Investigation of the Effect of Electrical Stimulation on Proteotoxic models of *Caenorhabditis elegans*

by

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ABSTRACT

Huntington's disease (HD) is one of the nine fatal neurodegenerative diseases in which increase in CAG repeats (CAGn) in coding region leads to polyglutamine (polyQ) expansion in protein sequence. PolyQ expansion is the key factor for misfolded protein formation in neurons, causing the disease progression. For example, individuals with less than 35 CAG repeats do not possess any of the symptoms whereas the ones with more than 40 repeats show variety of them. Up to date, no specific and well-defined treatment for HD has been developed. Patients in latter stages need assistance in their daily activities which put great economic burden of the societies. Therefore, more effective therapeutic interventions to attenuate the symptoms of HD are needed.

One approach to HD therapy is targeting misfolded protein through activation of the Heat-Shock Proteins (HSPs). They are conserved proteins, expressed in the presence of distinct stress conditions. They aid in protein assembly, proper folding and translocation. HSPs are divided into 5 groups based on their molecular weight: HSP100, HSP90, HSP70, HSP60, and the small HSPs. It is known that under stress conditions HSP expression is induced by transcription factors (Tf) like Heat Shock Transcription Factor-1 (HSF-1). Some of the current studies focus on augmenting HSP level to intervene aggregation-related diseases.

Recently it has been shown that mild electrical stimulation (MES) has positive impact on wound healing, psychological diseases, neuromuscular activity, decrease in inflammation and decrease of pain. Most importantly, ES also influences the expression of HSPs. For example, ES significantly increase the expression level of HSP70 and HSP72 in mouse myoblasts (C2C12) and adenocarcinoma (A549) cell lines, respectively. Similar observation has been made with ES treated rat brain, which shows an increase in the mRNA levels of HSP70 and HSP90.

C. elegans is a 1mm-long soil-nematode which is widely-used model organism, since their first introduction to research by Sydney Brenner in 1963. *C. elegans* has several advantages. For example, 83 percent of its genome including HSP genes is conserved among species. Additionally, transgenic insertion of corresponding disease gene like polyQs enables visualization of processes like aggregation, degeneration in this multicellular organism.

The purpose of the current study was to test whether ES can be used as a therapeutic intervention in proteotoxic models. For this purpose, we first examined the influence of ES on *C. elegans*. We investigated the ability of ES treatment to increase the HSP expression in nematodes and to alleviate the disease severity in various frequencies.

Key words: Mild electrical stimulation, *C. elegans*, Huntington's Disease, polyglutamine, lifespan, heat shock proteins.

ÖZET

Huntington Hastalığı (HD); DNA'nın kodlama yapan bölgesinde CAG tekrarı (CAGn) artışının protein sekansında poliglutamin (polyQ) genişlemesine neden olduğu, dokuz ölümcül nörodejeneratif hastalıktan biridir. PolyQ genişlemesi, nöronlarda proteinlerin yanlış katlanması sonucu degeneratif hastalıkların ilerlemesinde ana faktördür. Örneğin, 35'ten az CAG tekrarı olan bireyler HD semptomlarına sahip değilken 40'tan fazla CAG tekrarı olanlar belirtilerden birçoğunu göstermektedir. Bugüne kadar bu hastalığa özgü ve iyi tanımlanmış herhangi bir tedavi geliştirilememiştir. Ayrıca hastalığın ilerleyen aşamalarında hastaların günlük aktivitelerini gerçekleştirebilmek için yardıma ihtiyacı olması toplumu ekonomik olarak olumsuz etkiler. Bu nedenle, HD semptomlarını iyileştirmek için daha etkili tedavisel müdahalelere ihtiyaç duyulmaktadır.

HD tedavisine yönelik yaklaşımlardan biri yanlış katlanmış proteinleri ısı şok proteinleri (heat-shock proteins; HSPs) aracılığıyla hedeflemeyi amaçlamaktadır. HSPs farklı stres koşullarında aktivasyon gösteren türler boyunca genetik olarak korunmuş proteinlerdir. Proteinlerin toplanma, düzgün katlanma ve yer değiştirmesine yardımcı olurlar. Isı şok proteinleri moleküler ağırlıklarına bağlı olarak 5 gruba ayrılırlar: HSP100, HSP90, HSP70, HSP60 ve küçük HSPs. Stres durumunda HSP ekspresyonu Isı Şok Transkripsiyon Faktör-1 (Heat Shock Transcription Factor-1; HSF-1) gibi transkripsiyon faktörleri ile indüklendiği bilinmektedir. Güncel çalışmalardan bazıları HSP seviyesini arttırarak proteinlerin çökmesi ilişkili hastalıklara müdahale etmeyi amaçlamaktadır.

Son zamanlarda, hafif elektrik stimülasyonunun (Mild electrical stimulation; ES) yara iyileşmesi, psikolojik hastalıklar, nöromüsküler aktivitesi ve inflamasyon ve ağrı azalmasında olumlu etkiye sahip olduğu gösterilmiştir. Daha da önemlisi, ES HSP ifadesini etkilemektedir. Örneğin; ES, sırasıyla, fare miyoblast (C2C12) ve adenokarsinom (A549) hücre hatlarında HSP70 ve HSP72 ifade seviyesini arttırmaktadır. Benzer bir gözlem ES ile

tedavi edilen sıçan beyninde HSP70 ve HSP90 mRNA düzeylerinde bir artış olduğu gösterilerek yapılmıştır.

C. elegans 1963'te Sydney Brenner tarafından bilimsel araştırmalarda model organizma olarak sunulmasından sonra yaygın olarak kullanılan 1mm uzunluğunda toprak solucanıdır. Bu model organizmanın çeşitli avantajları vardır. Örneğin, HSP genleri de dahil olmak üzere genomunun yüzde 83'ü türler arasında muhafaza edilmiştir. Ayrıca, polyQ gibi hastalık genlerinin transgenik olarak eklenmesi, protein çökmesi ve nöral dejenerasyon gibi aşamaların çok hücreli bu organizmada gözlemlenmesini sağlar.

Bu çalışmanın hedefi ES uygulamasının proteotoksik modellerde tedavi amaçlı kullanılması için etkinliğinin test edilmesidir. Bu amaçla öncelikle *C. elegans* üzerinde ES etkisi incelenmiştir. ES tedavisinin değişik frekanslarda kurtçuklarda HSP seviyesini arttırmak ve HD hastalığının fenotipik etkilerini azaltma amacıyla kullanılabilecek ES uygulamasının değişik frekansları araştırılmıştır.

Anahtar Kelimeler: Hafif elektrik stimülasyonu, *C. elegans*, Huntington Hastalığı, poliglutamin, yaşam süresi, ısı şok proteinleri.

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ABBREVIATIONS

ABD	ATP-binding
AOs	Antisense Oligonucleotides
ATF6	Activating Transcription Factor 6
C2C12	Mouse Myoblasts Cell Line
CAGns	Genetic Expansion of CAG Triplet Repeats
DIC/Nomarski	Differential Interference Contrast
DRPLA	Dentatorubral-Pallidolusian Atrophy
ER	Endoplasmic Reticulum
ES	Electrical Stimulation
FC	Frontal Cortex
HD	Huntington's Disease
HS	Heat Shock
HSE	Heat Shock Sequence Element
HSF-1	Heat Shock Transcription Factor
HSPs	Heat Shock Proteins
HSR	Cytoplasmic Heat Shock Response
Htt	Huntingtin
IRE1	Inositol Requiring Enzyme 1
MES	Mild Electrical Stimulation
Ms	Millisecond
NaN3	Sodium Azide
NGM	Nematode Growth Medium

NLS	Nuclear Localization Signal
PERK	Protein Kinase RNA-Like ER Kinase
SBD	Substrate-Binding Domains
SBMA	Spinal and Bulbal Muscular Atrophy
SCA1	Spinocerebellar Ataxia
SCA17	Spinocerebellar Ataxia 17
SCA2	Spinocerebellar Ataxia 2
SCA3	Spinocerebellar Ataxia 3
SCA6	Spinocerebellar Ataxia 6
SCA7	Spinocerebellar Ataxia 7
SSRIs	Selective Serotonin Reuptake Inhibitors
Tf	Transcription Factor
TRAP1	Tumor Necrosis Factor Receptor Associated Protein 1
UPR	Unfolded Protein Response
wHtt	Wild type Huntingtin
XBP1	X-Box Protein1

CHAPTER 1: INTRODUCTION

1.1. Huntington's Disease

Huntington's Disease (HD) is a genetic neurodegenerative disease which has an onset in between ages of 35 and 50 years old. George Huntington made the first detailed description of autosomal dominant inheritance of this disease in 1872 (Huntington, 2003). Its symptoms which decrease the quality of patients' life can be divided into two categories: physical and psychological. Physical symptoms include involuntary movements, weight loss and speech and swallowing difficulties whereas psychological ones are depression, aggression and early onset dementia (Roos, 2010; Ross, 2002).

HD is an example of disease led by a single gene. The gene called *huntingtin* (*htt*) positioned at Chromosome 4. The discovery of *htt* was performed by creating DNA probes complementary to the sequence of chromosome 4. These probes were used to compare differences between the genome of normal and HD cell lines. The researchers also further illustrated the gene's novelty and protein coding capacity ("A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group,"1993). In the 5' terminus of the gene, there is a region composed of CAG nucleotide repeats. Since CAG sequence encodes a hydrophilic uncharged amino acid Glutamine (Q), the product from CAGns are referred as polyglutamine tracts or repeats (polyQ) (Green & Wang, 1994). As shown in **Figure 1.1.1**, wild type polyQ involves in protein-protein interaction via changing its conformation from "disordered" structure to alpha-helical form in order to extend neighboring coiled coil fragment in the presence of q interacting protein (Schaefer, Wanker, & Andrade-Navarro, 2012). This conformational alteration is confirmed by means of X-Ray crystallography of N-terminus of Htt (Kim, Chelliah, Kim, Otwinowski, Bezprozvanny,2009).

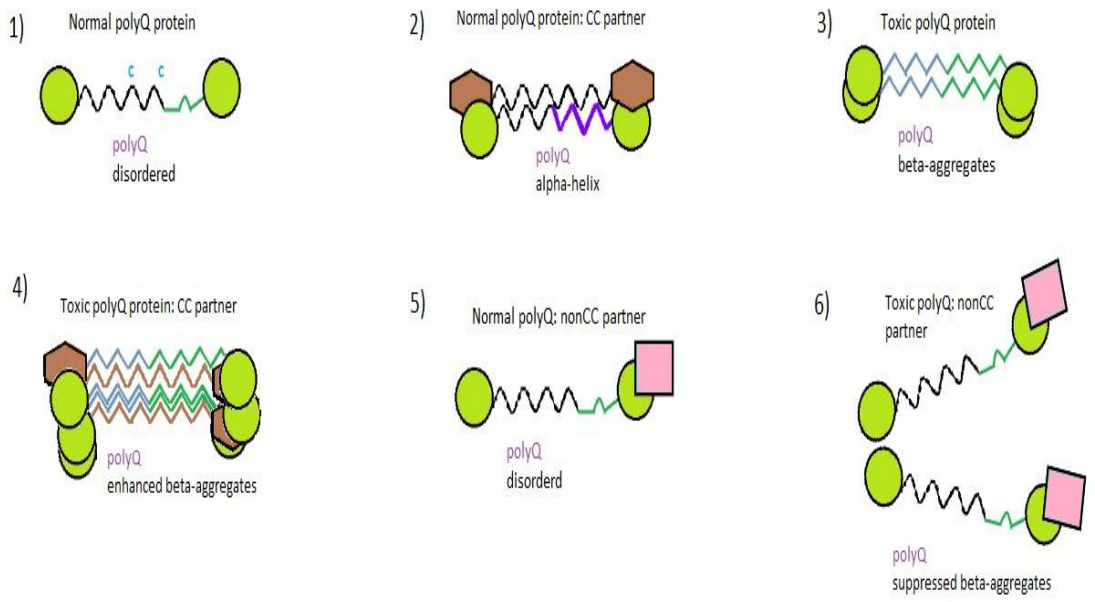


Figure 1.1.1 The conformational change of polyQ structure under different situations.
(Adapted from Petrakis, Schaefer, Wanker, & Andrade-Navarro, 2013)

Genetic expansion of CAGns is led by genetic instability. During germ line production, CAGns in the paternal allele of *htt* are augmented more than maternal ones. CAG expansion was also identified during embryogenesis, mitotic somatic cells and post-mitotic neurons (McMurray, 2010). This expansion can be disadvantageous for individuals' daily functioning. As it can be seen in **Table 1.1.1** individuals, having less than 28 CAGns has no abnormal phenotype. On the contrary, when the repeat number exceeds 40, individuals present disease symptoms. Increment in polyQs is critical as its increase favors β-sheet formation thermodynamically (Khare, Ding, Gwanmesia, & Dokholyan, 2005) and form insoluble protein aggregates (Scherzinger et al., 1997). The aggregates disrupt the cell homeostasis and results in cytotoxicity, which triggers dysregulation of different processes: transcription, protein quality control mechanism, mitochondrial biogenesis, oxidative stress and intracellular transport (Seidel et al., 2015; Williams & Paulson, 2008). In addition, MRI analysis of two different studies enlightened that HD patient suffer from neural degeneration

in striatum, caudate and putamen compared with control group (Yu, Li, Wu, Shen, & Tu, 2014; Harrington et al., 2014). Furthermore, when lentiviral transfection of polyQs Q82 and Q19 on neural cell cultures was compared, nuclear inclusions were observed when critical value of Q35 is exceeded. However, there was no observed aggregation when polyQ is below 19. Likewise, neurodegeneration in striatal neurons are apparent in Q82, correlating with the protein accumulation (Zala et al., 2005).

