2α can also increase the

translation of protective proteins, including activating transcription factor 4 (ATF4) (33). In mammals, ATF4 controls a range of target genes, including the pro-apoptotic transcription factor, C/EBP homologous protein (CHOP) (34-36). The downstream targets of ATF4 in *C. elegans* are, however, still not known.

2.5.3 ATF6 Pathway

ATF-6 is a transmembrane transcription factor that is activated upon accumulation of unfolded and misfolded proteins (34). Once activated, ATF-6 translocates to the Golgi apparatus, where it is cleaved by site-1 protease (S1P) and site-2 protease (S2P) to generate the cleaved form of ATF-6 (5). Once cleaved, ATF-6 upregulates a wide range of UPR^{ER} target genes, including chaperones, protein foldases, and components in the protein degradation ERAD pathway in mammals (5). However, in *C. elegans*, ATF-6 regulates fewer target genes than in mammals, and the specific role of ATF-6 branch in *C. elegans* is still to be determined (37).

2.6 Conditions related to ER Stress and the UPR^{ER}

2.6.1 Aging and Reduced UPR^{ER}

The ability of UPR^{ER} to respond to ER stress declines with age. This may allow for an accumulation of unfolded and misfolded proteins and contribute to age-related diseases (35). In addition, the UPR^{ER} response to protein chaperones and foldases also decreases with aging (38). Many of these chaperones suffer oxidative damage during aging, which can further imperil the function of the UPR^{ER} system (39). The decline in the protective role of the UPR^{ER} during aging is accompanied by an increase of pro-apoptotic markers in the UPR^{ER} (38). Expression of pro-apoptotic markers, including C/EBP homologous

protein (CHOP) and caspase-12, are upregulated in the aged, but not young rats, when treated with lactacystin, a proteasome inhibitor that disrupts protein homeostasis (40). Thus, overall, aging reduces the protective functions of the UPR^{ER} and strengthens its pro-apoptotic signaling, which further leads to age-related accumulation of unfolded and misfolded protein and cell dysfunction.

The homolog of insulin/insulin-like growth factor receptor (IIS), DAF-2, is well known to be associated with lifespan in *C. elegans* (41). The DAF-2 mutant has an extended lifespan that depends on the activation of the forkhead box transcription factor (FOXO). In addition, UPR^{ER} components *ire-1* and *xbp-1* are involved in lifespan extension, which occurs as *xbp-1* upregulates *daf-16* in the DAF-2 mutant (42).

In addition to IIS, protein skinhead-1 (*skn-1*), the mammalian homolog of nuclear respiratory factor 2 (Nrf2), has been associated with longevity in *C. elegans* (43). A recent report indicates that Vitamin D can extend lifespan through activation of *skn-1* in *C. elegans*, and the activation of *skn-1* requires UPR^{ER} components *ire-1* and *xbp-1* (44). Consistently, others have reported that *skn-1* contributes to longevity by activating the IRE-1 and PERK branches of the UPR^{ER} (33).

Improving ER protein homeostasis also contributes to longevity in *C. elegans* (35). As UPR^{ER} regulates many components that can improve ER protein homeostasis (41), upregulation of UPR^{ER} can improve age-related diseases (45). In fact, the activation of the hexosamine pathway, which has been linked to UPR^{ER} upregulation, can improve longevity and reduce protein aggregation by enhancing ER-associated protein degradation, proteasome function, and autophagy in *C. elegans* (45, 46).

2.6.2 Neurodegenerative disease

A common pathological marker for many neurodegenerative disorders is the accumulation of unfolded and misfolded proteins in neurons (47). Thus, studying stressors and potential therapies that target unfolded and misfolded proteins may provide a better understanding of these disorders and play a crucial role in the development of preventive and treatment strategies for these neurodegenerative disorders (47).

2.6.2.1 Alzheimer's Disease

Alzheimer's is a neurodegenerative disease exhibiting the presence of extracellular senile plaques and intracellular neurofibrillary tangles (17). The major component of senile plaques is β-amyloid (Aβ), while the neurofibrillary tangles are formed by aggregation of tau proteins (17). Many studies have reported that ER stress and the UPR^{ER} system are involved in the development of Alzheimer's disease (48, 49). Recent studies have shown that Aβ can accumulate at the ER lumen and increase free and bound Ca^{2+} in neurons (50). This causes ER stress and activation of the UPR^{ER} (50), which leads to impaired synaptic function and eventually induces apoptosis of neurons (49). In addition, a protein associated with Alzheimer's disease, mutated presenilin-1, can increase Ca^{2+} release in the ER and inhibit IRE-1 activity, which worsen ER homeostasis and reduce UPR^{ER} signaling (51).

