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Astragalus membranaceus root concentration and exposure time: Role in heat stress diminution in *C. elegans*

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Summary

***Astragalus membranaceus* (AM) root modulates polyglutamine aggregation in adult *C. elegans* via the DAF-16/IIS pathway. The effects of AM root on stress response in larval *C. elegans* through the HSF-1/IIS pathway are unknown. Herein, this study aims to elucidate AM root effects on heat-induced paralysis of first larval stage (L1) *C. elegans* during short-term and long-term heat shock. Wild-type (N2) *C. elegans* (n=100 per plate) were synchronized to the L1 stage to eliminate age-based variability, exposed to 0 mg/mL (control) or 2 mg/mL, 4 mg/mL, or 8 mg/mL AM root extract (experimental), and heat shocked at 37°C for either ten or twenty minutes. A harsh touch sensitivity assay was performed, and *C. elegans* responses were recorded. Paralysis rate after ten-minute heat shock decreased as AM root extract concentration increased, indicating that AM root may enhance HSF-1 activity in L1 *C. elegans* after short-term stress. Unexpectedly, longer periods of heat shock resulted in a lower percentage of worms becoming paralyzed in the control condition, possibly due to habituation to stress. Exposure to low concentrations of AM root extract promoted paralysis during long-term stress ($p<0.05$), possibly due to AM root interference in the habituation of C. elegans. Further investigations will be necessary to explore the role of AM root in *C. elegans* habituation and in other laboratory organisms. Deeper insight into molecular pathways altered by AM root during stress responses will not only help to elucidate such pathways in *C. elegans*, but will also have implications in the modulation of stress-response pathways in humans.**

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Introduction

Astragalus membranaceus (AM), a traditional Chinese medicinal plant of the Leguminosae family, enhances natural immune response mechanisms and is commonly prescribed for weakness, chronic illness, immune deficiencies, and stress-related illness (1, 2). Previous studies have revealed that AM enhances memory and reduces stress-induced anxiety in mice (3), improves antioxidant capacity in lambs (4), and decreases muscle atrophy in rats (5). The benefits of AM lie predominantly in its root, which contains favorable polysaccharides, flavonoids, amino acids, saponins, and astragalosides (1, 2). AM polysaccharides have advantageous biological functions, including immunomodulation and antioxidant activities (2, 3), prevention of aging (6) and stress associated with exercise in rats (7), inhibition of oncogenesis in rats (8), and improvement of health of diabetic hamsters (9). AM flavonoids have also been discovered to be able to stimulate and regulate the immune system in mice (10).

Furthermore, AM root reduces polyglutamine (polyQ) aggregation in *C. elegans*, which may lead to cellular dysfunction, and inhibits proteotoxic stress in nematodes (11). AM regulates a subset of abnormal dauer formation-16 (DAF-16) genes involved in DAF-16/Forkhead Box O (FOXO) transcription of the insulin-like growth factor-1/insulin-like signaling (IIS) pathway, and necessary in the development of stress resistance in *C. elegans* (11). Under stress, the dauer formation-2 (DAF-2) receptor signals for DAF-16 to activate gene expression of heat shock proteins (HSPs; **Figure 1**), which maintain proteostasis in the IIS pathway in *C. elegans* (12). Similarly, under stress, heat shock factor-1 protein (HSF-1) enters into the nucleus to activate stress resistance mechanisms via the IIS pathway (**Figure 1**; 12). While DAF-16 protects *C. elegans* from proteotoxicity during adulthood, HSF-1 is critical for proteotoxicity protection in the worms' larval stages (13).

Previous research has described the effects of AM on protein aggregation reduction and proteotoxicity mitigation through DAF-16/FOXO transcription in *C. elegans* (11). However, the effects of AM on HSF-1 activity for stress resistance in *C. elegans* have not been explored. Thus, this study aims to unravel the mechanisms of stress resistance imparted by AM in larval *C. elegans* to provide insight into the potential effects of AM on HSF-1. Because heat shock causes protein aggregation in *C. elegans* (12), and protein