Table 1.1.1 CAG repeat sizes and their relation with HD

# of CAG repeats	Consequence
<28	Normal Individual
29-34	Healthy but Carrier Individuals
35-39	Some HD patients and Some Healthy but Carrier Individuals
>40	HD patients

Wild type Huntingtin (wHtt) is predominantly located in cytoplasm but also found nucleus via its nuclear localization signal (NLS) (Tao & Tartakoff, 2001). Wild type as well as mutant Htt (mtHtt) is expressed in the all brain area throughout embryonic neural development (Bhide et al., 1996). On the other hand, the expression of wHtt is also detected in non-neural tissues (Sharp et al., 1995). Even though, the function of wHtt is not fully determined, it acts as a regulator of four important processes: cellular transport (Caviston, Ross, Antony, Tokito, & Holzbaur, 2007), transcription (Zuccato et al., 2001), apoptosis (Buren, Wang, Smith-Dijak, & Raymond, 2014) and embryogenesis (Sanders et al., 2015).

Whether nuclear or cellular assembly of polyQs induces cytotoxicity has been a critical question for HD studies. To answer this question, in a cell culture study, researchers utilized synthetically prepared and aggregated peptides which contains fluorescein residue with or without NLS. They identified that to cause severe toxicity, polyQs has to exceed critical value (>40) and it has to localize in the nucleus.

In addition to HD, 8 other neurodegenerative diseases are also linked with CAGns: Spinal and bulbal muscular atrophy (SBMA), Dentatorubral-pallidolusian atrophy (DRPLA), Spinocerebellar ataxia (SCA1), Spinocerebellar ataxia 2 (SCA2), Spinocerebellar ataxia 3 (SCA3), Spinocerebellar ataxia 6 (SCA6), Spinocerebellar ataxia 7 (SCA7), Spinocerebellar ataxia 17 (SCA17). Even though the causative protein and the regional locations are distinct, the extension of CAGns triggers similar consequences: protein aggregation and death of corresponding neurons (Gusella & MacDonald, 2006). For instance, similar with HD, in the brain areas, there are SCA3-induced nuclear protein accumulations. When the accumulation in frontal cortex (FC) and the pons were examined, HD samples contains either similar or more aggregation in pons compared to FC. Interestingly, degeneration occurs in FC. On the other hand, in the case of SCA3, neural loss is visible in the pontine nuclei, even though FC also includes accumulated protein formations. Therefore, it can be concluded that each disease influences distinct neural tissues (Paulson et al., 1997).

Table 1.1.2. Different potential interventions against target pathogens of HD (Adapted from Ross, 2002)

Target Pathogen	Possible Intervention
CAGn expansion	Gene therapy
PolyQ increase	
Misfolded Protein and change in PPI	Chaperone Activation
Protein Accumulation	Accumulation blockers
Disruption in transcription, mitochondrial or caspase activity	Blockers for exicitotoxins Stimulators for metabolism
Neural degeneration	Inhibitors for apoptosis
Disease symptoms and progression	Anti psychotic drugs, SSRIs

Individuals need to apply for genetic testing, based on detecting changes in the *htt* gene, for HD diagnosis. After being diagnosed with HD, treatments are selected based to manage patients' symptoms such as against movement or psychological abnormalities. **Table 1.1.2** determines some approaches to HD. For example, available medications against chorea are antipsychotic drugs such as haloperidol, olanzapine and benzodiazepines (Warby, Graham, & Hayden, 1993) and Selective Serotonin Reuptake Inhibitors (SSRIs) like imipramine are used to alleviate psychological symptoms. Yet, medications utilized for this disease are ineffective for terminating disease progression (Ghavami et al., 2014). For example, SSRIs failed to ameliorate cognitive decline (Tyagi et al., 2010). Therefore, more effective therapeutic interventions to attenuate the symptoms of HD are needed.

Other potential therapies, which are in either trial or experimental stage, target polyQ aggregation from various perspectives. To exemplify, gene therapy aims at suppression of mHtt expression through some molecular techniques as RNAi or antisense oligonucleotides (AOs). The delivery of AOs is done by lentivirus which have tendency to randomly insert its genome. As a result, this method brings safety concerns (Chen, Carter, Cho, & Chan, 2014). One of the very current and effective approaches is to target abnormal changes in protein tertiary structure and protein interactions

1.2. Targeting Protein Misfolding

In a cell, misfolded structure of a protein causes the non-native exposure of protein's hydrophobic residue to aqueous cytosolic side. This exposure triggers recruitment of other misfolded proteins or interaction with lipid residue of cellular membrane. Therefore, abnormal alterations in the tertiary structure of a protein cause gain-of-toxicity in addition to loss-of-functionality (Vabulas, Raychaudhuri, Hayer-Hartl, & Hartl, 2010). In order to eliminate this dangerous situation for cell, protein family named heat shock proteins (HSPs) are specialized for protein homeostasis by involving in protein assembly, proper folding and translocation. Heat shock proteins has role on intervention of protein misfolding via inducing either refolding or degradation of misfolded proteins. HSPs are essential for protein homeostasis, or in short, proteostasis (Ghavami et al., 2014).

HSPs are divided into 5 groups based on their molecular weight: HSP100, HSP90, HSP70, HSP60, and the small HSPs. In addition, they can be divided into 3 functionally distinct categories as 1) Holdases interacting with misfolded proteins without involving in its refolding, 2) Foldases regulating refolding by using ATP and 3) Disaggregases reversing protein aggregation by the help of ATP (Diaz-Villanueva, Diaz-Molina, & Garcia- Gonzalez, 2015).

In the presence of the stress, HSPs goes up in order to increase the folding capacity of the cell. This is mainly achieved by transcriptional upregulation of HSP genes by heat shock

transcription factor (HSF-1). HSF-1 recognizes the heat shock sequence element (HSE) containing the common motif nGAAn in HSP gene promoter. This motif is necessary for recognition of HSF-1 (Akerfelt, Morimoto, & Sistonen, 2010). Deletion of DNA binding domain of HSF-1 eliminates stress-mediated transcription of HSPs in the fibroblast cells (McMillan, Xiao, Shao, Graves, & Benjamin, 1998). Absence of stress, HSF-1 is kept as inactive monomer bound to HSP70 and HSP90. The presence of unfolded proteins causes trimer formation of HSF-1 with two other HSF-1 molecules. Trimerized HSF-1 translocates into nucleus and triggers the transcription of HSPs by binding HSE (Vihervaara & Sistonen, 2014).

There are three important HSF-1-mediated and HSP-related signaling pathways: Cytoplasmic heat shock response (cHSR), Endoplasmic Reticulum (ER) unfolded protein response (UPR), mitochondrial UPR. The localization of unfolded proteins determines which of these pathways will get activated.

1.2.1. Cytoplasmic Heat Shock Response:

HSP90 is one of the main components of cHSR. It has three important binding domains. Its ATP binding domain in N-terminus, substrate binding domain lies in the in the middle part of the protein. C terminus of the protein contains the dimerization domain. HSP90 can be found in either in open or close conformation. ATP-binding to N terminus of HSP90 turns the open conformation to the close one. Moreover, it is active as a dimer form with ATP binding. HSP90 interact with partially folded proteins (Hainzl et al., 2009) as well as fully folded ones which have instability and tendency to aggregate (Pratt & Toft, 1997). Its putative substrates play role in phosphorylation, transcription and nuclear export (Karagoz & Rudiger, 2015). Furthermore, it is associated with cancer progression so its inhibition is more favored. Besides, inactivate HSP90 facilitates the release of HSF-1 and upregulation of HSP expression. For this purpose, chemical named geldanamycin or its derivatives which attach to

ABD is frequently studied for cancer and neurodegenerative disease treatments (Lin et al., 2015; Luo, Sun, Taldone, Rodina, & Chiosis, 2010).

Another important cytoplasmic chaperone is HSP70. As HSP90, the activity of this chaperone is also ATP-dependent. It acts as a foldase which binds to misfolded protein from its hydrophobic region and help either refolding or inducing its destruction by proteosomal or lysosomal degradation (Bercovich et al., 1997; Chiang, Terlecky, Plant, & Dice, 1989). Enhanced HSP70 expression lessens polyQ aggregation, neural degeneration in *D. melanogaster* (Warrick et al., 1999). In molecular mechanism, the protein performs this attenuation by eliminating the interaction between polyQ and GAPDH (Guzhova et al., 2011). As a result, augmenting the HS70 activity can be beneficial for treatments of neurodegenerative diseases but augmentation of HSP70 is less characterized and studied contrary to its inactivation as a cancer therapy (Brandvold & Morimoto, 2015). Even though HSP70 has similar structural localization of ABD in amino-terminus, its SBD is located in carboxyl terminus rather than mid-domain. ATP binding is strengthened by binding of additional HSP, HSP40 (Turturici, Sconzo, & Geraci, 2011). HSP40 includes specific structure named J-domain which recruits substrate to either HSP70 or downstream degradation elements. Increased expression of HSP70 together with HSP40 has been shown to attenuate polyQ aggregation in neural cell culture (Kobayashi et al., 2000). Hence, HSP40 is considered as co-chaperone of HSP70.

1.2.2. Mitochondrial HSRs:

Second group of chaperones are mitochondrial HSPs. High proportion of the mitochondrial proteins are encoded in the nucleus and synthesized in cytoplasm by ribosome. Synthesized protein goes into mitochondria without going under folding procedure by the help of receptors embedded in mitochondrial membrane (Dudek, Rehling, & van der Laan, 2013). Mitochondria holds chaperones named tumor necrosis factor receptor associated protein 1 (TRAP1) and GRP7 which are the members of HSP90 and HSP70 family respectively and act similarly with cytoplasmic HSP90 and HSP70 (Brandvold & Morimoto, 2015). Additionally,

HSP60 is localized in the mitochondria. Like the client binding to HSP90 and HSP70, HSP60 is also attracted by hydrophobic part of its target; yet, instead of binding it engulfs the target protein. One of the most investigated HSP60 is bacterial homologue GroEL which has ring-like morphology comprised 7 subunits with apical, intermediate and equatorial region. On its own, GroEL carries out stabilization of clients (Bartolucci, Lamba, Grazulis, Manakova & Heumann, 2005; Fei, Yang, LaRonde-LeBlanc & Lorimer, 2013). Nevertheless, adherence of its co-chaperone GroES, member of HSP10 family, assists folding process with ATP-dependent manner and unfolded protein reaches its native form. GroES involvement causes altered conformation with removal of hydrophobic residue from facing the central cavity (Haldar et al., 2015). Unfortunately, the role of HSP60 on neurodegenerative diseases is unknown.

1.2.3. Endoplasmic Reticulum HSRs:

Endoplasmic Reticulum (ER) is another metabolic organelle where large variety of protein regulations like synthesis, folding, packaging and transfer take place. As a consequence, chaperone activity is predominantly required. Similar with mitochondria, HSP90 and HSP70 family members GRP94 and BiP/GRP78 are parts of chaperone-mediated proteostasis network in ER. The network is mediated by 3 distinct parallel pathway whose compounds are trans-membrane bound proteins inositol requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase RNA-like ER kinase (PERK). Under normal circumstances, these three trans-membrane proteins are attached to HSPs. Imbalance in proteostasis triggers HSP release from membrane-bound proteins and causes leaving these proteins alone. One of them, PERK embodies oligomer, which propagates phosphorylation of itself as well as initiating its kinase activity. Kinase phosphorylates eIF2α to block mRNA translation.