In humans, Aβ is produced when amyloid precursor protein (APP) is cleaved by β- and γ-secretase (17). However, the *C. elegans* homolog of APP, *apl-1*, does not contain the genetic information for β-amyloid (17). Thus, researchers have created *C. elegans* models of Alzheimer's disease by introducing human $A\beta_{1-42}$ to generate transgenic strains (52). They have applied these models to screen bioactive compounds

and discover contributors to the disease (52). Using this model, resveratrol is reported to reduce the toxicity of Aβ aggregation through upregulation of autophagy and proteasomal degradation (52). Ablation of the IRE-1 branch of the UPR^{ER} may reduce the toxicity of β-amyloid, although the role of IRE-1 in Alzheimer's disease has been controversial (53, 54).

2.6.2.2 Parkinson's Disease

Parkinson's disease is a neurodegenerative disorder marked clinically by tremors, bradykinesia, and impaired balance (14). These have been associated with two main pathological hallmarks: the formation of proteinaceous inclusions (Lewy Bodies) and the loss of dopaminergic neurons from the *substantia nigra* (14). Recent studies have identified a pathological role for ER stress in Parkinson's disease (55). Aggregation of α-synuclein in the ER can impact ER homeostasis, including depletion of ER chaperones and inhibition of ER-to-Golgi trafficking (56-58). Although it is generally agreed that ER stress is involved in the neuropathology of Parkinson's Disease, the exact mechanism for how induction of ER stress causes the loss of dopaminergic neurons in Parkinson's disease is not yet well understood (58).

In humans, α-synuclein aggregates are products of the *α-syn* gene (14). Because *C. elegans* does not possess this gene (14), *C. elegans'* models of α-synuclein are established with human α-synuclein expression in neurons or muscle promoters (19). In addition, neurotoxins like 6-hydroxydopamine can be used to degenerate dopaminergic neurons in *C. elegans*, which also induces ER stress (59). Consistently, mutation of leucine-rich repeat kinase 2 (LRRK2), a gene commonly associated with Parkinson's disease, has been shown to inhibit UPR^{ER} responses, leading to increased ER stress in *C.*

elegans (59). Moreover, a type of bacterial metabolite, phenazine derivatives, has been shown to exacerbate ER stress in the worm model of Parkinson's disease (13).

2.6.2.3 Huntington's Disease

Huntington's disease is an inherited neurodegenerative disease that results in cognitive, motor, and psychiatric changes (60). This disease results from expansion of CAG repeats in the *Huntingtin* gene that adds a long polyglutamine repeat to the Huntingtin protein (60). Recent studies have linked ER stress to the development of Huntington's Disease in several ways: [1] polyglutamine fragments can entrap ERAD proteins (Npl4, Ufd1, and p97) and induce ER stress (61), [2] overexpression of these ERAD proteins can reduce the toxicity caused by polyglutamine (61), and [3] polyglutamine indirectly increases accumulation of unfolded and misfolded proteins by disruption of vesicular trafficking and by causing defects in lysosome-mediated protein degradation (62).

In *C. elegans*, the human huntingtin-polyglutamine protein is incorporated in muscle or neurons to generate disease models for relatively easy detection of disease development (18, 63). Using these models, UPR^{ER} components and changes in the disease progression can be investigated. For example, manganese treatment can induce ER stress and increase polyglutamine aggregates as an indicator of Huntington's disease (64). This provides further evidence that ER stress is associated with the development of Huntington's disease (64).

2.6.2.4 Obesity and Type 2 Diabetes

Obesity is also known to be associated with increased ER stress and activated UPR^{ER} in many tissues, such as hypothalamus, liver, muscle, and adipose tissue (65). Treatment of

chemical chaperones, which improve protein folding and synthesis, can improve ER function and reduce ER stress markers in the liver and adipose tissue of obese mice (66). This indicates that obesity-associated ER stress may result from a high protein synthesis load (66). In addition, increased free fatty acid load, as well as changes in the composition of the ER membrane, can also activate the UPR^{ER}, which suggests that the UPR^{ER} responds to lipid imbalances (67-70).

The activated UPR^{ER} plays a significant role in the development of obesity-related disorders, particularly type 2 diabetes (65). Increased ER stress in obesity can lead to the induction of c-Jun N-terminal kinases (JNK), and thus reduce the activity of insulin receptor substrate-1 (IRS1) to down-regulate insulin signaling (66). The UPR^{ER} also interacts with hepatic gluconeogenesis by ATF-6, one of the three branches of the UPR^{ER}, to inhibit the activity of CREB-regulated transcription coactivator 2 (CRTC2), which results in down-regulation of gluconeogenesis in the liver (71). With increased insulin demand due to insulin resistance, the pancreatic β-cells can exceed their protein folding capacity and generate ER stress (5). Eventually, prolonged ER stress in the pancreas can trigger the UPR-mediated apoptosis in β-cells, exacerbate hyperglycemia, and eventually increase insulin deficiency (22).