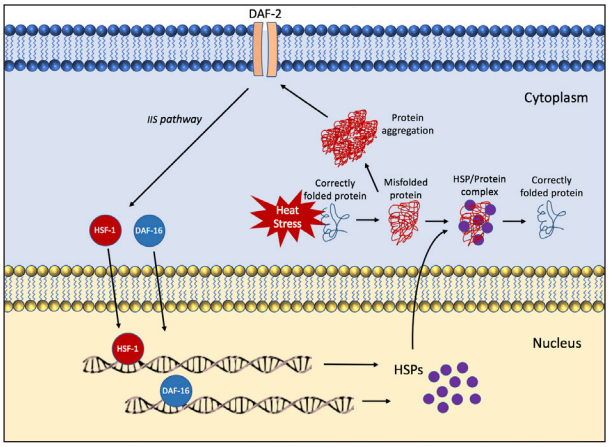

Figure 1. Heat stress response pathway in C. elegans. Heat stress leads to incorrect folding of proteins and aggregation of misfolded proteins. Misfolded protein aggregation causes osmotic stress and initiates dauer formation-2 (DAF-2) signaling for transcription factors heat shock factor-1 (HSF-1) and dauer formation-16 (DAF-16) to enter the nucleus, as part of the insulin-like growth factor-1/insulin-like signaling (IIS) stress response pathway. In the nucleus, HSF-1 and DAF-16 induce transcription of heat shock proteins (HSPs), which correct misfolded proteins to restore proteostasis.

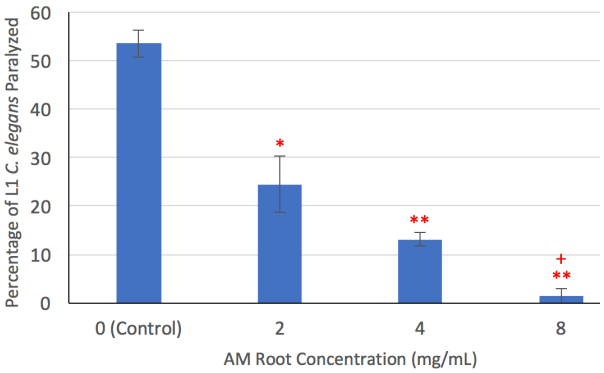

Figure 2. Percentage of L1 *C. elegans* paralyzed after ten-minute incubation at 37°C. Synchronized L1 N2 *C. elegans* (n=100) were treated with 0 mg/mL, 2 mg/mL, 4 mg/mL, or 8 mg/mL AM root extract and were subject to ten-minute heat shock at 37°C ten minutes after plating. Harsh touch sensitivity assay was conducted with a nickel worm pick. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test; *=p<0.05 and **=p<0.01 indicates statistical difference between 2, 4, or 8 mg/mL and 0 mg/mL, +=p<0.05 indicates statistical difference between 2 mg/mL and 8 mg/mL. Each value represents the mean of two plates in each experimental condition. All error bars indicate SEM.

aggregation leads to paralysis in *C. elegans* (14), the effectiveness of AM in the treatment of heat-induced stress was measured by the percentage of worms paralyzed following heat shock. A decreased percentage of worms paralyzed after heat shock and treatment with AM indicates increased activity of heat shock response pathways.

Herein, we investigated the effects of both concentration of AM root extract and time of exposure to heat shock on paralysis of first larval stage (L1) *C. elegans*. Increasing concentration of AM root extract was expected to decrease paralysis of *C. elegans* in both short-term (ten-minute) and long-term (twenty minute) heat shock. Although this hypothesis held true for the short-term heat shock experiments, long-term heat shock experiments unexpectedly showed that low to moderate AM root concentrations increased paralysis, suggesting that AM root extract may interfere with natural habituation processes. Overall, this study may serve to offer deeper insight into the molecular mechanisms of stress resistance in *C. elegans* through observations of larger-scale physiological features.

Results

AM root extract enhances heat shock response in *C. elegans* during short-term stress

To determine the effects of AM on *C. elegans* exposed to short-term stress, synchronized L1 *C. elegans* were treated with 0 mg/mL, 2 mg/mL, 4 mg/mL, or 8 mg/mL AM root extract and incubated at 37°C for ten minutes. A harsh touch sensitivity assay was conducted and worm activity was recorded, with unresponsiveness of *C. elegans* to the harsh touch stimulus indicative of paralysis. The percentage of paralyzed worms decreased with increasing AM root extract concentration (**Figure 2**). Accordingly, quantification revealed 24% ($p<0.05$), 13% ($p<0.01$), and 1% ($p<0.01$) paralysis in 2 mg/mL, 4 mg/mL, and 8 mg/mL AM root extract treatment groups, respectively, compared to 54% paralysis in control animals (**Figure 2**).