As in the case of PERK, oligomerization occurs in IRE1. Thanks to its endonuclease activity, translation of X-Box Protein1 (XBP1) is performed. As a Tf, XBP1 alert genes modulating the transfer of unfolded proteins from ER to cytoplasm for further degradation.

The third regulatory element of ER stress, ATF6 has distinct machinery after release of HSPs. This time, ATF6 moves to Golgi apparatus. In the golgi, ATF6 is cut to gain Tf property so that XBP1 transcription is initiated. In addition to enhancing XBP1 expression by promoting its transcription, endonuclase activity of ATF6 improves PERK's activity on XBP1 translation (Xu, Bailly- Maitre, & Reed, 2005; Pellegrino, Nargund, & Haynes, 2013). In the light of this information, it can be said that BiP/GBR78 has crucial impact on ER UPR.

1.3. Electrical Stimulation

Electrical stimulation (ES) or mild electrical stimulation (MES) has been studied for the treatment of distinct disorders. Previously, it has been shown that ES has positive impact on wound healing, psychological diseases, neuromuscular activity, decrease in inflammation and decrease of pain. Recently, couple of the researches demonstrates that ES is also influential for the expression of HSPs. To exemplify, on mouse myoblasts cell line (C2C12) were given with direct electrical stimulations having the same voltage (13V) with different frequencies (12 Hz, 100 Hz) and durations (11 min and 90 min). Then cells were collected specific times (0 h, 1 h, 4 h, 8 h and 12 h) after the treatment and the expression of HSP70 is examined in both mRNA and protein levels. The results indicate that the cells respond low frequency ES more in mRNA level whereas in protein level the change led by low frequency ES is less (Wang, Liu, Jin, & Steinacker, 2010). With similar cell culture technique, mouse astroglial and adipose cell lines were transfected with construct containing *hsp70* gene followed by *luciferase* gene. The luciferase activity and mRNA levels are quantified when alternating current with 10 Hz frequency and voltage ranging 0-0.3V is applied for 1 hour. The results displays that transcriptional expression of HSP70 is initiated 3 hours and reaches the higher value 6 hours after treatment. The protein level increases, starting from eighth hour and kept high till the 32^{nd} hour (Yanagida, Mizuno, Motegi, Kobatake, & Aizawa, 2000). Additionally, adenocarcinoma cells have elevated HSP72 expression in the presence of ES (Morino et al., 2008). As it can be seen, most of the ES-induced HSP experiments are

conducted through cell culture. Therefore, the effects of ES on multicellular organisms are not well studied.

One of the few researches investigated the role of electro-acupuncture on ischemic-reperfusion rat models. 30 min-long electro-acupuncture, in which the animals were given electrical stimulation ranging from 4 Hz to 16 Hz with voltage staring with 1 Volt and raise up to 3 Volt, causes augmentation in HSP70 and HSP90 of cortex and hippocampus. Furthermore, ES-treated rats show mitigated apoptotic pathways (Sun, Shi, Chen, Liu, & Guan, 2003). Another study focuses on Huntington mouse model, the researchers report that Htt mutant male mice containing Q87 have extended lifespan. ES treatment is based on weekly 50 mA stimulation for 0.2 s. ES-treated animals have increased HSP70 and HSP40 expression in striatal cells compared to control group (Mughal et al., 2011). Even though, this recent data gives us some insight about how the ES regulates this progression is not well-known. Also, they mainly focus on striatum but HSP expressions in other tissues are not investigated. Therefore, new researches are needed to understand ES effect on HSP levels in different tissues of multicellular organisms.

1.4. *Caenorhabditis elegans* (*C. elegans*) as a model organism

C. elegans is a 1mm-long soil-nematode which has been widely-used model organism since their first introduction to research by Sydney Brenner in 1963. The worms are generally found as self-fertilizing hermaphrodites. There are many beneficial features making *C. elegans* as one of the most commonly-used model organisms. First of all, it can be maintained on plates containing Nematode Growth Medium (NGM) agar, which displays its easy growth. Secondly, it has really short lifetime around 3 weeks per generation. This characteristic attracts studies focusing on aging and aged-associated-diseases (Stiernagle, 2006). Furthermore, worms have a well-characterized body anatomy. For instance, it is known that an adult hermaphrodite contains 302 neural cells out of 959 somatic cells (www.wormatlas.org). In addition to its anatomy, the development of *C. elegans* is fully described. Development can be outlined into 2 main stages: embryonic development and post-

embryogenesis. Embryonic development is based on proliferative process containing time interval between one-cell fertilized embryo and 30-cell staged gastrula (around 350 min at 22^{O}C) and differentiation process in which cells migrate through their final destination and form germ layers as mesoderm, ectoderm and endoderm and 3-fold-staged embryo (around 8 hours at 25^{O}C). Proliferative stage takes place in mother's uterus and differentiation stages generally starts when the embryo is laid. From the beginning of 1.5 fold worm starts to move while covered with egg-shell. Post-embryogenesis contains larval period from hatching to beginning of adulthood (around 50 hours), reproductive stage starting from the first fertilized egg formed to the last embryo laid (3-4 days at 25^{O}C) and post-reproductive period (around 15 days). Whole lifecycle of a worm is influenced by environmental factors like temperature, pH and food availability. To exemplify, in the absence of food, *C. elegans* goes under morphological changes at Larva 1 or L1 stage and forms dauer larva. These morphological alterations such as closure of mount by internal plug enable worm to resist harsh conditions like starvation up to 3-4 months (Sulston, Schierenberg, White, & Thomson, 1983; Byerly, Cassada, & Russell, 1976).

In addition to these advantages, its transparent whole body makes this model organism as a powerful tool to observe cellular formations under Differential Interference Contrast (DIC/Nomarski) Microscope. This feature also assists detecting expression pattern of genes, which or whose promoters are fluorescently tagged, in a living multicellular organism. For this purpose two distinct reporter gene methods can be used: transcriptional and translational reporters. In transcriptional reporter, the construct is composed of the promoter of gene of interest followed by reporter gene which is generally GFP or mCherry. On the other hand, in translational one, the gene of interest is fully included and the reporter gene is put into the gene without destroying the structure and function of the protein of interest (Boulin, Etchberger, & Hobert, 2006). By the help of translational reporters, Morley et al. reveals that the threshold of polyQs to form aggregates is also 40 for the worms expressing polyQs in the muscle tissues. Moreover, age induced aggregation is visualized by the same group. Similar with humans, polyQ mutant worms exhibit movement deficiencies when compared with wild

type individuals (Morley, Brignull, Weyers, & Morimoto, 2002). Similar aggregation pattern is also found in neuronally expressed polyQs. Unlike humans, *C. elegans* do not have tendency to have neurodegeneration induced by polyQ aggregation (Brignull, Morley, & Morimoto, 2007).

Another advantageous property of *C. elegans* is its conserved genome. Around 83 percent of *C. elegans* genes share homology between human genome (Lai, Chou, Ch'ang, Liu, & Lin, 2000). For example, over-expressed HSF-1 (identically named with its human homolog) is also found to lead to extension of lifetime of *C. elegans*. Furthermore, *hsp-4* is homolog of human BiP (Ient et al., 2012).

Even though there is not much study about the effect of ES on *C. elegans*, in a very recent research, worms are exposed to ES with 2 V/cm and 0.1 millisecond (ms) pulse duration for 20 min while they are in larval stage for 3 days. Then, various assays including heat resistance assay is performed. It is found that the ES enhances heat resistance and HSP expression as seen in cell lines (Matsuyama et al., 2014). This study is very informative about how ES given during development influences *C. elegans*. However, worms' HSP expression alteration by post-developmental ES is not investigated. In addition, even if post-developmental ES affects similarly, it would be interesting to analyze the impact of ES on some proteotoxic models like polyQ-aggregation-related ones. Hence, the purpose of the current study was to test whether electrical stimulation can improve the progression of polyQ model by altering the expression level of heat-shock proteins in *C. elegans*.

CHAPTER 2: MATERIALS AND METHODS

2.1. Nematode Growth Medium Agar Plate Preparation

After the NGM agar solution (500 ml) was taken out of autoclave the solutions below were added into it.

Compounds	Amount
Cholesterol (5 mg/ml)	0.5 ml
CaCl (1M)	0.5 ml
$MgSO_4$ (1M)	0.5 ml
KH_2PO	12.5 ml
Fungizone	200 µl

After stirring the mixture, 8ml for small plate and 15ml for large plate was poured.

2.2. Bacterial Media and Food Preparation

E. coli strain *HB101* was taken from its glycerol stock and streaked to LB agar plate containing streptomycin (100 mg/ml=1X) and the plate was kept in 37 °C incubator. Single colony was isolated by sterilizable inoculating loop and inoculated in autoclaved streptomycin (1X) containing B-Broth. The broth was incubated overnight at 37°C. The *HB101* liquid culture was kept at 4°C. The culture was spotted on 60 mm or 90 mm NGM agar plates and was allowed to dry and grow for at least 1 day at room temperature. The plate can be used to maintain worms.

2.3. Worm Maintenance and Strains

By using platinum wire or chunking the old agar containing *C. elegans,* worms were transferred onto the NGM plates previously-spotted with *E. coli HB101* strain and they were kept at 15^{O}C or 25^{O}C based on their temperature sensitivity. To prevent contamination and evaporation, the plates were parafilmed.

Table 2: The strains used in the experiments

Strains	Genotype	Comment
N2	*wild type*	Purchased from CGC
AM44	*rmIs190*[F25B3.3p::Q67::CFP]	Purchased from CGC
AM47	[F25B3.3p::polyQ40::cfp]	Purchased from CGC
AM49	*rmIs172*	Purchased from CGC
AM141	*mIs133*[unc-54p::Q40::YFP]	Purchased from CGC
AM138	*rmIs130*[unc54p::Q24::YFP]	Purchased from CGC
SJ4005	*zcIs4 [hsp-4::GFP]* V	Purchased from CGC
SJ4058	*zcIs9 [hsp-60::GFP + lin-15 (+)]*	Purchased from CGC
AM799	*hsp90p::mCherry*	Purchased from CGC
AM722	*hsp70p::GFP*	Purchased from CGC
SX1137	*pash-1 (mj100)*	Gift from Miska Lab
SX1359	*pash-1 (mj100) I; mjEx331*	Purchased from CGC

2.4. Cleaning and Storage of Strains

2.4.1. Spot Bleach Procedure:

On an empty plate, 30 µl drop of bleach solution was spotted out of food source. 30-40 gravid adult worms were placed in the drop and let to dissolve. When the spotted area dried the plate was put into 15°C or 25°C incubators.

2.4.2. Double Bleach Procedure:

Gravid adult worms on 90 mm-large plates were washed with M9 solution and collected into the 15 ml falcon tubes. The worms were spin down at 2000 rpm for 2minutes. The supernatant was discarded. The washing step was repeated 3 times to remove the bacterial food source. 6ml of bleach solution was added to pellet and vortexed until the worms were dissolved (less than 8 min). M9 was added up to 15ml. The sample was centrifuged at 2000 rpm for 2 min and supernatant was removed. The pellet was washed with M9 about 3 times. In the last step, 300µl of bleach solution was added to 100µl of the worm pellet. The sample was spotted on empty plates near the food source. After waiting for plates to dry, they were placed into 15°C or 25°C incubators.

2.4.3. Freezing Procedure:

Strains were grown until they exhaust all the food on the in 90 mm plates and enter into dauer stage. First, 5 cryotubes were labeled with the name of the strain, genotype and the date of freezing. Then, 2.5 ml of 2X freezer buffer is poured in 15 ml falcon tube. The plate was washed with 3 ml of M9. Then the whole solution with worms were collected and added into the falcon tube. Then, the sample was mixed. 5 ml of mixture was divided to each cryotubes equally and the tubes were kept at -80°C. One day later, one of the tubes was thawed to make sure that there are surviving animals after freezing.

2.5. Embryo Hatching Assay

Worms can be synchronized either by double bleach or picking 20 mothers and let them lay embryos for 2 hours. When the embryos reaches to L4 stage, animals were picked and overnight incubated at 15°C or 25°C depending on their temperature sensitivity to complete their developmental stage and reach young adults. That day is considered as Day 0.

All the animals were treated with ES at 2 Hz, 10 Hz or 80 Hz at Day 0 for 5 min. The worms were then placed to 6-well plastic plates with NGM agar spotted UV killed bacteria (*E. coli HB101* strain) and kept at 25 °C. Every 12 hours, the mothers were transferred into a new well and the hatched and unhatched embryos were counted. The ES procedure was repeated until the reproduction period ceased.