C. elegans is an established model for research in obesity and type 2 diabetes. The key pathways on energy metabolism, such as the lipid metabolism and insulin/insulin-like growth factor signaling (IIS), are conserved between mammals and C. elegans (72). For example, researchers have found that a change of phospholipid composition in the ER membrane in *C. elegans* can activate UPR^{ER} without disturbing ER proteostasis (68). This indicates that in C. elegans lipid imbalance may directly activate the UPR^{ER} (68),

which is consistent with observations in mammals (73). Thus, the worm model has tremendous potential for valuable research in obesity and diabetes, particularly research in ER stress- and UPR- mediated mechanisms.

2.7 Conclusion

The introduction of the *C. elegans* model of human diseases has provided a versatile and powerful platform for studying the pathology and potential treatments of these diseases. As one of the complex pathways in cells, UPR^{ER} is involved in the pathology of many diseases. Using the worm model to study the UPR^{ER} already has been proven to be fruitful, and continued discoveries using this worm model will provide important insights in research related to the UPR and to human diseases in the future.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

All the *C. elegans* strains and *Escherichia coli* OP50 used in the current study were obtained from *Caenorhabditis* Genetics Center (CGC), University of Minnesota. Strains used include SJ4005 [*zcIs4 (phsp-4::GFP)*]; AM141[*rmIs133 (unc-54p::Q40::YFP)*]. All the chemicals were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA) unless otherwise indicated. Household Clorox bleach (The Clorox Company, Oakland, CA, USA) was used for synchronizing the worms. Fluorodeoxyuridine (FUdR) and carbenicilin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

3.2 *C. elegans* Culture

C. elegans were cultured using standard methods as previously described (74). Nematode growth medium (NGM), M9-buffer solution, and S-complete solution were prepared as the previous protocols (74). Briefly, after a synchronous worm population was acquired, synchronized L1 worms were cultured in 12-wells plates at 25°C with treatments (25 µM deltamethrin or 0.1% DMSO as control).

3.3 Fluorescence Microscopy

Worms were immobilized in 2 mM levamisole and mounted on a slide with 3% agarose using a cover slip before imaging. After preparation of the slides, fluorescent images were taken using a Nikon Eclipse Ti-U Inverted Microscope (Micro Video

Instruments, Avon, MA). The average GFP intensity of SJ4005 worms was measured for ER stress response, and the average number and size of polyglutamine YFP inclusions were scored for AM141 worms. For each treatment, about 30 worms were used. Analysis of the images was performed using the ImageJ software developed by the National Institute of Health (NIH).

3.4 Growth Rate Assay

After 48 hours of treatment, worms were transferred from liquid media to NGM plates, and the worms at each developmental stage were counted for growth rate analysis.

3.5 Locomotion Assay

Locomotion activity of the worms was measured using the Wormlab tracking system (MBF Bioscience, Williston, VT, USA) as previously described (75, 76). Low-peptone nematode growth media (NGM) plates were prepared and used for tracking as previously reported (75). Worms from each treatment groups were transferred from liquid media to NGM plates and stabilized for about 15 minutes. After stabilization, a 1-minute video was captured at 7.99 frames per second. Videos were analyzed using the Wormlab software, and the Wormlab software generated data for worm size and locomotive behavior.

3.6 Statistical Analysis

Data values are given as means ± S.E. and analyzed using statistical software GraphPad Prism v.7.0 (GraphPad Software, La Jolla, CA, USA). Differences among groups were analyzed using one-way or two-way analysis of variance (ANOVA) and

Tukey's multiple-range test. The significance of differences was defined at the $P < 0.05$ level.

CHAPTER 4

RESULTS

4.1 Deltamethrin induces ER stress response in *C. elegans*

The induction of ER stress response in worms can be measured by using the SJ4005 strain with an *hsp-4p::GFP* reporter. *Hsp-4* is the homolog of mammalian ER chaperone BiP, and BiP is used as a general marker of ER stress response (5). During ER stress, *hsp-4* is upregulated and the GFP intensity in SJ4005 worms is higher than without ER stress (68). The expression of the *hsp-4* GFP reporter was measured to investigate whether deltamethrin can induce ER stress response in *C. elegans*. In this experimental, a 4-hour treatment of 3 mM dithiothreitol (DTT), an ER stress inducer, was used as positive control. As shown in Figure 4.1, 25 µM deltamethrin increased the ER stress response significantly by 44% compared to control. Since the induction of ER stress is associated with the accumulation of unfolded and misfolded proteins, the effect of deltamethrin on protein misfolding was examined using a *C. elegans* polyglutamine model (64).

