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Mechanisms of statin effects on muscle and neuronal proteostasis

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When I decided to take on this endeavor, little did I know about the wonder in the world of research I was about to be introduced to. These are the people that have impacted my research and career path, in the best way possible.

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ABSTRACT

Statins are widely prescribed and used chronically, but we know little about the effects on long-term protein homeostasis during stress and aging. Our aim was to quantify the effect of statins on stress-induced protein damage. We administered atorvastatin in a dose-response curve in *Caenorhabditis elegans* under naïve control conditions and in conditions of hypertonic and heat stress known to induce muscle damage measurable as countable puncta in a polyglutamine aggregation model of damage. We observed that there is significant polyglutamine aggregation variability among worms at baseline and thus further study requires within experiment baseline controls, per worm. Our results are that statins exacerbate ($p<0.05$) the amount of protein damage induced by hypertonic and heat stress in a muscle protein reporter of polyglutamine aggregation. Many patients taking statins long-term report adverse muscular side effects may be experiencing some tissue-level pathophysiology associated with statins; our results support an effect of statins on muscular protein damage during stress. Further study is required to understand the effects of statins on age-induced protein homeostasis, long-term effects of augmented statin-induced damage, and translation to human studies.

CHAPTER 1 – REVIEW OF LITERATURE

HISTORICAL INFORMATION ABOUT STATINS

Statins were discovered ~4 decades ago and are now the largest class of drugs prescribed, globally (1, Figure 1). Much remains unknown, however, about the side effects of chronic use. While the average person takes statins ~40 years to protect against cardiovascular diseases, 30% of patients discontinue statin use following an adverse side effect (2). Due to adverse effects of statins, the American Heart Association (AHA) recommends dynamic patient and physician relationships to find a statin that is tolerated and fits the patient's profile (Table 1, Table 2). With one in four adults over 40 using statins, ideal statin therapy should promise increase in quality of life, with minimal discomfort, muscle weakness, and pain (3).

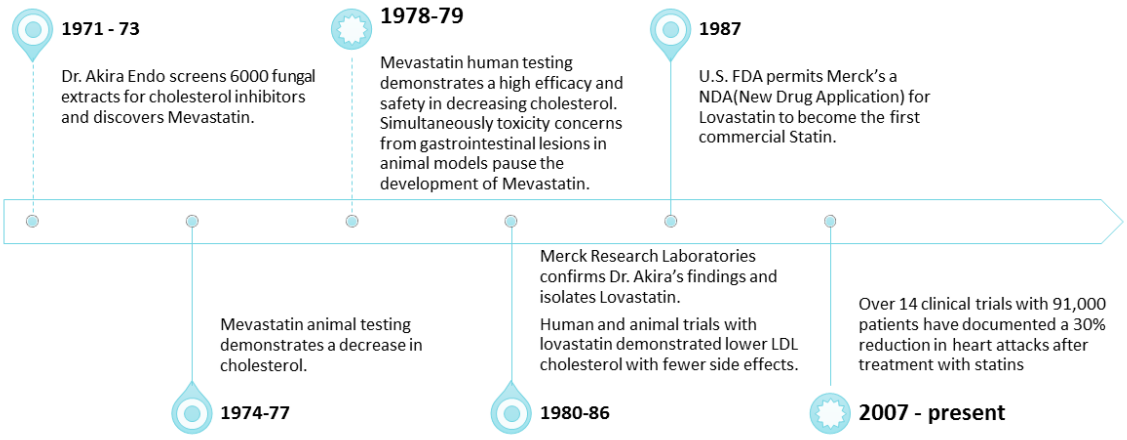

Figure 1: The Development of Statins (4)

Patient Condition	Treatment
20-75 and LDL-C ≥ 190 mg/dl	High-intensity statin without risk assessment
40-75 and LDL-C ≥ 70 mg/ dl	Moderate-intensity statin with risk assessment to consider high-intensity statin

Table 1: American Heart Association's Statin Guidelines (5)

Statin Type	Solubility, Half Life (hrs), Bioavailability (%)	Intensity (Low Intensity – Lowers LDL-C by <30%) (Moderate Intensity – lowers LDL-C by 30%-49%) (High Intensity – lowers LDL-C by ≥ 50%)
(Lovastatin)	Lipophilic, 13.37 hrs, 5%	20 mg = (Low Intensity) 40 mg = (Moderate Intensity)
Zocor (Simvastatin)	Lipophilic, 4.85, 5%	10 mg = (Low Intensity) 20-40 mg = (Moderate Intensity)
Pravachol (Pravastatin)	Hydrophilic, 1.8, 17%	10-20 mg = (Low Intensity) 40 mg = (Moderate Intensity)
Lescol (Fluvastatin)	Lipophilic, 3, 24%	20-40 mg = (Low Intensity)
Lipitor (Atorvastatin)	Lipophilic, 14, 14%	10 mg = (Moderate Intensity) 40 mg = (High Intensity) *Today's most popular statin.
Crestor (Rosuvastatin)	Lipophilic, 19, 20%	5 mg = (Moderate intensity) 20 mg = (High intensity) *Today's strongest statin.
Baycol (Cerivastatin)	Lipophilic, 2-3, 60%	(High Intensity) *Removed from the market following 50 deaths attributed to statin induced rhabdomyolysis.

Table 2: Statin Profile/Types (6, 27, 28)

Cardiovascular diseases result in one out of every three deaths globally and are the leading cause of death in both men and women in the United States (7, 8). Statins are among the most commonly prescribed drugs for reducing cholesterol levels and work mechanistically by HMG-CoA reductase inhibition in the mevalonate pathway (Figure 2). Through the mevalonate pathway, mevalonate, a precursor of cholesterol, is synthesized from HMG-CoA (9). Statins target the mevalonate pathway to regulate the production of cholesterol, but indirectly also regulate lipoprotein signaling (10). Data suggests that the indirect effects on lipoproteins may increase susceptibility to protein tissue and muscle damage with stress (e.g., exertional rhabdomyolysis) including exercise-induced stress (11, 12). Myopathy (muscle weakness) and myalgia (muscle pain) affect ~10% of the 35 million Americans taking statins (6). This pain-related side-effect contributes to some of the decisions of ~25-60% of patients to stop statin use (13).

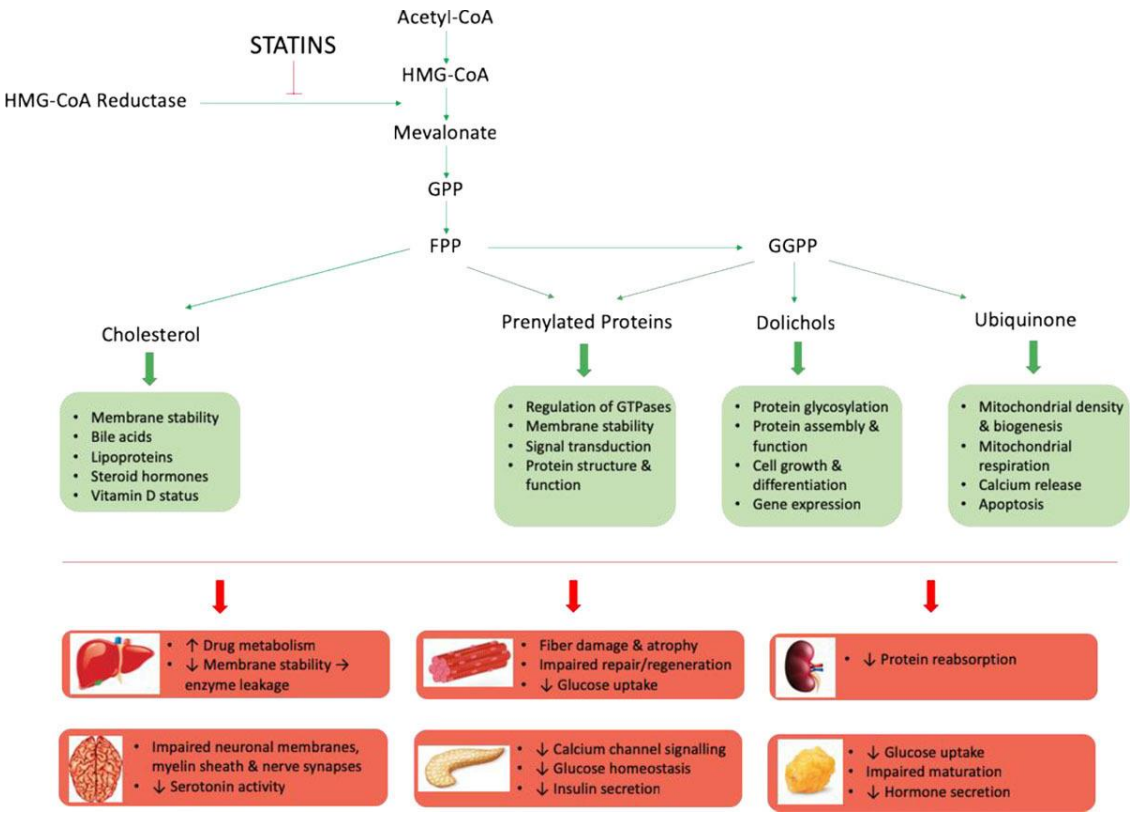

Figure 2: Statins Pathway (14)