2.6. Lifespan Assay

As explained in the embryo hatching assay, worms were picked at L4 stage and kept overnight at 15°C to complete their development. On Day 0, as young adults, they were divided into 4 experimental groups which are control group, 2 Hz, 10 Hz and 80 Hz. ES treatment was applied every day for 5 minutes at 2 Hz, 10 Hz or 80 Hz frequencies. **Figure 4.4.1** determines the system we used to stimulate worms. The worms were transferred to a new 90mm NGM plate ever day following ES treatment and their locomotion was recorded until all the animals die.

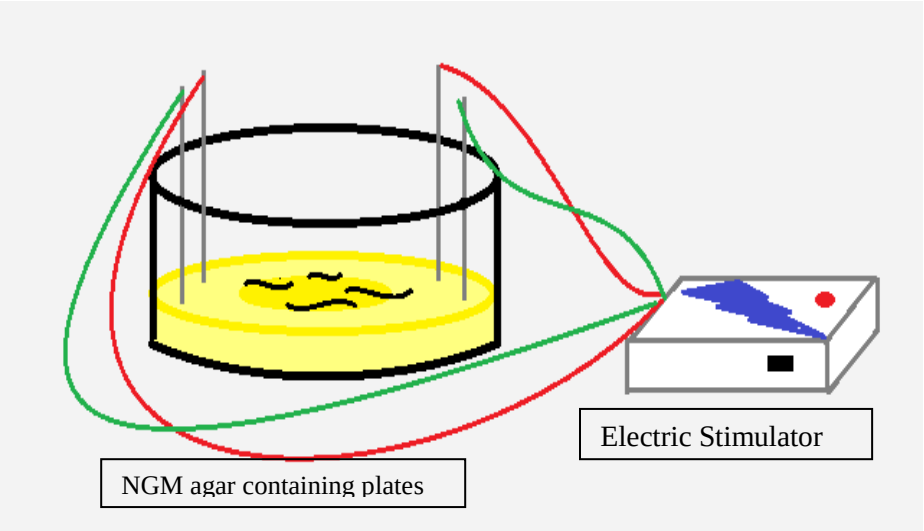


Figure 2.6.1 Representative image showing the experimental setup for ES treatment of worms in a petri dish.

2.7. Imaging Analysis

2 microscope slides were covered with a layer of tape and located near 2 long edge of an empty slide. 2% agarose was liquefied by alcohol burner and kept on heat block at 65°C. By the help of Pasteur pipette, a drop of agarose was placed to the mid-area of the slide. Right after dropping, a new clean slide was put on agarose drop. After waiting couple of minutes, the upper slide was moved. 5 µl of 100 mM sodium azide (NaN_3) was dropped on to the agarose pad. The experimental animals were put inside of the drop and a cover slip was placed onto them.

2.8. Experimental Setup for Imaging

2.8.1. Transgenic Heat Shock Protein Models

Worms were picked at L4 stage and placed into 15°C incubator overnight. The next day, animals were separated into three group electrical stimulation (ES), heat shock (HS) and control group. The HS group is placed at 35°C for 2 hours while other groups are at15°C. 10min before removing HS group from 35°C, the ES group was applied ES and control group was also treated similarly but without ES. The all three group is waited either room temperature or 15°C for 1 hour. Then put on agarose pad as previously described and 10x, 20x and 100x images were taken.

CHAPTER 3: SOLUTIONS

3.1. LB Agar:

For 1L LB agar (pH:7)

Compounds	Amount
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
NaOH (10M)	Few drops
ddH_2O	Up to 1 L

After setting pH to 7, the mixture was autoclaved.

3.2. Streptomycin (1000x):

For 15 ml

Compounds	Amount
Streptomycin	1.5 g
ddH_2O	15 ml

3.3. B-broth:

For 1 L

Compounds	Amount
Bacteriological Tryptone	10 g
NaCl	5 g
ddH_2O	Up to 1 L

After preparing mixture, it was autoclaved.

3.4. NGM Agar (500 ml):

Compound	Amount
Agar	8.5g
NaCl	1.5
Bactopeptone	1.25
ddH_2O	500ml

The solution was autoclaved.

3.5. 1M KH_2PO_4:

For 1 L solution:

Compound	Amount
KH_2PO	136 g
KOH Pellets	About 21 g
ddH_2O	Up to 1 L

The pH was set to 6 and solution was autoclaved.

3.6. 1M $MgSO_4$ (1 L):

Compound	Amount
$MgSO_4.7H_2$	246.48 g
ddH_2O	Up to 1 L

The mixture was autoclaved

3.7. 1M CaCl:

For 1 L

Compound	Amount
$CaCl.2H_2O$	147.02 g
H_2O	Up to 1 L

The solution was autoclaved.

3.8. Bleach Solution:

For 50 ml solution

Compound	Amount
NaOCl	4 ml
10M NaOH	4 ml
ddH_2O	Up to 50 ml

3.9. Freezer Buffer:

For 1 L solution

Compound	Amount
1M NaCl	100 ml
1M	50 ml
Glycerol	300 ml
ddH_2O	Up to 1 L

The buffer was autoclaved after mixing well. After cooling down, 3 ml 0.1M $MgSO_4$ is added into it.

3.10. 2% Agarose:

For 100 ml

Compound	Amount
Agarose	2 g
ddH_2O	100 ml

3.11. 200 mM Sodium Azide Stock:

Compound	Amount
Sodium Azide Powder	0.195 g
ddH_2O	100 ml

CHAPTER 4: RESULTS

Neurodegenerative diseases such as Huntington's disease (HD) are caused by protein aggregation leading to imbalance in protein homeostasis. Up to date, there is no specific and well-defined treatment for aggregation-related diseases. Depending on the location of the aggregation, cytoplasmic, endoplasmic reticulum and mitochondrial unfolded protein responses are activated, which leads to an increase in the levels of molecular chaperones. Elevated molecular chaperone (HSP) levels enhance the folding capacity of the cell and current studies have been focusing on the approaches to modulate the HSP expression to mitigate the number of protein aggregates. Very recently, at cellular level, mild electrical stimulation (MES) has been shown to increase HSP expression. However, the numbers of studies that investigate the effect of ES on multicellular organisms is very limited. Therefore, the aim of this study is to understand the effect of ES on multicellular organisms to evaluate its potential as a treatment for diseases caused by protein aggregation (proteinopathies). We applied ES to *C. elegans* model organism at three frequencies (2 Hz, 10 Hz and 80 Hz). Firstly, we investigated the effect of ES to physiology of wild type worms. We showed that ES does not negatively affect reproductive capacity or lifespan of the wild type animals. Secondly, we utilized the Huntington's disease models of *C. elegans* and examined how ES treatment affects the lifespan and locomotion of both wild-type and disease models. Lastly, we determined the changes of HSP expression in various tissues after ES.

4.1. Electrical Stimulation does not negatively affect progeny number and embryonic development of wild type worms.

Before starting to investigate the efficacy of ES to alleviate the symptoms of proteinopathies, we examined its effect on normal physiology of the worms. In order to understand this, firstly, the impact of ES on fecundity of *wild type* (wt) worms was determined. Fecundity of worms is determinant of maternal effect which is a response of mother to an unfavorable situation. Therefore, by comparing the progeny amount and the features of wt and treated worms, it is possible to test if ES has a toxic effect on worms. Since ES treatment on *C. elegans* has not been studied before, we applied ES for 5 or 10 minutes and determined the progeny numbers of both MES treated and control animals. **Figure 4.1.1** illustrates that when ES was applied for 5 minutes, in comparison to wt individuals, there is no significant change in the number of embryos laid by ES treated animals. 10 minute treatment of ES at 2 and 10 Hz frequencies caused a mild increase in progeny number, however the change was not statistically significant.

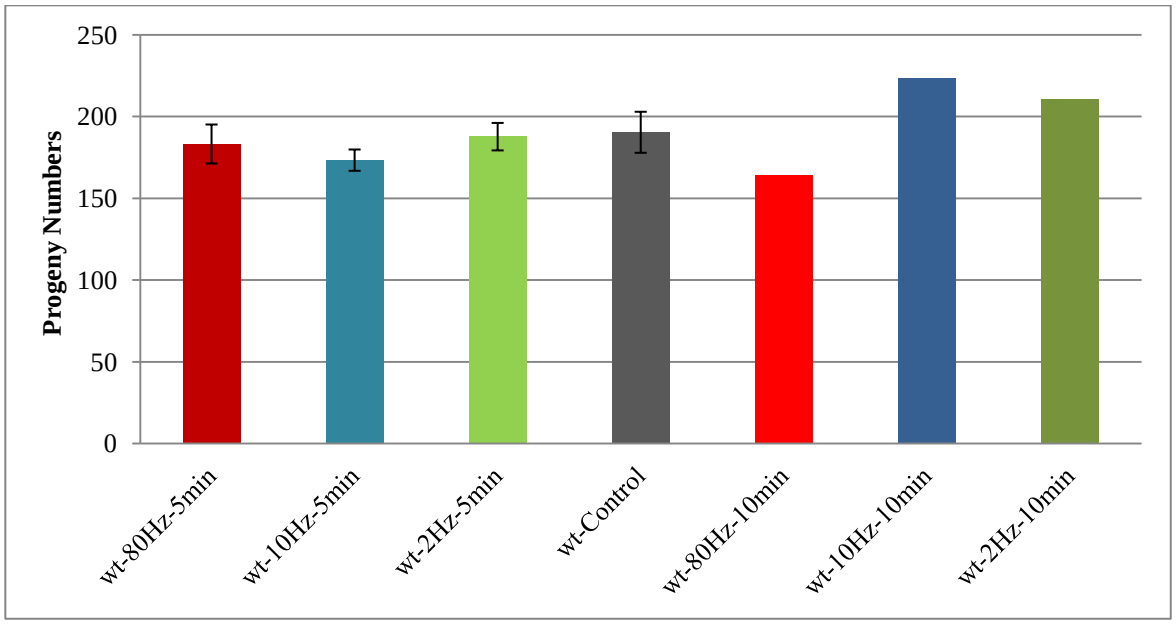


Figure 4.1.1 Amounts of total laid embryos in the presence of ES treatment. L4-staged wt worms were picked and transferred into 15°C incubator overnight. They were transferred to new UV-killed bacteria containing large NGM plates and treated with ES with 3 frequencies (80 Hz-10 Hz-2 Hz) for 5 min or 10 min. Non-treated worms were used as control groups. After treatment, the worms were placed at 25°C. ES was applied every day and hatched and unhatched progenies were counted until the reproductive stage ceased. Three biological replicates were performed with 6 worms.

After no toxic effect on embryo amount of ES-applied worms was observed, we analyzed whether these laid embryos can complete the embryonic development. It is well-characterized that at 22°C, wt embryo hatches around 840 minutes after fertilization, which is considered as the end of the embryonic development. Therefore, the laid embryos of the ES treated mothers were observed every 12 hours for 72 hours and the amount of hatched and unhatched embryos were recorded. In **Figure 4.1.2,** the number of embryos that completed embryonic development was shown as the percentage of the embryos that hatched after the ES treatment. When all the ES-treated groups were compared with control group, it can be seen

that similar to progeny numbers, there was no significant change in the percentage of hatched embryos.

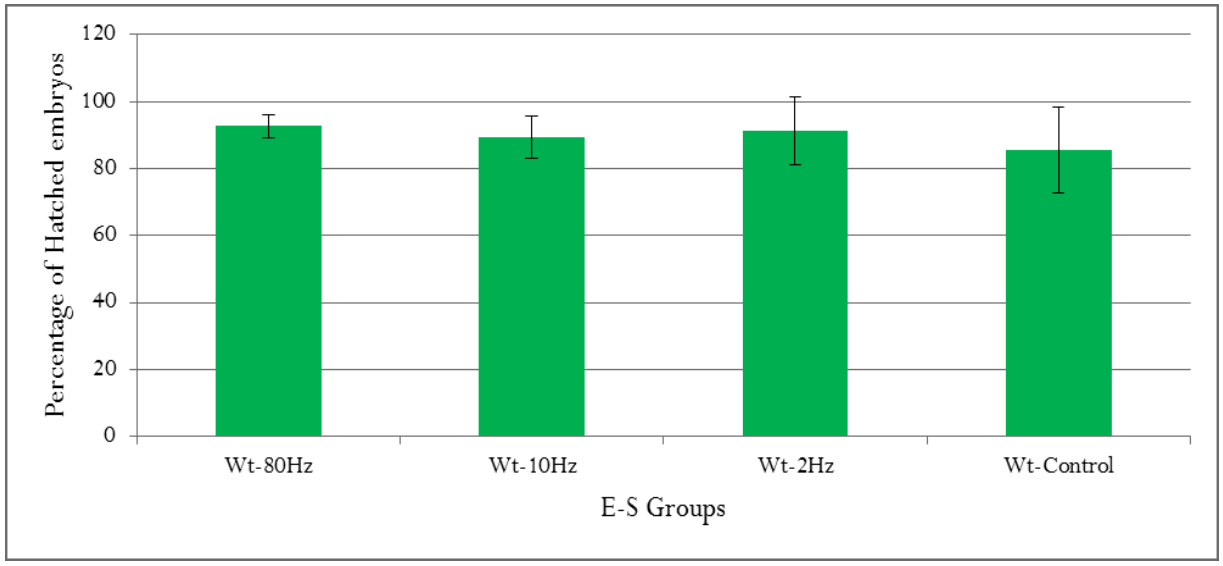


Figure 4.1.2: Embryo hatching percentages of wild type worms in response to ES. The experiment was performed as described in Figure 4.1.1. Error bars indicates the standard deviation.