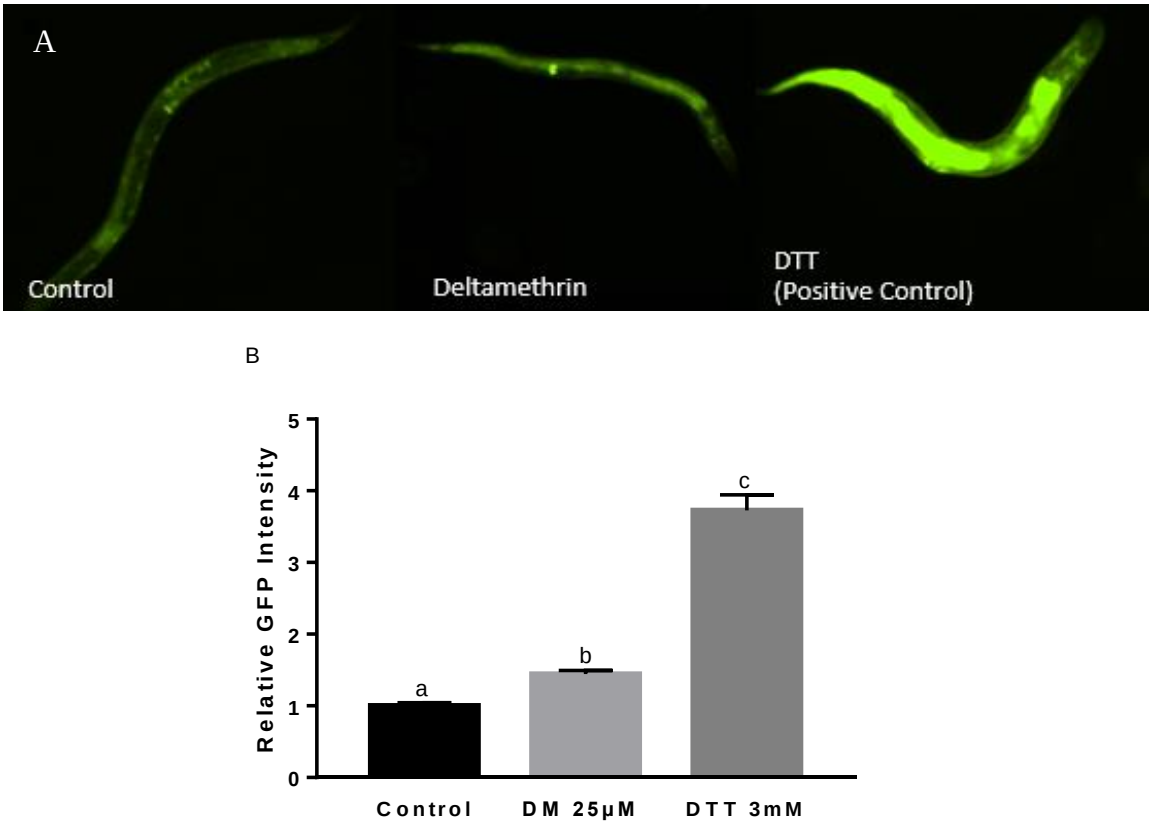

Figure 4.1 Effects of deltamethrin on ER stress response. (A) Representative images of SJ4005 worms on day 3 with control, deltamethrin and dithiothreitol (DTT) treatments. (B) Deltamethrin induced ER stress response in *C. elegans*. Treatment of deltamethrin started from L1 stage for three days. The data are described as means ± S.E. (control n=29, deltamethrin n=36, DTT n=69). Means with different letters are significantly different (P<0.05).

4.2 Effect of deltamethrin on locomotive activity, growth rate and worm size

Exposure to deltamethrin has been linked to the reduction of locomotive activities in *C. elegans* (77) and improper development in zebrafish and *Daphnia* (4, 78). However, treatment of deltamethrin at 25 μM had no significant effects on the size (Figure 4.2A&B), and locomotive activities (Figure 4.2C&D) or growth rate of *C. elegans* strain SJ4005 (Figure 4.2E).