STATIN SIDE EFFECTS

The most common adverse effect of statin use, is myopathy and myalgia (6). The mechanism may be related to how statin signaling pathways are related to protein homeostasis or proteostasis, the collection of processes that support protein synthesis and function. The mevalonate pathway produces ubiquinone (coenzyme Q), cholesterol, dolichol, and prenylated proteins. While statins limit LDL-cholesterol, they also affect the production of compounds crucial to the maintenance and structural integrity of proteins (15). In addition, CoQ10 is mainly carried by LDL cholesterol, thus the lowering of LDL cholesterols lowers CoQ10. Coenzyme Q is responsible for remodeling skeletal muscle and functions in electron transport chain of the

mitochondria in producing ATP, decreases under statin therapy (16). However, studies that supplement Coenzyme Q for patients with myalgia show little to no effects on pain management (17).

In some patients, statins reportedly cause myopathy and muscle damage, ranging from muscle weakness to rhabdomyolysis, a life-threatening syndrome caused by muscle breakdown (12). In the PRIMO Observational study, 832 out of 7924 patients reported pain with 315 patients reporting pain when using muscles and 31 patients being confined to bed from pain (18). While actual muscle ruptures are rare and often confused with tendon ruptures, a Netherlands pharmaceutical database has logged >165 cases of muscle rupture linked to statin use (19). The mechanisms by which statins increase muscle damage remains unknown. In a survey that reported 1,074 French statin users, 38% reported muscular symptoms that prevented everyday activities and a decrease in quality of life (13).

Statin induced muscle effects	Statin induced neurological effects	Additional general statin induced effects
-Difficultly walking, overall weakness, did not improve following resistance exercise, that dissipated following the stopped usage of statin. -Rhabdomyolysis leading to multiple organ failure and death is the most affects less than 0.1% of statin users. -Creatine Kinase levels, biomarkers of muscle damage, can rise up to 10 times the normal limit under statin therapy in around 7-29% of users.	-Mixed reports and studies on statins causing psychiatric disorders, memory loss, or slowing dementia progression. Regardless of positive or negative effects, there is a lack of causality and prevalence is rare. - A meta-analysis of 25 randomized controlled trials conclude that that statins have no effect on cognition. They neither decrease or increase cognitive performance	-Statin induced liver injury affects ~3% of statin users. -Statins can increase risk of diabetes by 9% following 4 years of statin adherence. -Statins can increase risk of acute kidney injury in the first 4 months of statin adherence -Sleep quality may be disrupted under certain stains but dissipate following a change in statins.

Table 3: Reported Side Effects when taking Statins (20-25)

CAENORHABDITIS ELEGANS ANIMAL MODEL

Caenorhabditis elegans have a conserved mevalonate pathway with cholesterol-independent, proteostatic branches of the statin signaling pathways. Less than half a dozen studies have studied statin effects in *C. elegans*, but it is known that worms lacking HMG-CoA lack protein prenylation and have an increased unfolded protein response (UPR) (15). *C. elegans* allows rapid alteration of gene expression through RNAi gene knockdown. In ongoing studies, genetic knockout of key components of statin signaling along the mevalonate pathway will allow determination of specific molecular regulators of statin effects on protein structure and function. *C. elegans'* ~40% genetic homology to humans (26), availability of reporter strains that allow easy visual assay of muscle and neuronal damage, genetic tractability, and short

lifespan make it a powerful comparative animal model for studying the effects of statins on muscle and neuronal proteostasis.

STATEMENT OF THE PROBLEM AND RESEARCH HYPOTHESIS

Despite the prevalence of statin use, little is known about the mechanisms and long-term impacts of statins on human aging, protein function, and muscular injury. Understanding the chronic implications of statin use and mechanisms by which statins may affect aging tissues is critical given the widespread use and potential impacts on individuals participating in physical activity, perhaps in stressful (hot, humid, with dehydration) environments. **The aim of the study was to use the tractable *Caenorhabditis elegans* animal model to 1) test the hypothesis that statin treatment will cause increased muscle damage during stress.**

CHAPTER 2 – METHODS

Materials and Methods

C. elegans strain and maintenance

AM140 rmIs132[*Punc-54*::Q35::YFP] worm strain was cultured in 20° C on 100mm petri dishes filled with 51mM NaCl NGM and coated with 350µL OP50 *E. coli*. Worms were maintained to avoid starvation by regular transfer to new food. The AM140 worm strain contains a fluorescent reporter of muscle damage that expresses a yellow-fluorescent-protein (YFP) in the promoter of a muscle-specific protein (unc-54).

NGM was made following a specific recipe of 1.49g NaCl, 1.25g bacto-peptone, and 8.5g bacto-agar and 500µL deionized water. These ingredients were combined in a 1000mL media bottle and after autoclaving on a 30-minute liquid cycle, the media was cooled at room temperature. After cooling, 500µL of cholesterol (to support reproduction), 500µL magnesium sulfate, 500µL calcium chloride, and 12.5mL of potassium phosphate were added to the media. Following the addition of these salts, the liquid media was poured into 100mm and 60mm petri dishes and allowed to solidify at room temperature.

OP50 *E. coli* for bacterial lawns were cultured in LB broth through the placement of a single colony of bacteria in 10mL of LB broth and incubated at 37° C for 12-18 hours in a shaking incubator (225rpm). Following incubation, 350µL of the overnight OP50 *E. coli* culture was pipetted onto the solidified media plate, spread using a sterile spreader, and allowed to grow into a bacterial lawn at 37° C.

To synchronize (to same developmental larval stage) and decontaminate worms for experiments, a bleach technique was used. A bleach solution kills all but *C. elegans* eggs which are transferred to new plates. The bleach technique includes washing the worms off of an NGM plate using 51mM NaCl buffer and pipetting the worms and buffer solution into a sterile 15mL conical tube. After spinning the worms down in a centrifuge at 2,000 rotations per minute (rpm) for one minute, the supernatant of buffer was removed and the conical tube was filled with additional buffer. This technique was repeated four times. After washing, a bleach solution of 1mL bleach, 250µL NaOH, and 3.75mL of autoclaved water was added to the conical tube containing the worms. The conical tube was shaken vigorously by hand for five minutes and the reaction was stopped with the addition of autoclaved water. After the addition of the autoclaved water, the conical tube was spun in the centrifuge for three minutes at 1,200 rpm. Following the centrifugation, the supernatant was removed and the conical tube was filled with NGM buffer and spun at 1,200rpm for 3 minutes. This wash was repeated two additional times.

After the final centrifugation, the remaining bleached eggs were pipetted to clean (no OP50 *E. coli*) 51mM NaCl NGM plates. These clean plates were left to incubate at 20 degrees Celsius overnight, allowing all eggs to hatch. The following day, the starved L1 worms are all synchronized because during starvation, all mixed populations of L1s will enter a dauer phase. They are fed by transferring them to NGM plates containing OP50 *E. coli.* After being fed, worms will grow together and reach the L4 stage, where they can be used for experiments.