4.2. ES does not change the lifespan of wild type animals.

Proteinopathy disease models of *C. elegans* have short lifespan. Before applying ES to disease models, we first determined the influence of ES to wild type animals. For this purpose, after wild type worms completed their development, they received daily treatment of ES. Two important values, namely maximum life and half-life can be used to understand if a treatment have an effect on lifespan. Maximum life refers to the day last individual of the population dies and half-life corresponds to the day at which half of the population is alive. **Figure 4.2.1** shows that all three ES frequencies have no significant influence on the maximum or half-life of the wt animals.

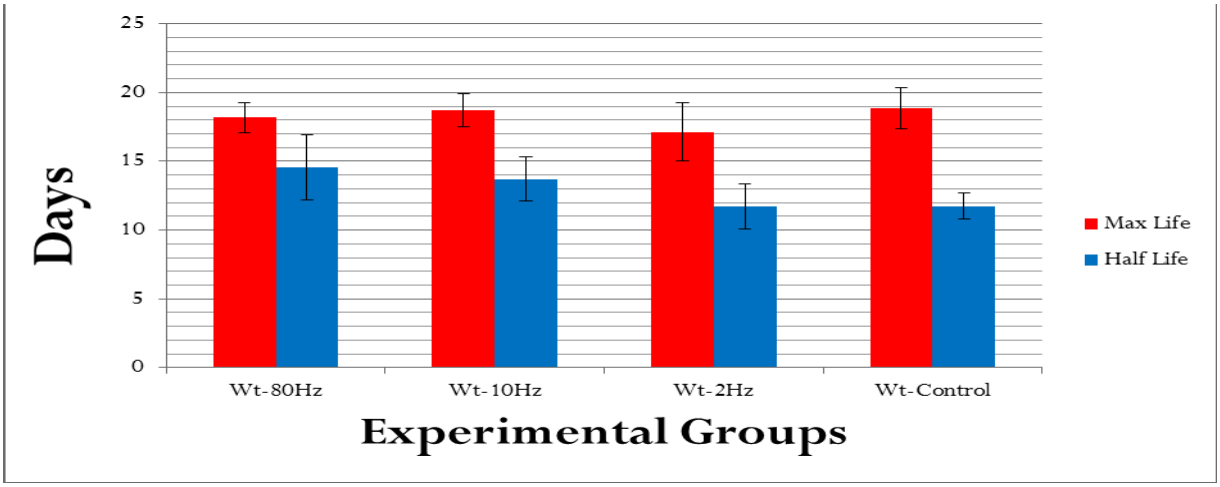


Figure 4.2.1: The lifespan analysis of ES treated animals. L4-syncronyzed worms were allowed to complete their development at 15°C. Then, young adults were placed onto NGM plates containing fresh UV-killed bacteria and exposed to 3 different ES with 80 Hz-10 Hz-2 Hz frequencies. Control group was not given ES treatment. At 25°C, every 24 hours ES treated and control animals were examined until all the animals were dead. Three biological replicates were carried out with around 30 animals in each trial. Error bars represents standard deviation.

4.3. ES extends the lifespan of transgenic worms expressing polyQ protein in their muscle cells, however do not affect the worms expressing neuronal polyQ protein.

After we established that ES treatment does not have a toxic effect on the physiology and lifespan of wild type animals, we evaluated the impact of ES on protein aggregation-mediated disease models as Huntington's disease model. To address this, we utilized *Caenorhabditis elegans* expressing polyQ protein fused either to cyan or yellow fluorescent protein, polyQ-CFP and polyQ-YFP, respectively. Previously, it has been shown that polyQ-mediated toxicity depends on the length of polyQ repeats. In *C. elegans*, polyQ35 in muscle cells and Q40 in neural cells have shown to be the critical value, polyQ proteins exceeding the critical value form in soluble aggregates in the cell. In our study, we

used transgenic lines differing in their lengths of polyQ expressed either in neuronal or muscle cells (Q19,Q40 andQ67 in neurons; Q24 and Q40 in muscle). Q19 in neurons and Q24 in muscles are both below the threshold for aggregation. Therefore, they were used as negative controls in the study. ES with three different frequencies 2 Hz, 10Hz and 80hz was applied daily and the lifespan of the animals was compared.

In **Figure 4.3.1** and **Table 4.3.1**, lifespan analysis showed that the half-life of almost all the groups in the first trial, except polyQ40 animals treated with 10 Hz had not change in comparison to control groups, where no ES was applied. There was slight decrease in the half-life of Q19 treated with 80 Hz ES. 10 Hz ES increased the max life of Q19, while Q19-2Hz group's max life is the same as control Q19 samples. However, when we compared the max-life of ES treated animals to controls we saw a decrease in the max life of Q40 worms treated with ES in all three frequencies (2 Hz-10Hz and 80Hz) compared to control Q40 group. Similarly, 80Hz ES treatment also lessened the max-life of Q19.

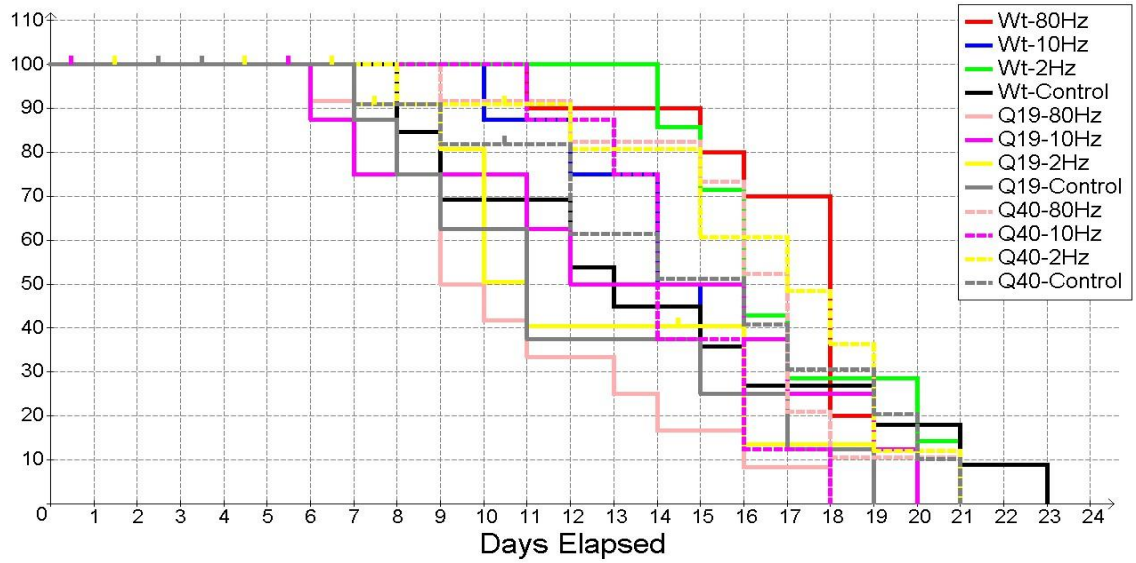


Figure 4.3.1: Survival data of first trial ES treatment on polyQ models. The experimental procedure is similar with the method described as Figure 4.2.1.

Table 4.3.1: Quantitative representation of Figure 4.3.1 showing the half and max-life differences of treated groups normalized with non-treated ones in the first trial ES treatment on neuronal polyQ models.

	Q40-80Hz	Q40-10Hz	Q40-2Hz	Q19-80Hz	Q19-10Hz	Q19-2Hz
Max Life	-4,54545	-18,1818	-4,54545	-5,26316	5,263158	0
Half Life	30,76923	0	15,38462	-10	10	10

To obtain statistically significant results, we conducted the second trial with 30 animals in each group. Moreover, we also included a strain that expresses polyQ67 in neurons. In humans, exceeding the critical value (>40) leads to more severe symptoms so we expected to observe similar pattern in worms too. However, as it can be seen in **Figure 4.3.2** and **Table 4.3.2**, we did not detect robust changes in control group of polyQ67 model compared to control group of polyQ19 and polyQ40. The results of this trial determined that the half-life of ES treated worms seems to lessen. When the max life of each groups were analyzed, 10 Hz and 2 Hz treatments for Q67 and Q40 shorten the max-life of each two groups. On the other hand, applying 80 Hz-treated Q67 and Q40 groups causes small extension, 4.35 and 4.76 percent respectively, in their max-life.

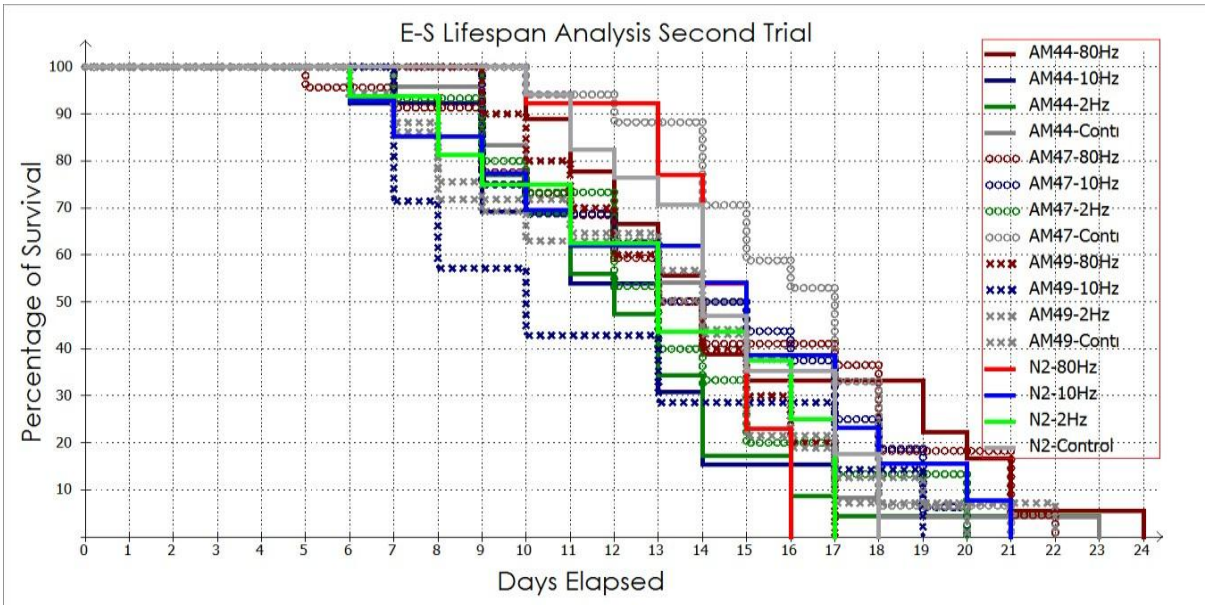


Figure 4.3.2: Survival data of second trial ES treatment on polyQ models. The experimental procedure is similar with the method described as Figure 4.2.1.

Table 4.3.2: Half and max-life of treated groups normalized to non-treated ones in the second trial of neuronally expressed polyQ models.

	Q67-80Hz	Q67-10Hz	Q67-2Hz	Q40-80Hz	Q40-10Hz	Q40-2Hz	Q19-80Hz	Q19-10Hz	Q19-2Hz
Max Life	4,35	-26,09	0	4,76	-4,76	-4,76	-22,73	-13,64	-9,09
Half Life	0	-7,14	-14,29	-23,53	-23,53	-23,53	0	-23,08	7,69

In order to make the lifespan experiment more reproducible, we increased the number of animals from 30 to 40. The result is represented in **Figure 4.3.3** with distinct lifespan graphs of four strains. Interestingly, in the third trial, ES increased the max life of each strain, but 2Hz in Q67 appears to be ineffective in longevity. Half-life of the Q40 treated with ES in

all frequencies demonstrates extension. On the contrary, ES treated groups of other strains show decreased half-life in comparison with their control samples.