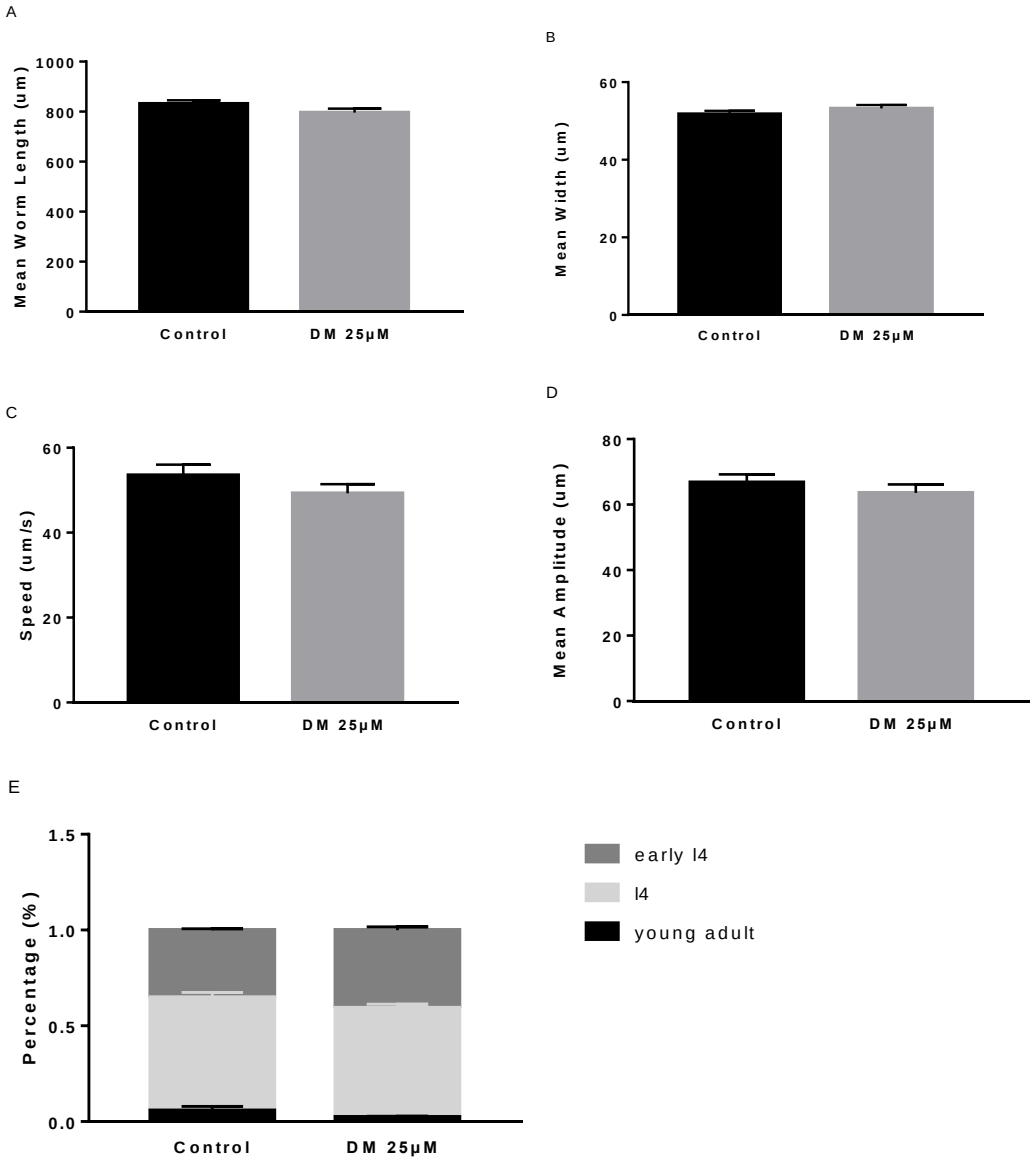

Figure 4.2 Effects of deltamethrin on the size, growth rate, and locomotive activity of SJ4005 worm. (A) Average body length of worms on day 4 of treatment. (B) Average body width of worms on day 4 of treatment. (C) Average moving speed on day 4 of treatment. (D) Mean amplitude of sinusoidal movement on day 4 of treatment. (E) Proportion of worms of each growth stage after 2 days of treatment. All worms were treated with deltamethrin from L1 stage. And the data were presented as mean means ± S.E. (n=184-191 for size and locomotion assay; n=3 plates and about 100 worms per plate for growth rate assay). Means with different letters are significantly different (P<0.05).

4.3 Deltamethrin increased polyglutamine aggregation in *C. elegans*

The effect of deltamethrin on the aggregation of polyglutamine is tested using the *C. elegans* transgenic strain AM141 expressing Q40::YFP in its body wall muscle cells (64). Expression of polyglutamine stretch leads to the formation of aggregation that can be visualized by fluorescent microscopy (79). Our results showed that treatment with 25 µM of deltamethrin had no significant effect on the average number of polyglutamine aggregates (Figure 4.3A), but significant changes in the average size of polyglutamine aggregates occurred (Figure 4.3B). According to previous studies, increased polyglutamine aggregation may result in polyglutamine-mediated motility defects (79).