Statin Administration

Lipitor (Atorvastatin), created by Pfizer, is the best-selling statin in the United States (1). In an efficacy study and economic analysis of statins, atorvastatin has significantly less mean total treatment costs, the highest success rate for patient outcomes, and significantly reduces triglycerides and LDL by at least 10% more than simvastatin, pravastatin, lovastatin, and fluvastatin (29, 30). We chose to use atorvastatin calcium for statin administration because it is widely used and well-tolerated, thus making it the most applicable to eventual translation in the human model. Atorvastatin pill drugs were provided by Dr. Beth Taylor's laboratory. The pure chemical form was purchased from Fisher Scientific (CATALOG INFO) and stored at room temperature 20° C. 80mg atorvastatin pills were pulverized using a mortar and pestle and dissolved in methanol (MeOH) to create weight by volume solutions of varying concentrations. For solutions made from pure atorvastatin powder, powder was directly added to MeOH. Atorvastatin liquid solution was added directly to the in 3mL solid media agar (NGM) to final mM concentrations 0.1, 0.125, 0.25, 0.375, 0.5, 0.75, and 1.0mM. Synchronized worms, grown on control, isotonic media (51mM NaCl NGM) are placed on 12-well plates for growth and drug control. Columns treated with solvent only (methanol or water) served as no-drug and vehicle only controls.

Quantification of Q35::YFP aggregates for Atorvastatin, Hyperosmotic, and Heat Stress

Q35::YFP aggregates were counted manually. During imaging, worms were live and moving. 8-hour statin survival assays of *C. elegans* were completed using 12 well petri plates. 3mL of media in 51mM and 200mM concentrations of NaCl were pipetted into the wells and allowed to solidify (one column in each well plate used per stress condition). After adding 20µL

of OP50 *E. coli*, 3 x AM140 L4 staged worms were staged with a worm picker to each well. For Atorvastatin stress assays, worm was screened at baseline, 0h (hour), and at every 2-hour interval for 8 total hours. This procedure was completed with 0mM (no additive), 0mM (MeOH), 0mM (H_2O), 0.1, 0.125, 0.25, 0.375, 0.5, 0.75, and 1.0mM. Atorvastatin-supplemented media and control media for 51mM and 200mM NaCl media concentrations. The worms were screened under a fluorescent microscope to count the number of polyglutamine (polyQ) aggregates in each stress condition.

For the heat stress assays, 3 x AM150 L4 worms were screened at baseline, 0h (hour), and for 5 hours on 51mM NaCl NGM plates at 35° C, 37° C, and 40° C for without heat recovery. This procedure was further carried on 51mM NaCl NGM plates at 35° C with 5 hours recovery. 1-hour heat stress at 40° C with 4 hours recovery was ran in conjunction with 0mM (MeOH), 0.25mM, 0.375mM, 0.5mM, 0.75mM, 1mM atorvastatin supplemented media and control media. After heat and statin stress, worms were taken out of incubator and placed in 20 °C incubator to allow for worm heat recovery and prevent worm death. Following recovery, worms were placed under the microscope for imaging. A fluorescent filter was used to allow for the visualization of Q35 fluorescent aggregates (puncta). These puncta were either counted manually live.

Statistical analysis

All data is presented as means ± SD. Pairwise comparisons were assessed for statistical significance ($p<0.05$) using paired t tests.

CHAPTER 3 – STATIN (PILL) EFFECTS ON HYPERTONIC STRESS-INDUCED PROTEIN DAMAGE

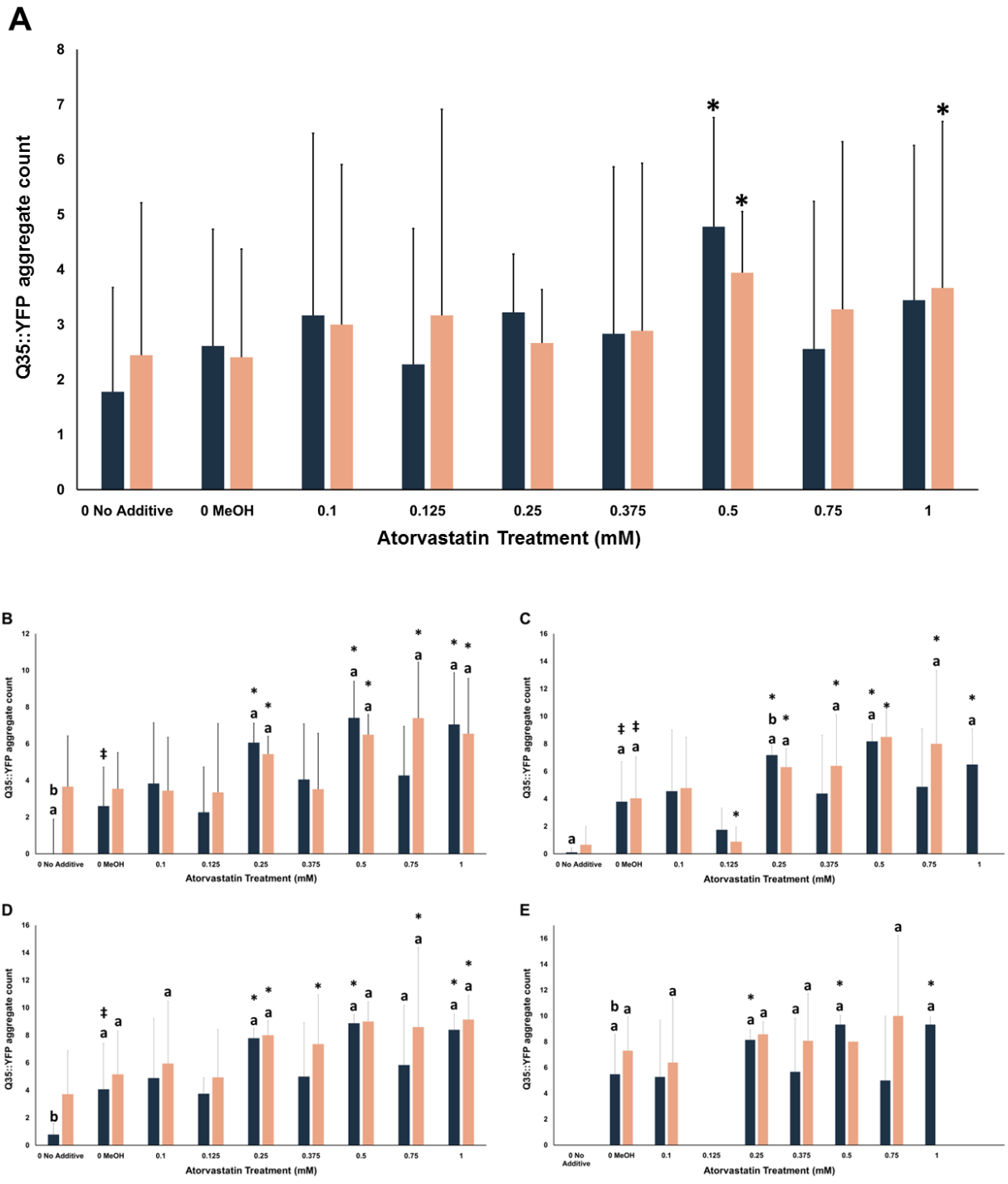

Figure 3. *Effect of pulverized atorvastatin pill and hypertonic stress on glutamine aggregate formation.* A. 0h, baseline, aggregate count. B. 2h, aggregate count. C. 4h, aggregate count. D. 6h, aggregate count. E. 8h, aggregate count. ***** represents statistically significant ($p<0.05$) differences between pulverized atorvastatin pill dose and respective 0 dose control to vehicle. **ǂ** represents statistically significant ($p<0.05$) differences between 0 MeOH and 0 no additive. **a** represents statistically significant ($p<0.05$) differences between experimental conditions and respective baseline (0h). **b** represents statistically significant ($p<0.05$) differences between isotonic/dehydrated (51mM) vs. hypertonic/dehydrated (200mM) conditions. Navy bars represent worms cultured and maintained on 51mM NaCl isotonic NGM plates and salmon bars represents worms cultured on 51mM NaCl NGM and exposed to 200mM NaCl NGM at the start of the experiment. Number of worms screened at baseline, start of experiment (n=432 total): n=36 (0 no additive), n=108 (0 MeOH), n=36 (0.1mM), n=36 (0.125mM), n=36 (0.25mM), n=36 (0.375mM), n=36 (0.5mM), n=36 (0.75mM), n=72 (1mM) (Table 4,5)

Results

Figure 3A. *Glutamine aggregation varies at baseline, requiring 0 time point controls within experiments, per worm.*

With first exposure (<5 min) of drug and concomitant environmental stress (*i.e.*, hypertonic stress), worms exhibit varying numbers of puncta. Statistically significant ($p<0.05$) differences between 0.5mM and 1mM atorvastatin treated worms were observed at time 0. It is unlikely that <5 min induced 2-fold increase in aggregates, and thus important that 0 time point controls be included for all worms in experiments testing drug and/or stress exposure.