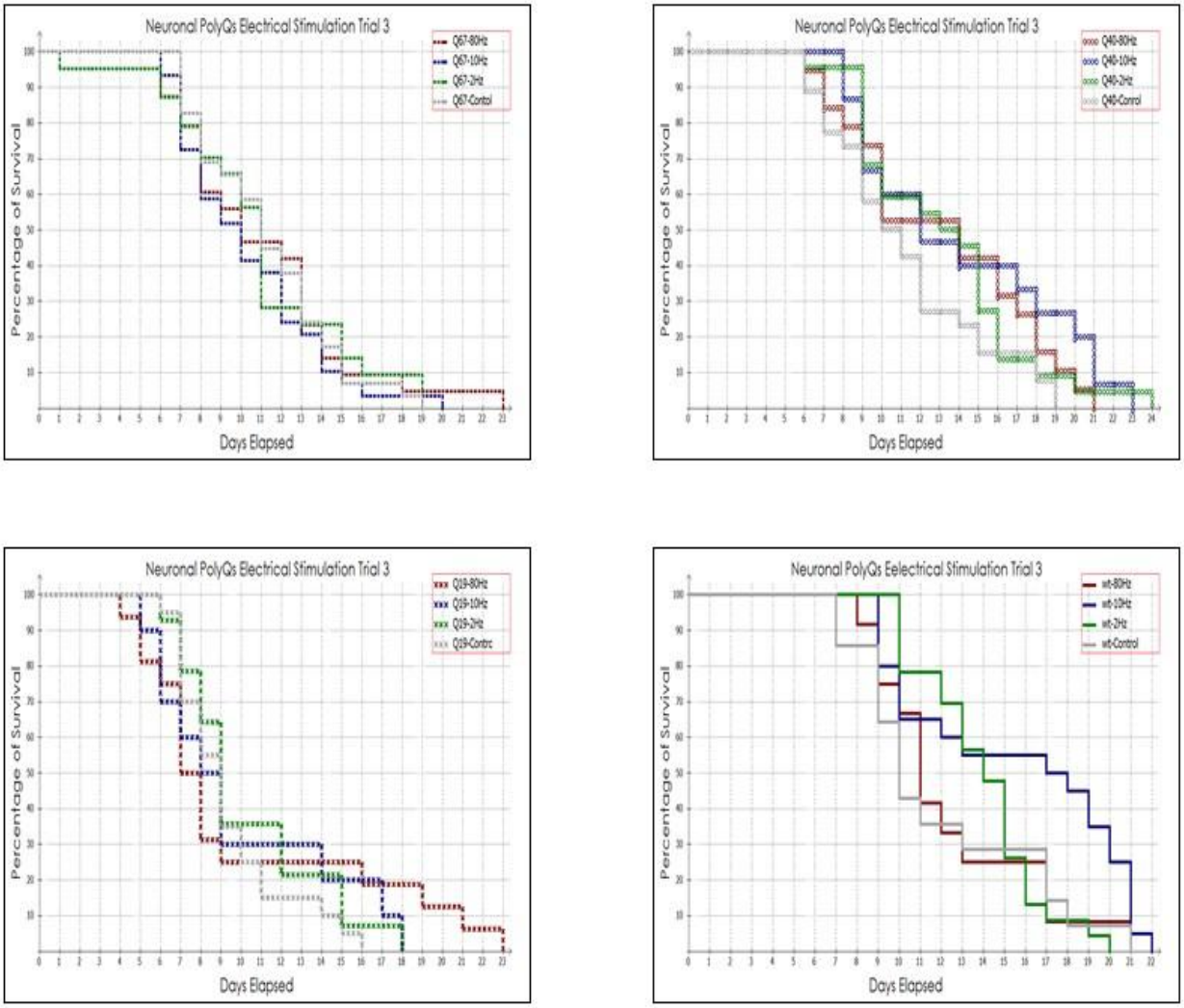


Figure 4.3.3: Lifespan data of third trial ES treatment on polyQ models. The experimental procedure is the same as the method explained as **Figure 4.2.1**.

Table 4.3.3: Quantitative analysis of Figure 4.3.3 showing changes of max and half life of the experimental groups in the presence of ES

	Q67-80Hz	Q67-10Hz	Q67-2Hz	Q40-80Hz	Q40-10Hz	Q40-2Hz	Q19-80Hz	Q19-10Hz	Q19-2Hz
Max Life	21,053	5,263	0	10,526	21,053	26,316	43,75	12,5	12,5
Half Life	-9,091	-9,091	0	40	20	30	-22,22	-11,11	0

Since all of the three trials shows different pattern in response to ES, representative graph was drawn. We were unable to detect robust changes in these phenomena when the representative data in the **Figure 4.3.4** and **Table 4.3.4** were analyzed.

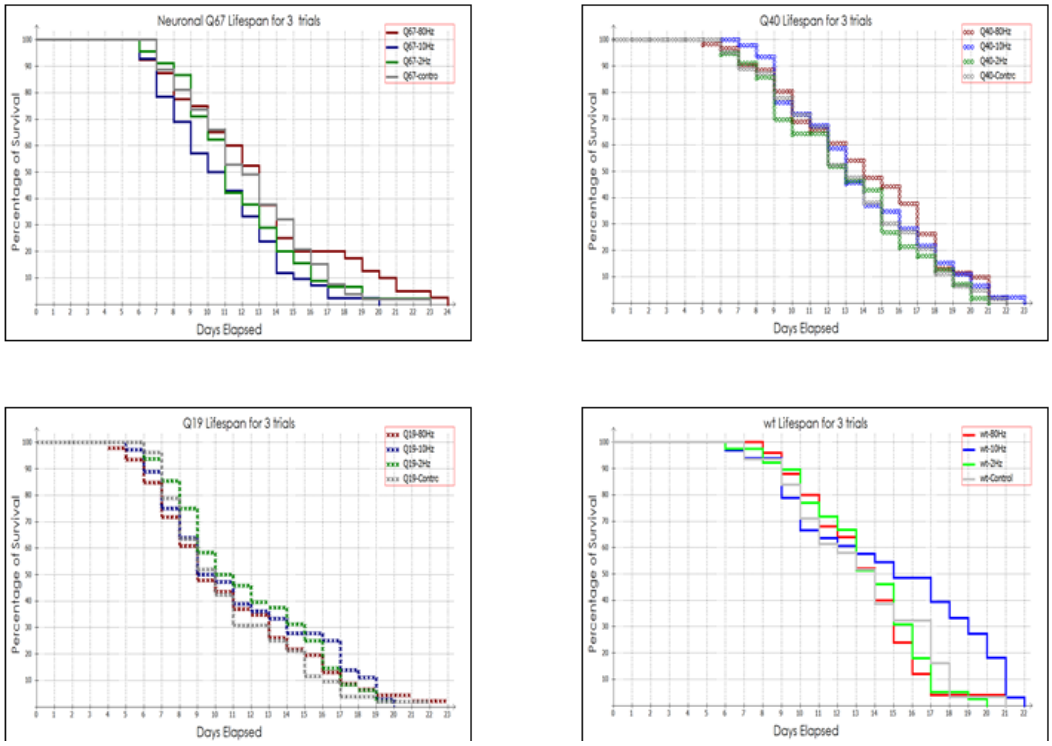


Figure 4.3.4: Combined lifespan result from all 3 trials of neuronally expressed polyQ models undergone ES treatment.

Table 4.3.4: Max life and half life differences of ES treated group relative to control groups for 3 trials.

	Q67-80Hz	Q67-10Hz	Q67-2Hz	Q40-80Hz	Q40-10Hz	Q40-2Hz	Q19-80Hz	Q19-10Hz	Q19-2Hz
Max Life	4,34783	-13,044	0	-4,3478	0	-4,3478	4,54546	-9,0909	9,09091
Half Life	8,33333	-16,667	-8,3333	7,69231	0	0	-10	-10	0

Lifespan analysis of *C. elegans* has been a widely-utilized measurement for aging-related studies. However, looking at only lifespan changes caused by treatment may not show us the all dimensions of the treated worms' aging progression. Therefore, a recent study subdivided this phenomenon into two important concepts: healthspan and gerospan. Healthspan is determined by the time interval in which animals have more than 50 percent of maximum functionality, whereas gerospan is rest of the lifespan in which animals show functionality less than 50 percent. Analysis of these two terms can be made by either chronologically or physiologically. As **in Figure 4.3.5**, chronological analysis is based on the actual days of lifespan. Physiological analysis takes into account the differences of maxiımum lifespan of the examined animals. Therefore, it enables to analyse the animals that has differences in their maximum lifespan. In physiological analysis, the normalization of the healthy and frailty periods to maximum life is considered as 100%. **Figure 4.3.6**. They found that four distinct mutants of *C. elegans*, which are known to extend lifespan compared to wild type worms, have no alterations in healthspan, but increased gerospan. As a result, even though we did not detect robust changes in lifespan in the presence of ES, analyzing the health or healthspan of these worms can make us to see whether ES is effective in changing the individuals' quality of life. **Figure 4.3.5** displays the healthspan and gerospan data based on locomotion assay and **Figure 4.3.6** is for the percentage of healthspan-gerospan. By looking at **Figure 4.3.5** it can be seen

that 80 Hz treatment lessened the healthspan of Q19-expressing worms even though the lifespan of these individuals were increased. Moreover, proportional decrease in the healthspan of 80 Hz treated-worms expressing Q19 can be found in **Figure 4.3.6**. On the contrary, the data in the **Figure 4.3.5** points out that 10 Hz for Q40 and 80 Hz for Q67 extended the number of healthy days in comparison with control group of each strain. Since the lifespan of 80Hz for Q67 was expanded in the control group of Q67, the proportion of healthy days for Q67-80Hz group was decreased. On the other hand, 10 Hz treatment caused proportional extension in healthspan of Q40 expressing worms.

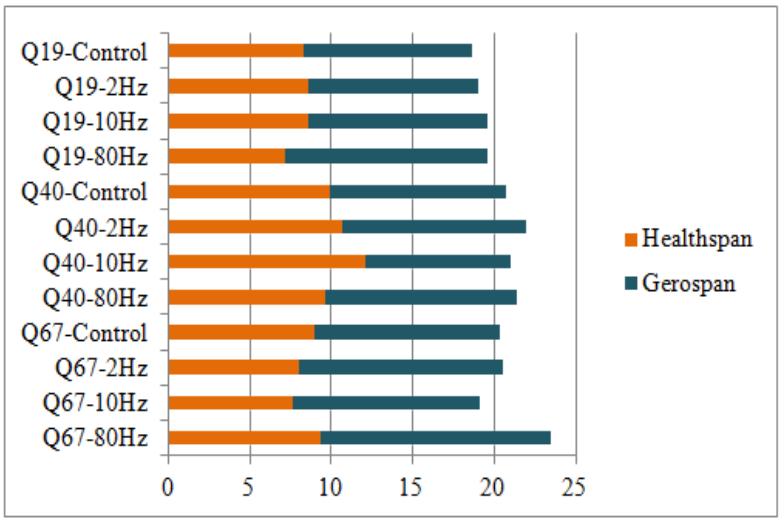


Figure 4.3.5: Chronological of healthspan-gerospan of neuronal polyQ-expressing worms treated with ES and their control samples. The experimental process was similar with the one in **Figure 4.2.1.**

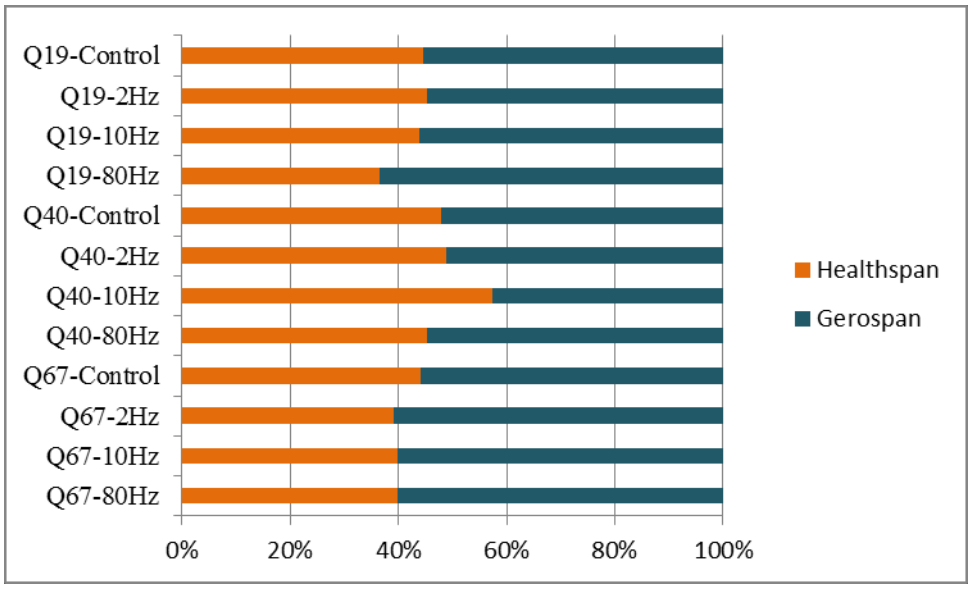


Figure 4.3.6: Physiological of healthspan-gerospan of polyQ-expressing worms treated with ES and their control samples. The experimental process was similar with the one in **Figure 4.2.1**. The calculation is based on max lifespan normalized to 100%.