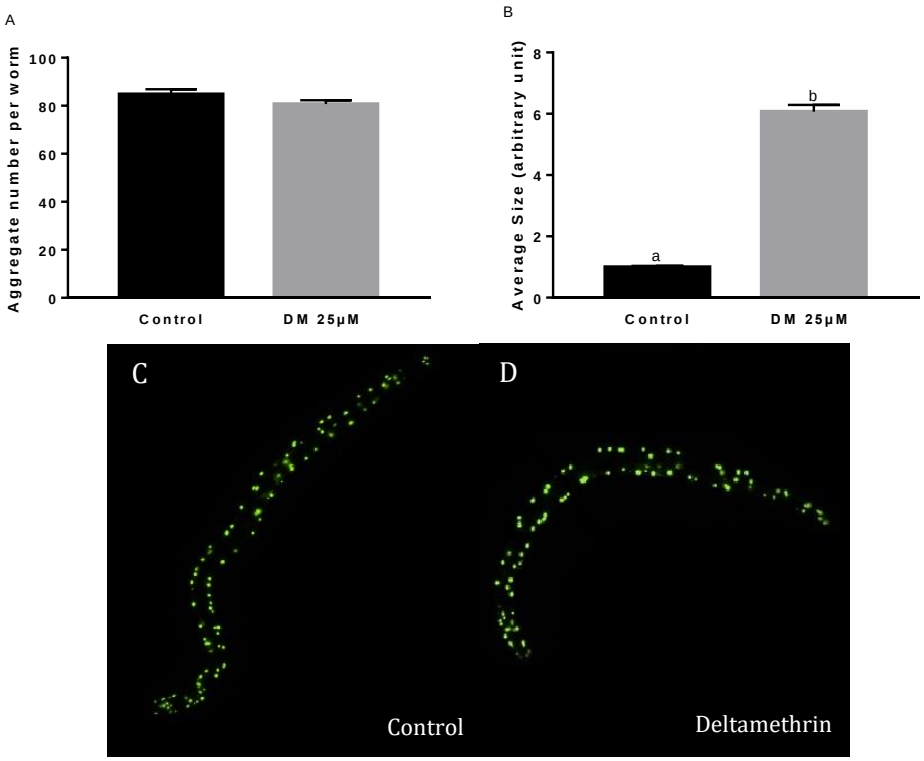

Figure 4.3 Effects of deltamethrin on the number and size of polyglutamine aggregation in *C. elegans*. (A) The number of polyglutamine aggregation was counted from YFP images using ImageJ. (B) The average size of polyglutamine aggregation was counted from YFP images using ImageJ. (C) Representative images of AM141 worms treated with 0.1% DMSO in control group. (D) Representative images of AM141 worms treated with 25 μM deltamethrin. Treatment of deltamethrin starts from L1 stage and lasts for six days. The data are described as means ± S.E. (control n=33, deltamethrin n=37). Means with different letters are significantly different ($P<0.05$).

4.4 Deltamethrin increased the proteotoxicity of polyglutamine in *C. elegans*

Prior studies have shown that polyglutamine aggregates can induce proteotoxicity in muscle cells of *C. elegans* by disrupting the actin and myosin myofibrillar networks (79). As a result of the polyglutamine-mediated proteotoxicity, the worms show a reduction in locomiton (79). In the current study, the locomotive activity was measured using WormLab system on day 4 with or without deltamethrin to investigate whether deltamethrin would exacerbate the proteotoxicity of polyglutamine. Average moving speed means the average distance the worm moves during a specified time period, and amplitude refers to the distance of vertical movement over the sinusoidal track of *C. elegans* (75). As for body size, treatment of 25 μM deltamethrin resulted in a significant reduction in body length by 7% (Figure 4.4A), but result in no significant changes in body width (Figure 4.4B). In terms of locomotive activities, deltamethrin treated worms had a significant reduction in average moving speed (~31%) (Figure 4.4C) and a significant reduction in mean amplitude (~11%) (Figure 4.4D). Furthermore, the growth rate (Figure 4.4E) of worms was not significantly different between control and deltamethrin-treated groups. Overall, these results indicate that deltamethrin treatment exacerbates the proteotoxicity of polyglutamine aggregation.