There were no significant differences between worms placed on 51mM NaCl NGM vs. 200mM NaCl NGM ($p>0.11$) at baseline. In addition, there were no significant differences between no additive and vehicle (MeOH: methanol) for 51mM NaCl NGM ($p>0.14$) and for 200mM NaCl NGM ($p>0.95$).

Figure 3B. *Glutamine aggregation and worm deaths occurs within 2 hours of exposure*. Within 2 hours of drug and concomitant environmental stress (e.g., hypertonic stress, atorvastatin stress), worms exhibit an increase in glutamine aggregation from protein damage and a decrease in lifespan survival. Some worms at higher atorvastatin and hypertonic stresses, are scored as dead within 2 hours. There are 21 worm deaths in the 51mM group and 38 worm deaths in the 200mM group, at high stress 0.5mM, 0.75mM, and 1mM atorvastatin groups. While there were not a significant number of puncta accumulated between the 51mM NaCl NGM vs 200mM NaCl NGM hyperosmotic group ($p>.06$), we do see more deaths accumulated in the hyperosmotic group within 2 hours of exposure to the stresses.

In addition, there are significantly less puncta ($p<0.01$) between the 0 No additive 51mM group at 2h than at 0h. This is highly unlikely and was so because (n=9) less worms with a higher aggregate count were not counted due to time constraints; the remaining (n=9) worms there were counted had a significantly smaller aggregate average leading to an ostensibly smaller amount of aggregates. Excluding the 0 No Additive group, due to missed worms for the count, there were no significant ($p>.06$) differences in aggregate counts between 51mM NaCl NGM and 200mM NaCl exposure 2 hour at every time point with the same atorvastatin treatment.

Figure 3C. *Glutamine aggregation and worm deaths skew the dose response curve within 4 hours of exposure.* High counts of worm deaths at high atorvastatin and osmotic stress, shifts the aggregate count. Anything beyond 0.375mM pulverized atorvastatin pill is too toxic of a dose as it leads to worm deaths. There are 5 deaths in the 0.25mM concentration and then 16 deaths in the 0.5mM concentration. Due to time constraints, experiment 2 did not screen at 4h for both the 51mM and 200mM for the no additive, MeOH vehicle, 0.125mM, and 1mM groups. The worms in 1mM atorvastatin and 200mM NaCl all died from every experiment except for the ones in experiment 2 that were not screened. Within 4 hours of worm exposure there are 60 worm deaths in the 200mM group and 28 worm deaths in the 51mM group, at high stress 0.25mM, 0.5mM, 0.75mM, and 1mM atorvastatin groups.

Figure 3D. *Higher osmotic stress in the same drug concentration does not significantly increase glutamine counts within 6 hours of exposure*. 200mM NaCl causes double the amount of deaths than 50mM NaCl conditions at time point 6. Within 6 hours of worm exposure there is double the amount of worm deaths in the 200mM compared to the 51mM NaCl NGM group without significant ($p>0.08$) differences in glutamine aggregates, at high stress 0.125mM, 0.25mM, 0.375mM, 0.5mM, 0.75mM, and 1mM atorvastatin groups. Even with the deaths, there are more total alive worms at 6h than 4h because experiment 2 was not screened at hour 4. While the data suggests that “0 MeOH” leads to significantly more puncta aggregates than “0 no additive,” the sample ($n=43$) size for 0 MeOH is quadruple that of the sample ($n=9$) size of no additive for no additive in the 51mM NaCl treatment. The larger sample size in MeOH

ostensibly has higher glutamine aggregates but this is so because of the sample size. Similarly, it appears as if 0.25mM has similar counts of puncta to 0.5mM, 0.75mM, and 1mM, but it is important to note that doses higher than 0.375mM pulverized pill cause more > 50% worms deaths. A consistent sample size is needed to truly determine the dose-response curve.

Figure 3E. *0.25 and 0.375mM atorvastatin doses at 8 hours of exposure contribute to the most aggregates and least deaths.* 0.5mM, 0.75mM, and 1mM Atorvastatin concentration have a 70-90% death rate by hour 8 of hypertonic and atorvastatin stress. While there was a total of 32 worms for each of the 0.25mM, 0.375mM, 0.5mM, 0.75mM, and 1mM conditions, there were only 10, 9, and 3 worms alive for the 0.5mM, 0.75mM, and 1mM conditions. With a 70 – 90% death rate, any atorvastatin from pill concentration above 0.5mM is much for the survival of AM140 worms. Glutamine aggregation occurs within 8 hours of exposure at a significant difference specific to the 0 MeOH condition for the 51mM NaCl NGM in the 0.25mM, 0.5mM, and 1mM NaCl group. With experiments from 5 different pooled experiments in the previous time marks, the 8 hours of worm exposure only holds data from experiments 3, 4, 5 and not from 1 and 2, as experiments 1 and 2, stopped screening at 6h. The lack of data from experiments 1 and 2 decrease the total worm count conditions for 0 No additive, 0 MeOH, 0.125, and 1mM.

Discussion

8-hour pulverized atorvastatin pill and hyperosmotic stress experiments were accumulated from 5 experiments. With atorvastatin stress and concomitant hypertonic stress,

worms exhibit an increase in protein damage and a decrease in lifespan survival. Experiments indicate a decrease in survival of worms at the hypertonic dose (200mM) compared to the isotonic dose (51mM), but no significant increase in puncta.

While glutamine aggregation varies at baseline, the protein aggregation forms are rates specified to their experimental condition. Within 2 hours, we noted that many worms are dying before they develop puncta. At high statin concentrations, (0.5mM, 0.75mM, and 1mM), the dehydrated (200mM NaCl NGM) group had significantly more deaths than the hydrated (51mM NaCl NGM) group, at all drug conditions without a significant increase in puncta. It is possible that the atorvastatin pill is too toxic at higher doses, leading to death unassociated or before protein damage occurs. Within 4 hours of exposure, doubling the statin dose from 0.25mM to 0.5mM triples the amount of deaths. At 6h, we note that moving from 50mM to 200mM causes double the amount of deaths at high statin concentrations. Thus, we conclude, that when administering drug stress with pulverized statin pills, the doses should not exceed 0.5mM as it leads to deaths before an adequate development of puncta over time. While the worms that do survive in those conditions do develop a significant number of puncta in compared to their baseline, the sample size dramatically decreases. 0.25 and 0.375mM atorvastatin doses appear to cause to the most aggregates and least deaths. 0.5mM, 0.75mM, and 1mM Atorvastatin concentration have a 70-90% death rate by hour 8 of hypertonic and atorvastatin stress.