Observing that ES has no significant effect on the lifespan of the worms expressing polyQs in their neurons may have 2 explanations. First of all, the lifespan of the animals expressing polyQ in neurons do not differ significantly from wild type animals. Therefore, ES may not have an impact on animals with wild type lifespan. Secondly, ES may not cause an upregulation of HSPs in the neurons of *C. elegans*. It is shown that unlike the neuronal polyQ expressing animals, transgenic worms expressing polyQ in their muscle tissues have motility defects and shortened lifespan. Therefore, we investigated the influence of ES on transgenic worms that express polyQ24 or polyQ40 in their muscle cells and determined the changes in their lifespan after ES treatment. **Figure 4.3.7** and **Table 4.3.7** illustrate that ES causes an increase in the half life of both Q24 and Q40 expressing worms. The effect of stimulation is more apparent in 10 Hz treated muscle-polyQ models. There is augmentation in max life of Q40 samples treated with ES but we observed opposite effect for max life of Q24 ones.

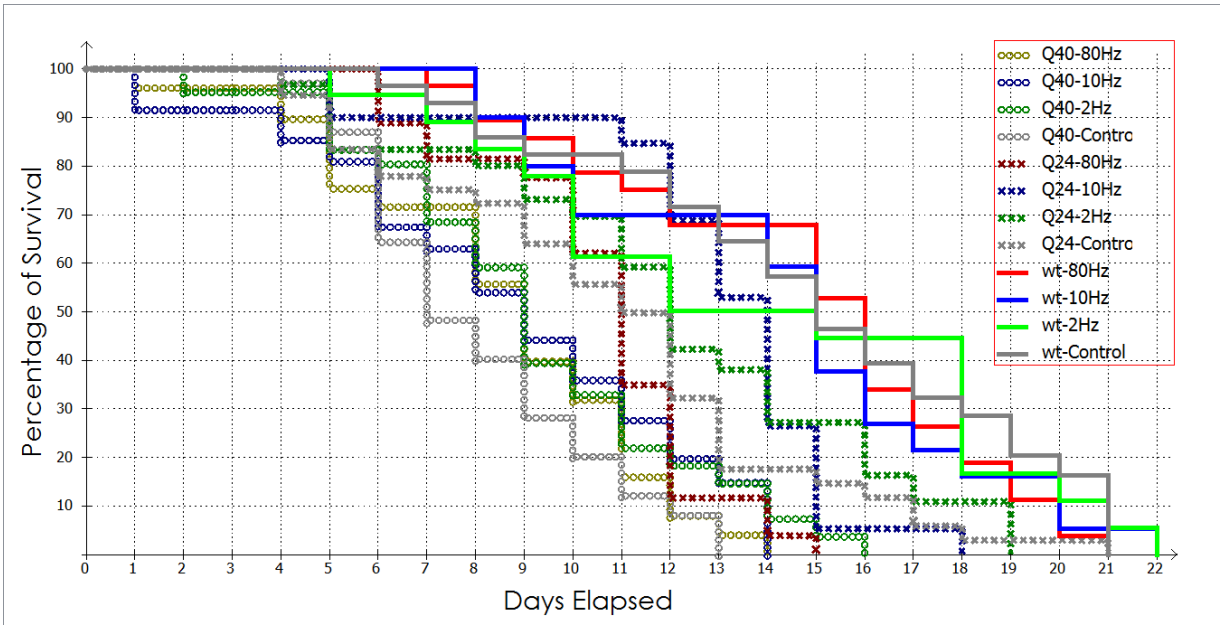


Figure 4.3.7: Kaplan Meier survival data of first trial ES treatment on worms expressing polyQ in their body wall muscle cells. The experimental procedure is similar with the method described as **Figure 4.2.1**. The experiment was performed with about 50 animals and the data represent only the first trial.

Table 4.3.7: Quantitative data of figure 4.3.7 showing max life and half life differences of ES treated group relative to control groups.

	Q40-80Hz	Q40-10Hz	Q40-2Hz	Q24-80Hz	Q24-10Hz	Q24-2Hz	wt-80Hz	wt-10Hz	wt-2Hz
Max-Life	15,38462	7,692308	23,07692	-28,571	-14,2857	-9,52381	0	0	4,761905
Half-Life	28,57143	28,57143	28,57143	0	27,27273	9,090909	6,66667	0	0

4.4. Mild Electrical Stimulation does not alter the expression levels of HSPs.

Previously it has been shown that ES induces HSP expression in cell lines (Wang, Liu, Jin, & Steinacker, 2010). We wanted to see whether the effect of ES would be similar in

multicellular organisms. It is known that HSP expression is regulated mainly by transcriptional activation. For this reason, transcriptional reporters in which the promoter sequence of the gene of interest located upstream of fluorescent protein such as GFP can be used in order to visualize and quantify the changes in HSP expression. Such transcriptional reporter lines have been obtained for mitochondrial, ER and cytoplasmic HSPs. We utilized these lines in order to determine the levels of HSP throughout the worms after the ES treatment.

We first determined the effect of ES on HSP-4 expression level. HSP-4 resides in the ER and folds in this organelle. Hsp4::GFP transgenic lines were treated with ES at 10 Hz frequency for 5 minutes. Then GFP expression controlled by *hsp-4* promoter activity was visualized using a fluorescent microscope (Nikon 80i) 1 hour after ES treatment. As a positive control heat shock was applied for 2 hours at 35^{O}C, which has been shown to induce the HSP expression. The ES-treated worms were compared with both control worms and heat shock applied (HS) worms. We initially quantified the GFP levels throughout the worms. In addition, we quantified only anterior and posterior parts of the worms. Our result represented in **Figure 4.4.1** highlights that there is no difference in transcriptional expression levels of HSP4 of 10 Hz ES treated individuals and control ones for all these three areas after 1 hour. Moreover, we detected increased expression level for these areas in HS group which is used as positive control.

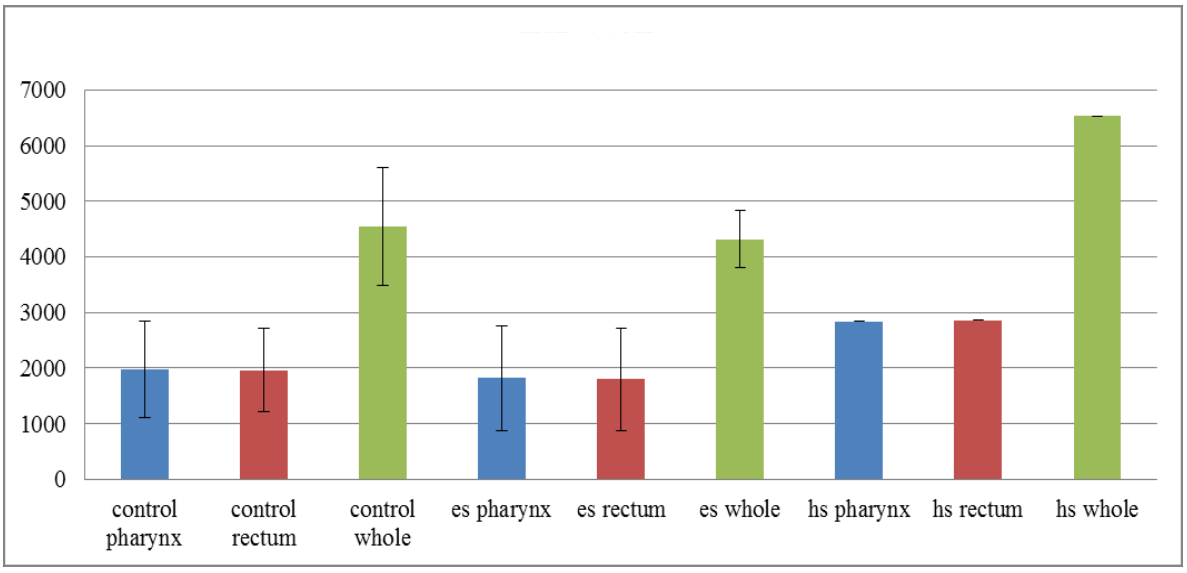


Figure 4.4.1: Transcriptional expression of HSP-4 in hsp-4p::gfp reporter strain treated with ES or HS. Worms were picked at L4 stage and allowed to become young adult with overnight 15oC incubation. Then HS worms were placed into 35^{O}C incubator for 2 hours while other groups were kept at 15^{O}C. 10 min before the HS application finished, other groups were removed from 15^{O}C incubator and 5min 10Hz ES treatment was applied to ES group while control group remain at room temperature as ES group without any treatment. 1 hour after the end of each treatment worms were visualized under the fluorescent microscope.

After examining expression level in HSP-4 transcription, the transcriptional regulation of HSP60, which is a mitochondrial chaperone, was examined. Hence, we used transgenic line expressing GFP under the control of *hsp*-60 promoter. We performed the procedure as in the case of HSP-4. As it can be seen **Figure 4.4.2**, there is no change in transcription levels of HSP-60 when treated with ES.