Influence of AM root extract on paralysis after long-term stress is concentration-dependent

To determine the effects of AM on *C. elegans* exposed to long-term stress, synchronized L1 *C. elegans* were treated 0 mg/mL, 2 mg/mL, 4 mg/mL, or 8 mg/mL AM root extract and incubated at 37°C for twenty minutes. Compared to the 54% of worms paralyzed from ten-minute incubation in absence of AM root, after twenty-minute (long-term) heat shock, only 12% of control worms were paralyzed. This may be due to habituation to the stress from the longer duration in the presence of intense heat (15).

Unlike results from short-term heat shock, as concentration of AM root extract increased from 0 mg/mL to 4 mg/mL for the twenty-minute incubation, the percentage of paralyzed *C. elegans* increased over two-fold in 2 mg/mL AM root extract and nearly four-fold in 4 mg/mL AM root extract (**Figure 3**; $p<0.05$). However, at the highest concentration of AM root extract (8 mg/mL), the percentage of *C. elegans* paralyzed after heat shock for twenty minutes (25%) was approximately half of the

percentage of *C. elegans* paralyzed in 4 mg/mL AM root extract (45%; **Figure 3**). The highest concentration (8 mg/mL) reduced paralysis in *C. elegans* compared to the control, although it did not totally decrease the percentage of worms paralyzed to the control percentage. For 8 mg/mL AM root extract, there was still a two-fold increase inparalyzed *C. elegans* compared to the control.

Discussion

Although the effect of AM on the DAF-16/IIS stress response pathway in adult *C. elegans* has been previously characterized, the effect of AM on the HSF-1/IIS pathway in larval *C. elegans* has not been well understood (11). This study served to reveal the effect of AM root extract on heat-induced paralysis in larval *C. elegans*, illuminating the potential effects of AM root on the HSF-1/IIS stress response pathway.

AM root extract may increase HSF-1/IIS pathway activity during short-term stress

Stress resistance of larval *C. elegans* involves the entrance of HSF-1 into the nucleus to instigate transcription of HSPs to reduce protein aggregation (12; **Figure 1**). Results of short-term stress assays indicate that AM root may play a key role in increasing DAF-2 signaling to HSF-1 in the HSF-1/IIS pathway in larval *C. elegans*, leading to increased transcription of regulatory HSPs. In turn, this may allow for reduced protein aggregation after short-term heat stress, as DAF-16 does in adult *C. elegans*.

Similarly, previous research has shown that 0.25 mg/mL, 1.0 mg/mL, and 2.5 mg/mL of astragalan, an AM polysaccharide, all decrease polyQ-mediated neuronal death in *C. elegans* via the DAF-16/IIS pathway (11). Thus, AM polysaccharides may be responsible for the decrease in protein aggregation observed with increasing AM root extract concentration during short-term stress. Additionally, in consensus with results of the experiments of short-term stress, AM has also been shown to normalize the number of tyrosine hydroxylase-immunoreactive neurons in the locus coeruleus of mice, activation of which signifies extreme stress (3). Tyrosine hydroxylase is altered by alpha-synuclein aggregation (16), which has similar effects as polyQ aggregation (17), further corroborating the effects of AM on mitigating protein aggregation shown in this study.

AM root extract may have a concentration-dependent effect on habituation to long-term stress

Results of the long-term stress experiments indicate that AM root extract at low concentrations may interfere with habituation through hindrance of the HSF-1/IIS pathway during long-term heat stress, thus decreasing the transcription of HSPs and allowing for an increased

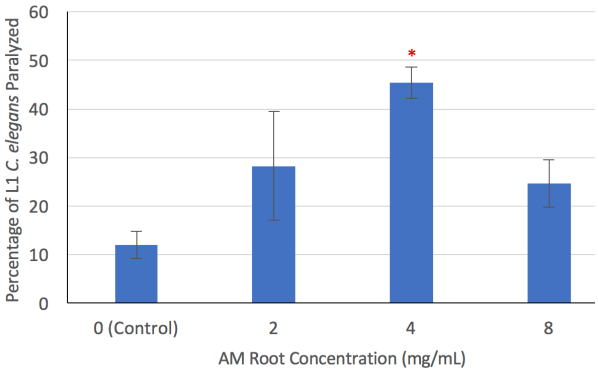

Figure 3. Percentage of L1 *C. elegans* paralyzed after twenty-minute incubation at 37°C. Synchronized L1 N2 *C. elegans* (n=100) were treated with 0 mg/mL, 2 mg/mL, 4 mg/mL, or 8 mg/mL AM root extract and were subject to ten-minute heat shock at 37°C ten minutes after plating. Harsh touch sensitivity assay was conducted with a nickel worm pick. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test; *=p<0.05 indicates statistical difference between experimental groups and the control group. Each value represents the mean of two plates in each experimental condition. All error bars indicate SEM.