CHAPTER 4 – PURE STATIN EFFECTS ON HYPERTONIC STRESS-INDUCED PROTEIN DAMAGE

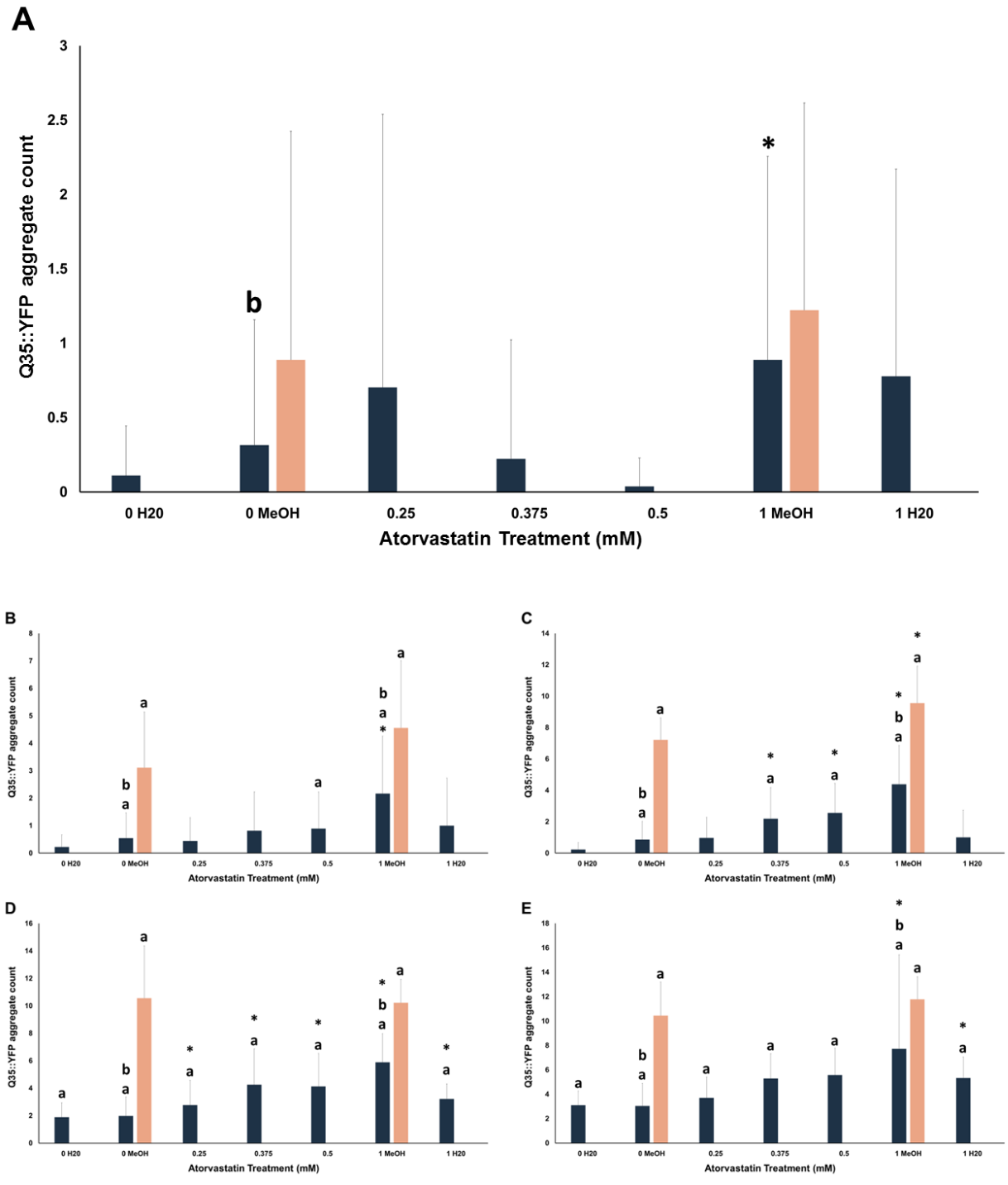

Figure 4. *Effect of pure atorvastatin salt and hypertonic stress on glutamine aggregate formation.* A. 0h, baseline, aggregate count. B. 2h, aggregate count. C. 4h, aggregate count. D. 6h, aggregate count. E. 8h, aggregate count. ***** represents statistically significant ($p<0.05$) differences between pulverized atorvastatin pill dose and respective 0 dose control to vehicle. **‡** represents statistically significant ($p<0.05$) differences between 0 MeOH and 0 no additive. **a** represents statistically significant ($p<0.05$) differences between experimental conditions and respective baseline (0h). **b** represents statistically significant ($p<0.05$) differences between isotonic/dehydrated (51mM) vs. hypertonic/dehydrated (200mM) conditions. Navy bars represent worms cultured and maintained on 51mM NaCl isotonic NGM plates and salmon bars represent worms cultured on 51mM NaCl NGM and exposed to 200mM NaCl NGM at the start of the experiment. Number of worms screened at baseline, start of experiment (n=180 total): n=9 (0 H_2O), n=54 (0 MeOH), n=27 (0.1mM), n=27 (0.25mM), n=27 (0.375mM), n=27 (0.5mM), n=27 (1mM MeOH), n = 9 (1mM H_2O) (Table 6,7).

Results

Figure 4A: *Glutamine aggregation varies at baseline, requiring 0 time point controls within experiments, per worm.* There is little to no variability at baseline, puncta differences were less than one. Regardless, baseline variability was determined to interpret any effects observed with drug and/or stress treatments on glutamine aggregation. The significant ($p<0.02$) differences between worms placed on 51mM NaCl NGM vs. 200mM NaCl NGM at 1 MeOH is 0.6 of a difference, which is not even countable as a single puncta. There is a difference in

decimal figures because averages were taken for the worms. There are no significant ($p>.6$) difference between the methanol or water vehicle at baseline.

Figure 4B. *Glutamine aggregation without worm deaths occurs within 2 hours of exposure to Atorvastatin salt*. At hour 2, there are no deaths at hour 2 even at high concentrations of 1mM atorvastatin for both 51mM and 200mM NaCl. In addition, the 1mM H_2O vehicle did not have a significant ($p>0.77$) number of puncta compared to the 0 H_2O concentration while the 1mM MeOH did have a significant ($p<0.37$) number of puncta compared to its 0 MeOH concentration. This may be so because atorvastatin is not soluble in water. The H_2O vehicle wells are also very cloudy and thus more difficult to see the worms. Future research should use methanol.

Figure 4C. *Glutamine aggregate increases accordingly with increased dose at 4 hours of stress exposure*. Glutamine aggregation occurs within 4 hours of exposure at a significant ($p<1.7x10^{-5}$) difference specific to time point 0 and the 51mM 0 MeOH group for the 0.375mM, 0.5mM, and 1mM MeOH groups. The 1mM MeOH has the highest aggregate count and the 0.25mM has the lowest aggregate count. At hour 4, there are still no noted deaths but 4 of the worms in the 0.5mM group may have climbed out of wells due to the atorvastatin stress. The 200mM group has significantly more puncta than both the 51mM NaCl 0 MeOH and 1 MeOH group ($p<8.5x10^{-20}$) and ($p<2.3x10^{-5}$).

Figure 4D: *200mM hyperosmotic stress increases the amount of aggregates compared to 50mM of the same dose*. The data suggests that 1mM atorvastatin salt administered in MeOH for

51mM may be the optimal dose to yield the most glutamine aggregates without worm deaths. The 200mM 1 MeOH, while it does yield much aggregates, it is unclear whether the aggregates form due to hypertonic stress or the atorvastatin dose. This is so because there is no significant difference between the 1MeOH and 0MeOH concentration for 200mM at time point 6 ($p>0.81$).

Figure 4E: *Glutamine aggregates are greater in 1 MeOH than 1* H_2O*.*
Glutamine aggregation occurs within 8 hours of exposure at a significant difference specific to hour 0 in all groups tested ($p<1.4x10^{-6}$). The 1 MeOH and 1 H_2O have a significant difference to their specific controls at hour 8 ($p<0.0002$) and ($p<0.006$) respectively. The 1 MeOH has greater significant difference from its control possible because atorvastatin is more soluble in methanol than water. The significant difference between the 51 and 200mM group for both 0 MeOH and 1 MeOH ($p<1.06x10^{-13}$) and ($p<3.86x10^{-7}$) demonstrates that 200mM does increase the aggregate counts.

Discussion

8-hour pure atorvastatin salt and hyperosmotic stress experiments were accumulated from 4 experiments. The pure atorvastatin salt was administered to rule out any factors the other compounds in the pill may have caused. With atorvastatin salt stress and concomitant hypertonic stress, worms exhibit increases in protein damage and without killing as many deaths. There may be a compound in the pulverized pill that leads to more worm deaths. Identical pure atorvastatin salt doses do not cause deaths as seen when using pulverized atorvastatin pill. With atorvastatin pill the 200mM group increases the risk of worm death

without an increase in glutamate aggregates; however, with atorvastatin salt, the 200mM group, increases the aggregate count without an increase in worm death. Thus, we conclude that atorvastatin salt is better than pulverized atorvastatin pills at creating a dose-response curve that increases glutamine aggregates without decreasing the sample size. There is a significant increase in puncta with the increase in statin dose, seen in all time points except for the baseline.

The 1mM H_2O group has more puncta compared to the 0 H_2O group at all time points, excluding the baseline. This may be so because the atorvastatin was not soluble in the water. If it was not soluble in the water, it is not evenly dissolved in the agar and accessible by the AM140 worms. The 1 MeOH and 1 H_2O have a significant difference to their specific controls at hour 8 of ($p<0.0002$) and ($p<0.006$) respectively. However, the 1 MeOH causes a greater significant difference from its control than the 1 H_2O because atorvastatin is more soluble in methanol than water.