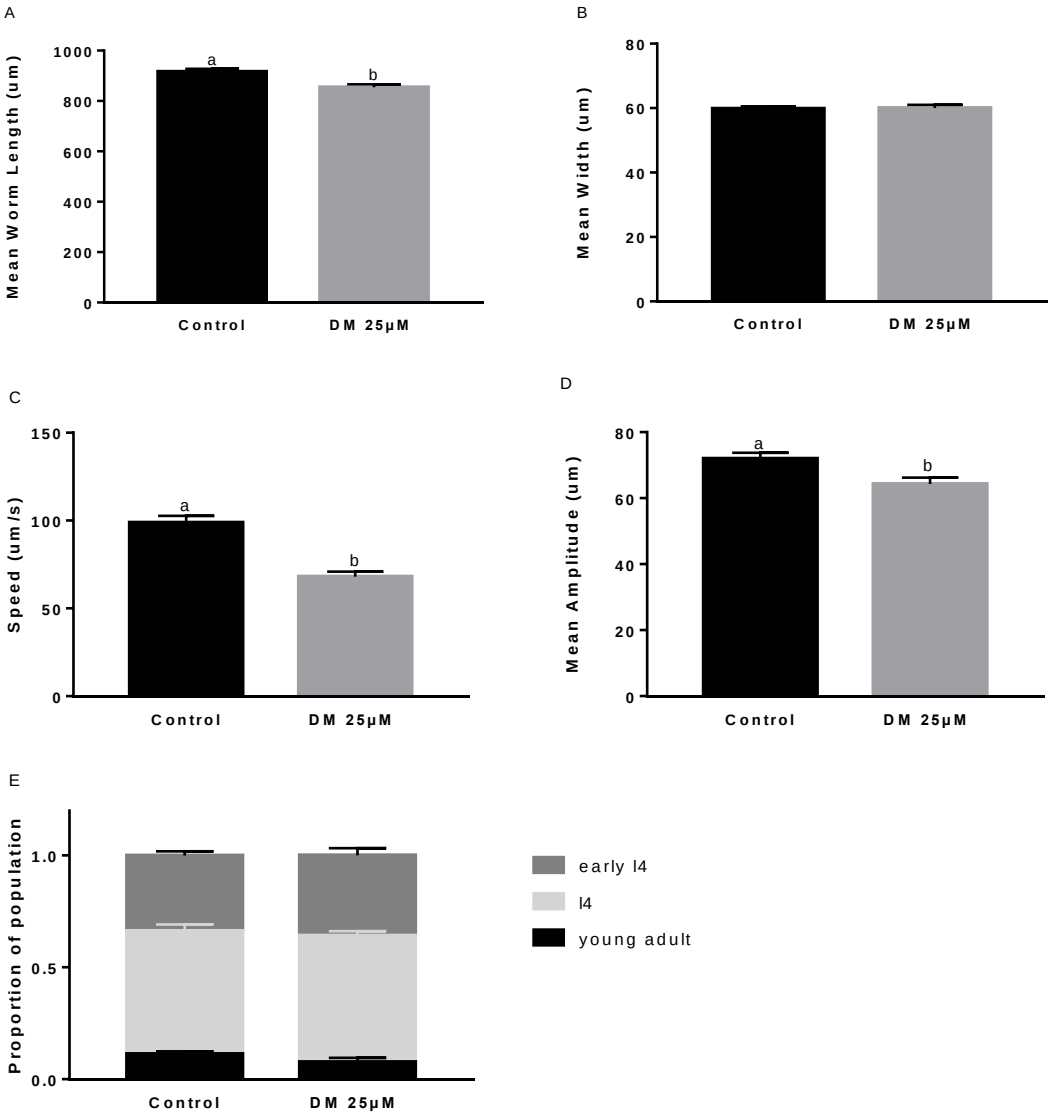

Figure 4.4 Effects of deltamethrin on the size, growth rate, and locomotive activity of AM141 worm. (A) Average body length of worms on day 4 of treatment. (B) Average body width of worms on day 4 of treatment. (C) Average moving speed on day 4 of treatment. (D) Mean amplitude of sinusoidal movement on day 4 of treatment. (E) Proportion of worms of each growth stage after 2 days of treatment. All worms were treated with deltamethrin from L1 stage. The data were presented as mean means ± S.E. (n=187-194 for size and locomotion assay; n=3 plates and about 100 worms per plate for growth rate assay). Means with different letters are significantly different (P<0.05).

CHAPTER 5

DISCUSSION

Previously, others have reported that deltamethrin induces ER stress response/ unfolded protein response (UPR^{ER}) in nerve cells and mice (4, 11). In the current study, deltamethrin treatment induced ER stress response in *C. elegans*, and is the first report that deltamethrin increased protein misfolding and exacerbated proteotoxicity in vivo using *C. elegans*.

ER is the organelle that synthesizes, folds and processs proteins and ER stress is defined as a condition where unfolded and misfolded proteins accumulate in the ER lumen and activate the ER stress response/UPR^{ER} (5, 68). Disruption of the protein folding and processing in ER can lead to accumulation of unfolded and misfolded proteins in the ER and trigger a specific stress response named the UPR^{ER} (5). Accumulation of unfolded and misfolded proteins plays a pathological role in many conditions, including aging, obesity, diabetes, and neurodegeneration diseases (2, 13, 65). For example, under ER stress, overaccumulated unfolded and misfolded proteins can form inclusions in neuron cells, and many of these inclusions (e.g. α-synuclein and β-amyloid) have been proven to be neurotoxic (2, 58). Previous reports have indicated that deltamethrin can induce ER stress response/UPR^{ER} (4, 11). However, induction of ER stress response/UPR^{ER} alone may not directly indicate accumulation of unfolded and misfolded proteins, because UPR^{ER} can be activated by other conditions without accumulation of unfolded and misfolded proteins, such as change of ER membrane phospholipid composition, oxidative stress, and metabolic stress (5, 33, 68). In the current study, we confirmed that deltamethrin caused protein misfolding and

proteotoxicity along with induction of ER stress response/UPR^{ER} in *C. elegans* by using a protein misfolding model based on polyglutamine aggregation.