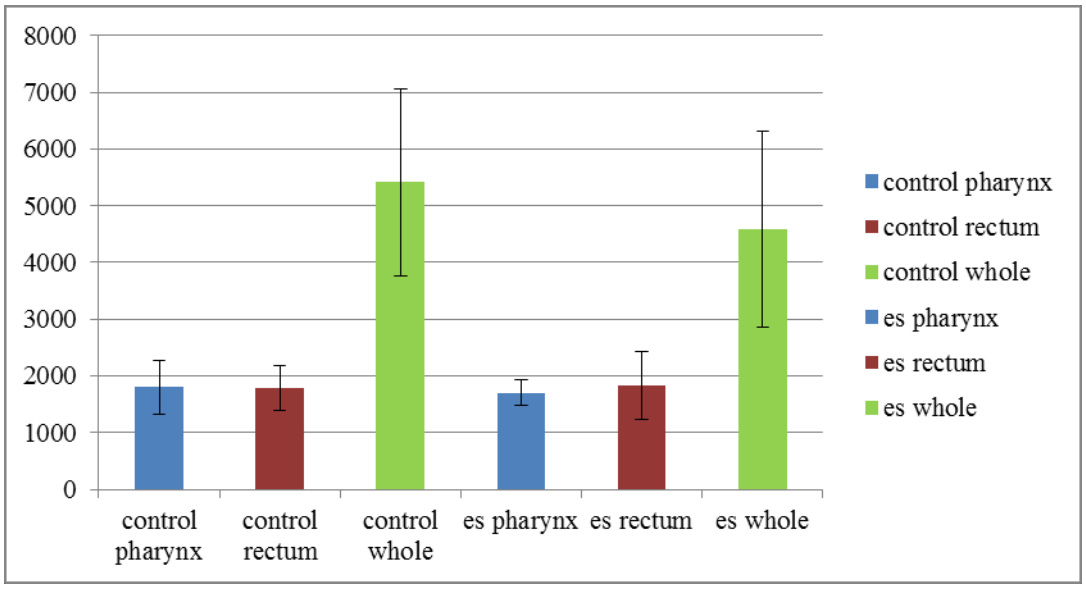
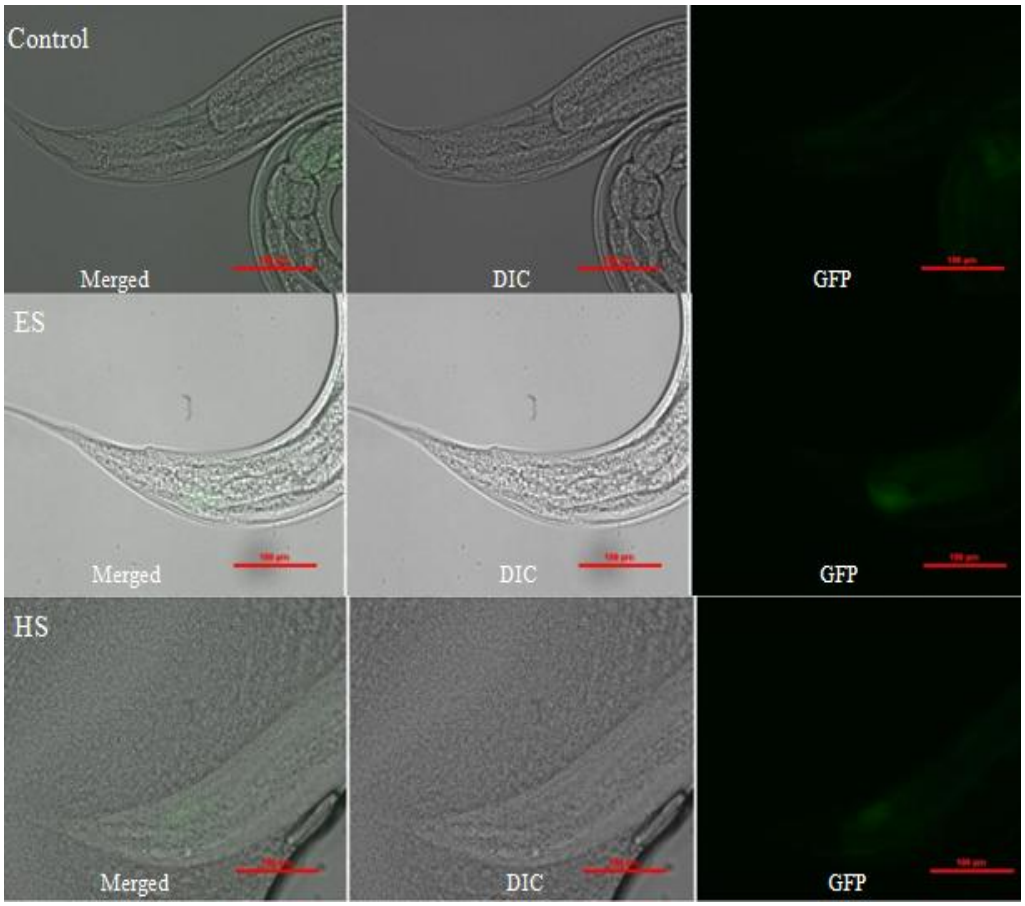


Figure 4.4.2: Transcriptional expression of HSP-60 of hsp-60p::gfp reporter strain after treated with ES. The experimental procedure was similar with the one explained in **Figure 4.4.1**.

Lastly, we focused on one of the main chaperones of cytoplasmic stress response, HSP90. Interestingly, we observed elevated HSP90 expression at the posterior end of the intestine after ES treatment. **Figure 4.4.3** indicates the quantification results of HSP90 transcriptional expression level of three groups in the posterior part of the intestine. We found that HSP90 transcription is increase by 23% with electrical stimulation compared to control animals, which is statistically significant ($p<0.05$). Heat shock applied animals also showed a similar level of increase in HSP-90 expression (30% increase).



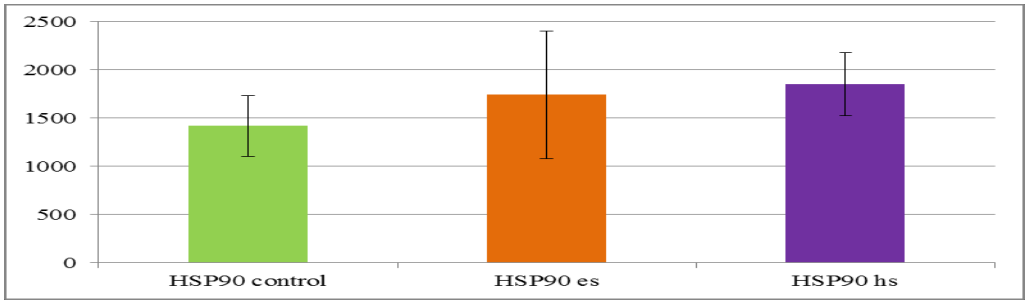


Figure 4.4.3: Differential expression of HSP-90 in the posterior part of the intestine 1 hour after exposure to ES or HS. The experimental procedure was similar with the one explained in **Figure 4.4.1**.

CHAPTER 5: DISCUSSION

In this project, we investigated the influence of electrical stimulation on proteotoxic models of *C. elegans*. For this purpose, we used Huntington's Disease models, expressing fluorescently labeled polyQ protein that differ in the lengths of glutamine tracts. Using these transgenic animals, we performed lifespan assay to understand whether ES treatment can ameliorate disease phenotype caused by polyQ aggregation.

Before examining polyQ models, we first examined the impact of three different frequencies (2 Hz-10 Hz-80 Hz) on wild type animals. The frequencies used in this study were previously applied to both humans and cell lines and they were shown to have no toxic effects to cell lines (Matsuyama et al., 2014). To confirm ES does not have any toxic effect to a multicellular organism, we applied ES to worms and two crucial processes of the worms: fertility and aging. When we applied ES to adult worms, we can directly see its influence on reproductive system of the worms by counting the embryos laid by the hermaphrodite worms. We found that all the frequencies of the ES did not lead to any significant changes in the fecundity of wild type worms. In addition, we examined the effect of ES on the embryonic development. The embryos laid by ES-treated mothers completed their development, hatched and progressed into next developmental stage at a level comparable to wild type worms.

In addition to reproduction of wt worms, we then test the influence of ES on aging by the analyzing the lifespan of the animals after ES treatment. Lifespan analysis enabled us to see whether ES has any toxic effect on wild type worms' survival or locomotion compared to non-treated ones. We observed that the maximum and half-life of the treated wt animals were similar with non-treated worms. Hence, we can conclude that the frequencies and the duration of the application did not cause any change in the lifespan of the wild type animals.

After making sure that ES does not negatively affect the animals. We directed our focus to HD models of *C. elegans*. We utilized transgenic wormsthat have neuronally expressed polyQs which are either below (Q19) or above (Q67) or the same with the threshold polyQ value (Q40). Survival assay for these strains was conducted with 3 biological replicates. By dividing the lifespan into several categories, we broadly analyzed the survival data. We

determined half-life and max life of all the groups. When the survival data of Q67 control group and wild type control group analyzed, we saw Max life of Q67 control samples is day 23 whereas the last member of wt control group died in day 21. The difference was not statistically different. As a result, it can be said that that neuronally expressed Q67 did not negatively alter max life. Additionally, half-life of non-treated Q67 expressing worms is around 15% less than half-life of wt non-treated ones. Therefore, Q67 aggregation might be influential for half-life of the population. Interestingly, lifespan graph of Q19 control worms in comparison with other control groups illustrates that Q19 strain is more vulnerable to age-related changes. This is an interesting observation because we would expect the opposite due to tendency of Q67 to aggregate and cause toxicity. Our observation might be due to the fact that the background mutation of Q19 strain might be effective the aging.

The max-life and half-life table for three trials enlightens that ES treated groups in all strains did not significantly make changes in any of these two phenomena. As it can be seen in the table of **Figure 4.3.7** the highest extension is in half-life of Q67-expressing worms treated with ES at 10Hz for 5 min, which corresponds to about eight percent of Q67 control group. Moreover, the greatest decrease is in the half-life of Q67-10Hz group, that of which mitigated by 16 percent compared to its control group. However, these changes are statistically negligible. This result illustrates that electrical stimulation is ineffective to enhance max life and half-life of the animals expressing polyQs of of different lenghts.

Previous study done with HD model of mice observed extension in survival time (Mughal et al., 2011). However, we did not observe similar results. This can be the result of the difference between phisiolgy of *C. elegans* and mice. For example, aggregated Htt protein hastens neural cell death in mouse hippocampal cell lines (Jiang, Nucifora, Ross, & DeFranco, 2003). On the contrary, even though CAG expansion has cytotoxic effect, is not associated with neurodegeneration in *C. elegans* (Brignull, Morley, & Morimoto, 2007).

Even though, lifespan is considered as the main determinant of aging, living long does not always mean living healthier. Therefore, a new concept for *C. elegans* aging studies that has started to emerge: Healthspan (Bansal, Zhu, Yen, & Tissenbaum, 2015). This concept is characterized as the time interval when the population has more than 50% of max functional

capacity of wild type animals. We also identified the healthspan-gerospan of all the experimental groups. Our findings suggests that rather than extending lifespan, ES treatment makes polyQ expressing worms live healthier and we can see in 10Hz ES group for Q40 and 80Hz ES group for Q67.

Current studies demonstrate the ES induced HSP expression in cellular level (L. Wang, Liu, Jin, & Steinacker, 2010; Yanagida, Mizuno, Motegi, Kobatake, & Aizawa, 2000). Therefore, we questioned whether ES treatment we applied has similar enhancement in HSPs. For this purpose, we examined 3 different HSPs belongs to three distinct chaperone-mediated stress response pathway. Firstly, we focused on ER unfolded protein response chaperone HSP-4 and benefited from a transgenic strain of *C. elegans* which expresses GFP under the control of HSP-4 promoter. By the help of this strain, we were able to indirectly detect alterations in transcriptional expression of HSP-4 by quantifying the GFP expression. We took microscopy images of worms, 1 hour after ES treatment and look at the differences in expression between control and ES group. We treat a group of worm with HS so that we could use them as positive control. The expression was analyzed in whole body as well as specifically focusing on anterior and posterior part of the intestine. Similar approach was used for mitochondrial chaperone HSP-60 and cytoplasmic HSP-90. Our findings point out that the mitochondrial and ER chaperone levels do not change after are not affected by ES treatment. However, we detected tissue specific increase in the HSP90 expression, especially in the dorsal part of the posterior intestine.

In the light of these result, we can speculate that ES treatment increases the healthy over frailty period of polyQ models by modulating the cytoplasmic heat shock response. However, in order to prove this hypothesis, further investigations should be done because electrical stimulation might activate some other pathways which are also crucial for healthspan of worms. For example, previous studies show that ES triggers Smad signaling(Y. Wang, Rouabhia, Lavertu, & Zhang, 2015) as well as NF-κB and interleukin-6 activation(Henriquez-Olguin et al., 2015). Therefore, this healthspan extension might be caused by activation of a parallel pathway rather than cytoplasmic HSRs.

CHAPTER 6: CONCLUSION

Huntington's disease is a neurodegenerative disorder progressively decreasing the patients' quality of life. It is a consequence of the mutation in a single gene named *huntingtin*. The mutation results in CAG expansion in coding region and polyQ increase in encoded protein. The aim of this project was to examine the potential role of ES on proteotoxic models to understand whether it can be used as therapeutic intervention of proteotoxicity-related diseases. Thus, our first intention was testing its bio-toxic feature by treating wt worms with ES. Our results displays that ES treatment does negatively influences the wt worms' reproduction and aging. Then, we focused on HD-related strains of *C. elegans* expressing polyQs and performed survival assay to both treated and control animals. The survival data determines that there is no extension in lifespan of ES-treated individuals but their healthspan is prolonged.

The induction of HSP expression by ES is previously shown. Therefore, we wanted to see if ES treatment we applied has similar effect on ER-specific HSP-4, mitochondrial HSP-60 and cytoplasmic HSP90. We found that HSP90 but not others showed tissue specific increase in transcriptional expression. As a result, we speculate that prolongation in healthspan might be related to cytoplasmic HSR. However, out hypothesis needs further investigation to be proven.

CHAPTER 7: FUTURE DIRECTIONS

In this project, I mainly focus on strains neuronally expressing polyQs and their phenotypical changes in the presence of ES treatment compared with control. There are also strains expressing polyQ in their muscle. The effect of ES on HD models of *C. elegans* will be understood better if these strains are investigated in a similar manner.

Since we did not observe increase in lifespan of worms with ES treatment we used; the frequency, application times or currents can be changed. By the help of these

changes, the extension in lifespan of *C. elegans* might be possible because maybe any of these variables we used may not be sufficient enough to provide an extension.

Additionally, since we observed enhancement in the transcriptional expression of cytoplasmic HSP90 by ES application, we also want to investigate ES-related alterations in transcriptional expression of other cytoplasmic chaperones like HSP-70. Furthermore, in addition to transcriptional expression, translational levels of HSPs will be examined by either Western blot or imaging the translational reporter strains of these HSPs.

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