Hypertonic stress alone and paired with statins cause a higher count of glutamine aggregates than isotonic conditions or isotonic statin conditions. With the pure atorvastatin salt, the 200mM group doubles the number of puncta in the 1MeOH 51mM vs 1 MeOH 200mM group. The osmotic stress causes more protein aggregation than the statin does, this is evident as the 0mM statin + 200mM NaCl group has a similar number of puncta as the 1mM + 200mM NaCl group. Introducing hyperosmotic stress to a statin group significantly ($p<3.8x10^{-7}$) increases the amount of protein aggregates. Thus, dehydration significantly increases protein damage in statin users.

CHAPTER 5 – COMBINED HEAT AND HYPERTONIC STRESS-INDUCED PROTEIN DAMAGE

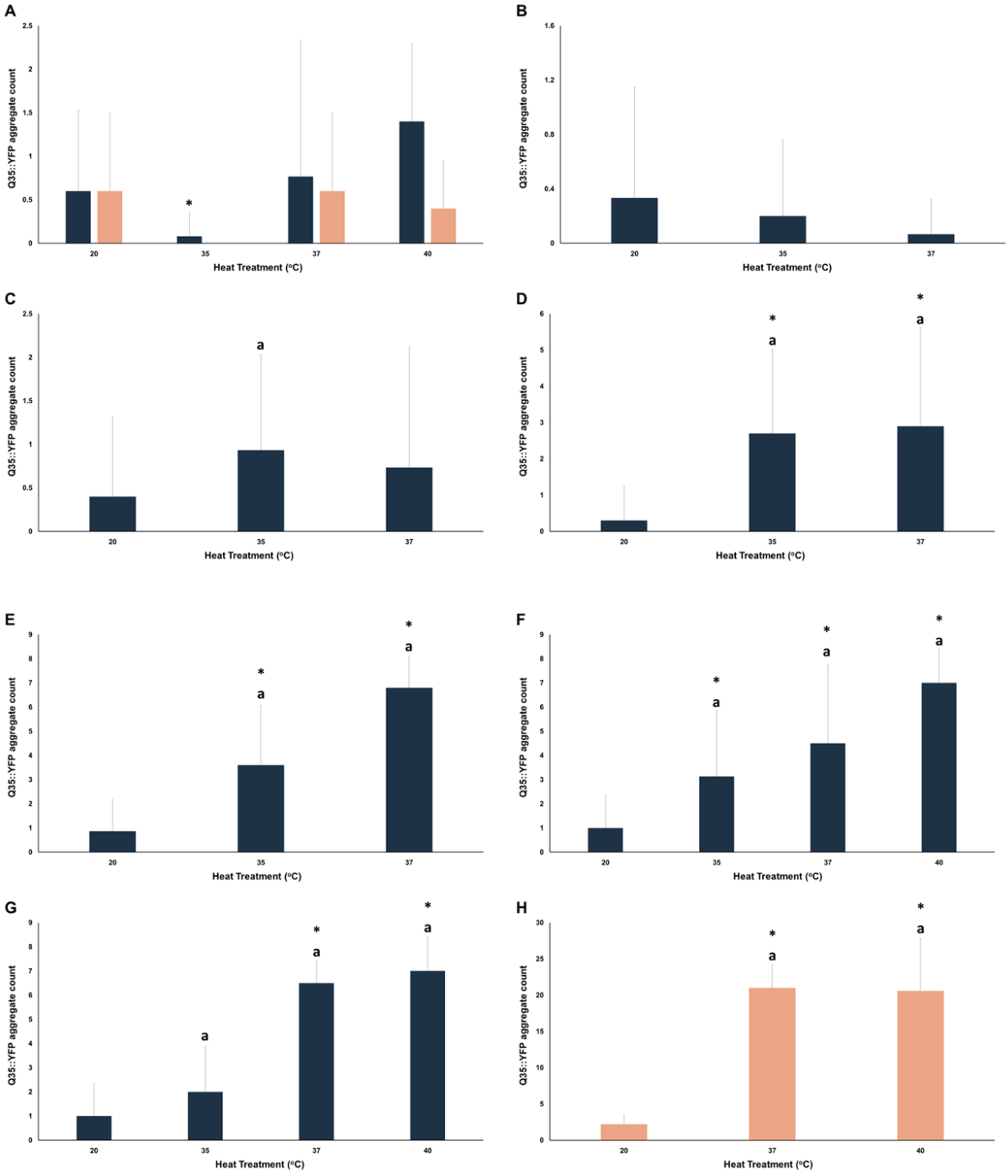

Figure 5. *Effect of varying heat stress on glutamine aggregate formation in isotonic and hypertonic conditions.* A. 0h, baseline, aggregate count. B. 1h, aggregate count. C. 2h, aggregate count. D. 3h, aggregate count. E. 4h, aggregate count. F. 5h, aggregate count. G. 6h, aggregate count. H. 7h, aggregate count. ***** represents statistically significant ($p<0.05$) differences between varying heat stress conditions and the respective room temperature (20 ^{o}C) control. **a** represents statistically significant ($p<0.05$) differences between experimental conditions and respective baseline (0h). Navy bars represent worms cultured and maintained on 51mM NaCl isotonic NGM plates and salmon bars represents worms cultured on 51mM NaCl NGM and exposed to 200mM NaCl NGM at the start of the experiment. Number of worms screened at baseline, start of experiment (n=105 total): n=35 of (20^{o}C), n=25 of (35^{o}C), n=35 of (37 ^{o}C), and n=10 of (40 ^{o}C) (Table 8,9).

RESULTS

Figure 5A. *Glutamine aggregation varies at baseline, requiring 0 time point controls within experiments, per worm.* Glutamine aggregates were counted to determine whether there was significant variability per worm in different experimental conditions at the start of the experiment (within 5 minutes of treatment). This is critical to determine the baseline variability in interpreting any effects observed with drug and/or stress treatments on glutamine aggregation and the greater concept of body muscle damage acutely, and chronically. Worms were grown on control, isotonic media (51mM NaCl NGM) and placed onto either 51mM NaCl control wells or 200mM NaCl and then administered heat stress. Within 5 minutes, baseline numbers of puncta were counted. There is minor variability, all within 1 puncta of each other,

in number of puncta among healthy worms placed onto experimental conditions and thus we conclude that this data should serve as baseline for each respective experiment. There were no significant differences between worms placed on 51mM NaCl NGM vs. 200mM NaCl NGM ($p>0.07$). The data for heat and osmotic stress are pooled from multiple experiments that administer heat at different lengths and with different recovery times, hence why there may not be data points for each condition at each 1 hour mark.

Figure 5B. *1 hour of heat stress is not enough time for glutamine aggregation.* No worms die within 1 hour of heat administration, there is a decreased number of total worms, because not every experiment screened at 1 hour after we noted that there is no observable difference ($p>0.2$) within 1 hour of heat stress.

Figure 5C. *Significant increase in puncta, within 2 hours, is still less than an overall increase of 1 puncta. 2 hour of heat stress without recovery, is not adequate time to observe a true development of puncta*. Within 2 hours of exposure to heat conditions, the 51mM worms in the 35 ^{o}C have an increase in number of puncta ($p<0.001$) to their baseline by 0; however, their overall puncta count is still less than 1. No worms die within 2 hours of heat administration, there is a decreased number of total worms, because not every experiment screened at hour 2, after we noted that there is no observable differences between the temperature conditions ($p>0.16$) within 2 hours of heat stress.

Figure 5D. *Glutamine aggregation occurs within 3 hours of 35 °C and 37 °C heat stress.* 3 hours of environmental stress (*i.e.*, heat stress), worms exhibit varying numbers of puncta. Within 3 hours of exposure to heat stress without recovery is an adequate time to observe significant ($p<0.004$) development of puncta in 35 °C, and 37 °C baseline, 0h. Additionally, they are also significantly ($p<.01$) different compared to the control of 20 °C at the same time point. No worms die within 3 hours of heat administration, there is a decreased number of total worms, because not every experiment screened at hour 3.

Figure 5E. *Glutamine aggregation and worm deaths occur at 4 hours of heat exposure*. Within 4 hours of exposure to heat conditions, the 51mM worms in the 35 °C and 37 °C have a significant difference ($p<0.01$) to their baseline. There are initially 15 worms screened at 37 °C but 10 of the worms died from the heat stress. The surviving 5 in the 37 °C have a significantly ($p<5.92x10^{-8}$) higher aggregate number than the control 20 °C at the same time point. The worms in the 35 °C are also significantly ($p<0.001$) greater than the control 20 °C at the same time point.