In the current study, deltamethrin increased polyglutamine aggregation and resulted in proteotoxicity, as determined by reduced average moving speed and amplitude (80). Increased polyglutamine aggregation seen in the presence of deltamethrin may have occurred due to a slowing or disruption of the removal of unfolded and misfolded proteins in the lysosome-mediated protein degradation process (62, 79). A previous study implied that deltamethrin activated the UPR^{ER} and impaired the hippocampal function possibly by UPR^{ER}-mediated apoptosis (4). Others suggested that deltamethrin activated general ER stress response and induced apoptosis through activation of the PERK branch of the UPR^{ER} in SK-N-AS neuroblastoma cells (11). Another pyrethroid insecticide, cypermethrin, impaired proteostasis by alterations to protein synthesis, maturation, and degradation genes in the brain of mice (81). Our results are consistent with these reports that deltamethrin may induce ER stress and protein misfolding. The alteration of calcium homeostasis and oxidative stress associated with deltamethrin, however, may also contribute to its effects on ER stress (77, 82). Thus, it is still not clear how deltamethrin increased polyglutamine aggregation and proteotoxicity.

Our results also showed that the worm length of AM141 worms was slightly, but significantly, reduced (7%) by deltamethrin and may indicate that deltamethrin had affected the development of *C. elegans*. Interestingly, deltamethrin did not influence the growth rate of AM141 worms, nor did it reduce the worm length in SJ4005 worms. These results are consistent with a previous report that deltamethrin has little to minimal impact on the growth rate of *C. elegans* (6). It has been previous shown that extreme ER stress

can lead to developmental arrest and larvae lethality in *C. elegans*, whereas moderate level of ER stress has no significant effect on the development of worms (68, 83). Based on the above evidence, it appears that deltamethrin treatment at 25 μM had a moderate effect on ER stress but had limited influence on the development of *C. elegans*.

Pyrethroids have been reported to affect locomotive activity in many animal models as it targets neurons (84). Nonetheless, our results implied that deltamethrin of 25 μM had no significant effect on body size, locomotion, or growth rate of SJ4005 worms. However, deltamethrin showed a dose-dependent effect on the locomotion of wild-type *C. elegans* (75). It is possible that the difference in *C. elegans* strain may be the reason why no significant changes on locomotive activity by deltamethrin in the current study with SJ4005 worms. In fact, it has been reported that the insertion of the GFP reporter into the *hsp-4* gene intragenetically affected the locomotive behavior of SJ4005 worms, as the average speed of SJ4005 worms is slower than that of the N2 worm used in the previous study (6, 85).

Overall, the current results suggest that deltamethrin induces ER stress response in *C. elegans* and increases accumulation as well as the proteotoxicity of polyglutamine aggregates. Further studies are necessary, however, to determine how deltamethrin induces ER stress response and increases protein misfolding by examining potential mechanisms, including calcium homeostasis and oxidative stress.

CHAPTER 6

FUTURE DIRECTIONS

Currently, there are limited reports on the role of deltamethrin in ER stress and ER stress-related diseases. In the present study, we discovered that deltamethrin can induce ER stress, increase accumulation of unfolded and misfolded proteins, and increase the proteotoxicity of polyglutamine aggregation in *C. elegans*. However, the exact mechanism how deltamethrin induces ER stress and disturbs protein homeostasis is not known. Since deltamethrin alters the activity of both voltage-gated sodium and calcium channel, deltamethrin may disturb ER protein homeostasis and triggers UPR^{ER} through increasing the calcium concentration (5, 77). In order to test this hypothesis in *C. elegans*, the expression level of ER Calcium transporter SERCA (Sarco/Endoplasmic Reticulum Calcium ATPase) may be evaluated to determine if deltamethrin disturbs the calcium homeostasis in the ER (86). The expression of SERCA is expected to increase if deltamethrin increased ER Ca^{2+} level (86). Alternatively, deltamethrin may also impact protein folding and processing through reduction of proteasomal and ribosomal activities, which can be examined by analyzing proteasomal activities using a fluorogenic peptide substrate assay and quantifying ribosomes in *C. elegans* (52). Moreover, the effect of deltamethrin has not been tested on all three branches of UPR^{ER}. The IRE-1 and the PERK branches are expected to be activated with deltamethrin treatment, as the activation of both branches was reported in the neurons (11).

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