accumulation of misfolded proteins in *C. elegans*. Low concentrations of AM root extract are not sufficient to exhibit the beneficial functions of AM root during long-term stress. While AM root extract in high concentrations may also interfere with the natural habituation to stress of the worms, these high concentrations may be sufficient to manifest beneficial properties of AM root extract, as percent paralysis was increased insignificantly with 8 mg/mL AM root extract compared to the control.

Through analyses of *C. elegans* at the level of the organism, we have illuminated phenotypic changes that may be indicative of the biomolecular mechanisms with which AM root extract interferes. Our study depicts the overall favorable characteristics of AM root with regards to short-term stress. The effect of AM root on habituation to long-term stress in *C. elegans* requires further research.

Future investigations and applications

To more concretely determine the effect of AM treatment on HSF-1 activity, future investigations should measure expression of HSPs preceding and following AM root exposure, as HSPs are transcriptionally regulated by HSF-1 (12). Likewise, HSF-1 binding to DNA may be analyzed (e.g. through chromatin immunoprecipitation assays) to determine the change in transcriptional activity of HSF-1 following AM root exposure.

Additional future investigation may yield insight into whether AM reduces proteotoxicity in larval *C. elegans*, as it does in adult worms (11). Further studies of AM root with regards to various cell lines and animal models, varying concentrations of extract, and different types

of stress (e.g. oxidative, osmotic, etc.) may provide additional elucidation of the function of AM root in stress relief. Previous research shows that the IIS pathway, involving DAF-2 and DAF-16, is maintained from *C. elegans* to *Drosophila melanogaster* and mammals (18); since the IIS pathway also regulates the activity of HSF-1 (19), the effects of AM root in other model organisms should be investigated to further determine its stress-reducing effects. Additionally, since stress response mechanisms in *C. elegans* and humans are very similar, as humans have homologs of the *hsf-1* and *daf-16* genes in *C. elegans* that are involved in human age-related diseases (12, 20, 21). AM root may have similar stress-relieving effects in humans as in *C. elegans*. Not only will unraveling the effects of AM root on *C. elegans* offer better understanding of the model nematode, but this advancement may also inform the treatment of human diseases.

Materials and Methods

Synchronization of *C. elegans*

Wild-type (N2) *C. elegans* (Ward's Biological Supply Company, Rochester, New York) were grown on Nematode Growth Medium (NGM) agar plates (Ward's Biological Supply Company, Rochester, New York) until adulthood. M9 buffer was created as previously described (22). After egg-laying, *C. elegans* were synchronized using M9 buffer. Eggs were hatched at 22°C and L1 worms were starved for 24 hours to arrest development.

Preparation of Nematode Growth Medium (NGM) agar plates

AM root powder (100% purity; Item #VS-1499/1050314) was obtained from The Vitamin Shoppe, Syosset, New York. AM root powder was boiled in distilled water to create 2 mg/mL, 4 mg/mL, and 8 mg/mL AM root solutions, respectively. Sterile petri dishes were filled with NGM and either distilled water (control; 0 mg/mL AM root) or respective AM root extract solution (experimental) in a 3:2 ratio, for a total of 5 mL solution in each petri dish. Solutions solidified for 30 minutes at 22°C. Plates were each seeded with 7 µL E. coli OP50 (Ward's Biological Supply Company, Rochester, New York).

Transferring *C. elegans*

L1 *C. elegans* were transferred to all control and experimental plates (n=100 per plate per experimental group) using the chunking method, whereby a sterilized scalpel was used to move a 2 mm x 2 mm chunk of agar containing *C. elegans* from an old plate to a fresh plate (23).

Heat shock assay and data collection

Ten minutes after plating, control and experimental *C. elegans* were incubated at 37°C (Quincy Labs Digital Incubator 12-140E) for ten or twenty minutes and observed under a compound microscope. Harsh touch sensitivity qualitative assay was performed using a nickel worm pick, as previously described (24), and the percentage of paralyzed worms in each group was recorded. All experimentation was performed in duplicate.

Statistical analysis

Statistical comparisons were performed on results of the heat shock assay using one-way ANOVA followed by Tukey's post hoc test, with a significance threshold set at $p<0.05$.

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