Figure 5F. *Glutamine aggregation increases in relation to heat*. Within 5 hours of exposure to heat conditions, the 51mM worms in the 35 °C, 37 °C, and 40 °C have a significant ($p<0.0001$) increase in protein aggregates compared to their baseline, 0h. 37 °C *heat administration for 5 hours causes aggregation and worm death.* Of the 20 worms screened for 37 °C, 13 of the worms died from heat stress. 40 °C may also cause deaths, but the sample (n=5) size was much lower than that of the 37 °C. In addition, within 5 hours of exposure to heat conditions, the

51mM worms in the 35 °C, 37 °C, and 40 °C have a significant difference ($p<0.0001$) to their baseline, 0h. Additionally, 35 °C, 37 °C, and 40 °C are still significantly ($p<.0104$) different compared to the control of 20 °C at the same time point.

Figure 5G. *Glutamine aggregation increases in relation to heat*. Within 6 hours of exposure to heat conditions, the 51mM worms in the 35 °C, 37 °C, and 40 °C have a significant difference ($p<0.0001$) to their baseline, 0h. *Glutamine aggregation increases in relation to heat*.
6 hours of heat stress without recovery, is an adequate time to observe a significant ($p<7.3x10^{-6}$) development of puncta at 37° C, and 40° C compared to 20° C baseline. In the experiments that made up this data set, there were 10 worms screen in 37° C conditions and none were dead. However, 2 were missing and may have climbed out due to the stressful conditions.

Figure 5H. *Hypertonic stress and heat stress significantly increase glutamine aggregation compared to just heat stress.* Within 6 hours of exposure to heat conditions and 1 hour of recovery, the 200mM worms in the 37 °C and 40 °C have a significant difference ($p<0.0002$) to their baseline, 0h. At 6 hours of exposure to heat conditions and 1 hour of recovery, the 200mM worms in the 35° C, 37° C, have a significant difference ($p<0.0002$) to their baseline at Hour 0. Additionally, 37° C, and 40 °C are still significantly ($p<.0005$) different compared to the control of 20 °C at the same time point. None of the worms died over the administration. It is possible that the “dead worms” from the 51mM up to 6 hours of heat administration were still alive, but just shocked from the heat and did not move when prodded with the worm pick with no response.

DISCUSSION

7 experiments were pooled together. The data demonstrates that there is a significant increase in the number of protein aggregates with the increase in temperature in isotonic conditions. The number of protein aggregates in osmotic and heat stress is triple the amount of just heat stress. This matches the results of previous experiments that showed an increase in the number of protein aggregates in the hyperosmotic group. *Hypertonic stress and heat stress significantly increase glutamine aggregation ($p<2.^{01x10^{-}}7$, $p<0.008$) compared to just heat stress* at 37 ^{o}C, and 40 ^{o}C.

CHAPTER 6 – STATIN EFFECTS ON COMBINED HEAT AND HYPERTONIC STRESS-INDUCED PROTEIN DAMAGE

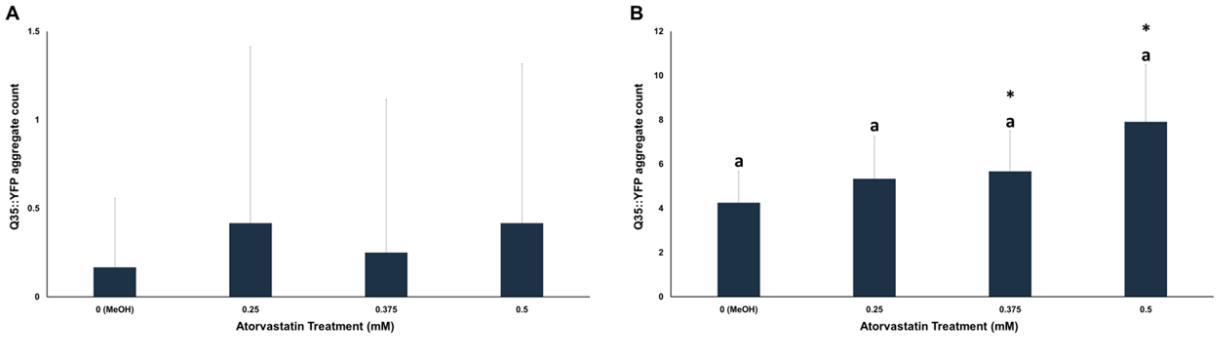

Figure 6. *Effect of pure atorvastatin salt and heat stress(40°C) on glutamine aggregate formation in isotonic conditions.* A: H*our 0, aggregate count.* B: *Hour 7, aggregate count.* Number of worms tested (n=90 total): n=45 (0 MeOH), n=41 (1mM) (Table 7). ***** represents statistically significant ($p<0.05$) differences between atorvastatin salt dose and respective 0 MeOH control. **a** represents statistically significant ($p<0.05$) differences between experimental conditions and respective baseline (0h). Number of worms tested (n=48 total): n=12 (0 MeOH), n=12 (0.25mM), n=12 (0.375mM), n=12 (0.5mM) (Table 10).

RESULTS

Figure 6A. Glutamine variability is not present at baseline, 0h. There is no significant difference between any condition and control, 0 MeOH.

Figure 6B. Following 1 hour of heat exposure at 40°C and 6 hours of recovery, there is significant ($p<2.56 \times 10^{-8}$) difference between every condition and their baseline. In addition,

0.375mM and 0.5mM statin doses have significantly ($p<0.04$) more puncta at hour 7 compared to the 0mM statin dose in concomitant stress of 40°C.

DISCUSSION

As demonstrated in previous experiments, paired hyperosmotic (200mM) and Statin stress increase more protein aggregation over time than only statin stress. Similarly, statin paired with heat stress (40°C) increases ($p<0.04$) more protein aggregation over time than only heat stress. Statin stress in conjugation with heat stress, increase protein aggregation. In isotonic conditions (51mM NaCl), higher statin administration (0.5mM) significantly ($p<0.0004$) increases protein aggregation compared to no statin administration. With atorvastatin salt stress and concomitant heat stress, worms exhibit an greater amount of protein damage than just concomitant heat stress. Statins exacerbate protein aggregation during heat stress. This matches our previous data that shows individual heat and individual statin stress experiments increase protein aggregation.

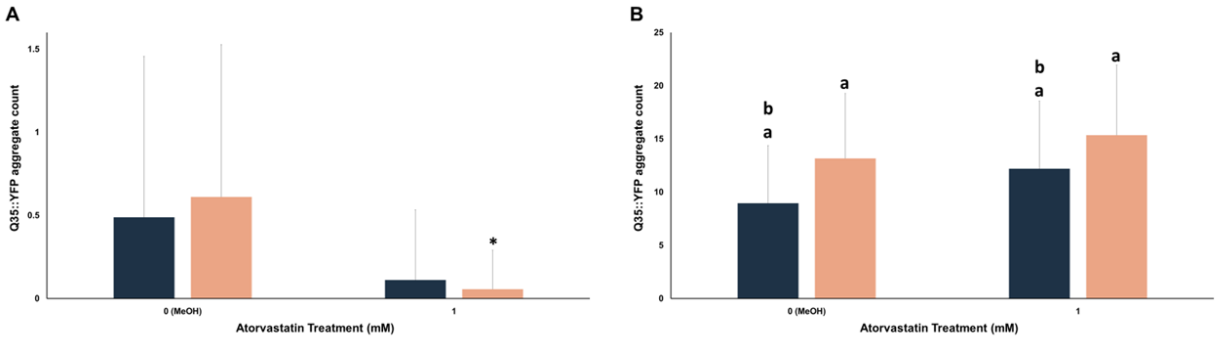

Figure 7. *Effect of pure atorvastatin salt and heat stress (*37°C*)on glutamine aggregate formation in hypertonic conditions*. A: 0h*, aggregate count.* B: *5h, aggregate count.* *****

represents statistically significant ($p<0.05$) differences between atorvastatin salt dose and respective 0 MeOH control. **a** represents statistically significant ($p<0.05$) differences between experimental conditions and respective baseline (0h). **b** represents statistically significant ($p<0.05$) differences between isotonic/dehydrated (51mM) vs. hypertonic/dehydrated (200mM) conditions. Number of worms tested (n=90 total): n=45 (0 MeOH), n=45 (1mM) (Table 11).

RESULTS

Figure 7A. *Glutamine aggregation varies at baseline, requiring 0 time point controls within experiments, per worm.* Glutamine aggregates were counted to determine whether there is significant variability among worms in different experimental conditions at the start of the experiment (within 5 minutes of treatment). There is variability in number of puncta among healthy worms placed onto experimental conditions, thus it is important to determine the baseline variability in interpreting any effects observed with drug and/or stress treatments on glutamine aggregation. The hypertonic 1mM atorvastatin group is significantly ($p<0.02$) smaller than the isotonic group 1mM atorvastatin group within 5 minutes of putting the worms onto plates.

Figure 7B. *Hypertonic stress and heat stress significantly increase glutamine aggregation compared*. Following 5 hours of heat exposure at 37^{o} C, there is a significant ($p<4.^{49x10-}11$) increase in all groups with respect to their baseline, 0h. In addition, there is also a significant

($p<0.006$) increase from the isotonic to the hypertonic groups for both 0 MeOH and 1 MeOH groups.

DISCUSSION

Statin administration in conjugation with both heat stress and hypertonic stress, leads to the greatest amount of protein aggregation. With concomitant hypertonic (200mM NaCl) and heat stress, statin administration leads to the greatest amount of protein aggregation. The dehydrated (200mM NaCl) condition has significantly ($p<0.006$) more protein aggregates than the hydrated (51mM NaCl) condition with the same statin and heat stresses. This matches our data that even without statin administration, there is also significantly more protein aggregates ($p<2.03x10^{-6}$) in the dehydrated state compared to the hydrated state under heat stress. When statin administration is further paired with hypertonic stress under hot conditions, there is the greatest amount of protein damage.

CHAPTER 7 –SUMMARY AND CONCLUSIONS

Our results show that heat and dehydration stress, exacerbates muscular damage in *C. elegans* administered statins. Protein aggregation is dose related to both heat, hypertonic environment, and statins. In summary, statins cause proteins to unfold and aggregate leading to an increased number of puncta that quantify muscular damage. Heat and hypertonic environments also lead to an increased number of puncta. When statins are combined with heat and hypertonic environments, there is the greatest amount of muscular damage.

Appendix

Table 4. Survival of worms of osmotic and atorvastatin pill stress for Hour 0 – Hour 4.

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 0	51	200	51	200	51	200	51	200
0 additive	18	18	0	0	0	0	18	18
0 MeOH	54	54	0	0	0	0	54	54
0.1	18	18	0	0	0	0	18	18
0.125	18	18	0	0	0	0	18	18
0.25	18	18	0	0	0	0	18	18
0.375	18	18	0	0	0	0	18	18
0.5	18	18	0	0	0	0	18	18
0.75	18	18	0	0	0	0	18	18
1	36	36	0	0	0	0	36	36

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 2	51	200	51	200	51	200	51	200
0 additive	9	18	0	0	0	0	9	18
0 MeOH	54	54	0	0	0	3	54	51
0.1	18	18	0	0	0	0	18	18
0.125	18	18	0	0	2	1	16	17
0.25	18	18	0	0	2	0	16	18
0.375	18	18	0	0	0	0	18	18
0.5	18	18	0	8	6	0	12	10
0.75	18	18	7	7	0	0	11	11
1	36	36	14	23	5	2	17	11

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 4	51	200	51	200	51	200	51	200
0 additive	9	9	0	0	0	0	9	9
0 MeOH	45	45	0	0	2	4	43	41
0.1	18	18	0	0	0	0	18	18
0.125	9	9	0	0	1	0	8	9
0.25	18	18	0	5	2	0	16	13
0.375	18	18	0	0	0	0	18	18
0.5	18	18	1	16	6	0	11	2
0.75	18	18	10	13	0	0	8	5
1	27	27	17	26	3	0	7	1

Table 5. Survival of worms in osmotic and atorvastatin pill stress conditions for Hour 6 – Hour 8

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 6	51	200	51	200	51	200	51	200
0 additive	9	18	0	0	0	0	9	18
0 MeOH	45	54	0	0	2	4	43	50
0.1	18	18	0	0	0	0	18	18
0.125	9	18	1	0	0	0	8	18
0.25	18	18	0	10	4	0	14	8
0.375	18	18	0	3	0	1	18	14
0.5	18	18	4	16	6	0	8	2
0.75	18	18	12	14	0	0	6	4
1	27	36	19	29	3	0	5	7
			36	72				

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 8	51	200	51	200	51	200	51	200
0 additive	no data for 0 additiv		0	0	0	0		
0 MeOH	36	36	0	0	1	4	35	32
0.1	18	18	0	0	0	0	18	18
0.125	no data for .125 adc		0	0	0	0		
0.25	18	18	0	11	4	0	14	7
0.375	18	18	0	3	0	1	18	14
0.5	18	18	4	17	5	0	9	1
0.75	18	18	12	15	0	0	6	3
1	18	18	13	18	2	0	3	0

Table 6. Survival of worms in osmotic and atorvastatin salt stress conditions for Hour 0 – Hour 4

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 0	51	200	51	200	51	200	51	200
0 H20	9						9	
0 MeOH	45	9					45	9
0.25	27						27	
0.375	27						27	
0.5	27						27	
1 MeOH	18	9					18	9
1 H20	9						9	

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 2	51	200	51	200	51	200	51	200
0 H20	9						9	
0 MeOH	45	9					45	9
0.25	27						27	
0.375	27						27	
0.5	27						27	
1 MeOH	18	9					18	9
1 H20	9						9	

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 4	51	200	51	200	51	200	51	200
0 H20	9						9	
0 MeOH	45	9					45	9
0.25	27						27	
0.375	27						27	
0.5	27				4		23	
1 MeOH	18	9					18	9
1 H20	9						9	

Table 7. Survival of worms in osmotic and atorvastatin salt stress conditions for Hour 6 – Hour 8

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 6	51	200	51	200	51	200	51	200
0 H20	9						9	
0 MeOH	45	9					45	9
0.25	27						27	
0.375	27						27	
0.5	27		3		1		23	
1 MeOH	18	9					18	9
1 H20	9						9	

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 8	51	200	51	200	51	200	51	200
0 H20	9						9	
0 MeOH	45	9					45	9
0.25	27						27	
0.375	27						27	
0.5	27		5		1		21	
1 MeOH	18	9					18	9
1 H20	9						9	

Table 8. Survival of worms in osmotic and heat stress conditions for Hour 0 – Hour 3

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 0	51	200	51	200	51	200	51	200
20° C	30	5					30	5
35° C	25						25	
37° C	30	5					30	5
40° C	5	5					5	5

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 1	51	200	51	200	51	200	51	200
20° C	15						15	
35° C	15						15	
37° C	15						15	
40° C								

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 2	51	200	51	200	51	200	51	200
20° C	15						15	
35° C	15						15	
37° C	15						15	
40° C								

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 3	51	200	51	200	51	200	51	200
20° C	10						10	
35° C	10						10	
37° C	10						10	
40° C								

Table 9. Survival of worms in osmotic and heat stress conditions for Hour 4 – Hour 7

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 4	51	200	51	200	51	200	51	200
20° C	15							
35° C	10							
37° C	15		10				5	
40° C								

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 5	51	200	51	200	51	200	51	200
20° C	20							
35° C	15							
37° C	20		13				7	
40° C	5				1			

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 6	51	200	51	200	51	200	51	200
20° C	10							
35° C	10							
37° C	10				2			
40° C	5				1			

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 7	51	200	51	200	51	200	51	200
20° C		5						
35° C								
37° C		5					5	
40° C		5						

Table 10. Survival of worms in atorvastatin salt stress conditions for at 40°C.

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 0	51	200	51	200	51	200	51	200
0 MeOH	12						12	
0.25	12						12	
0.375	12						12	
0.5	12						12	

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 7	51	200	51	200	51	200	51	200
0 MeOH	12						12	
0.25	12						12	
0.375	12						12	
0.5	12				1		11	

Table 11. Survival of worms in osmotic, and atorvastatin salt stress conditions for at 37°C.

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 0	51	200	51	200	51	200	51	200
0 MeOH	27	18					27	18
1	27	18					27	18

	Total Worms		Dead		Can't Find		Total Alive Screened	
Hour 5	51	200	51	200	51	200	51	200
0 MeOH	27	18					27	18
1	27	18			3	1	24	17

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