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Lycium extracts protect against β amyloid-induced pathological behaviors through UPR^{mt} in transgenic *Caenorhabditis elegans*

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Abstract

Lycium barbarum, a classic Chinese medicine, has a large variety of biological activities, including improvements in immunity, as well as anti-aging and anti-oxidation activities. It has been used to improve or restore deteriorating functions related to aging and diseases. Although its nerve protection effects also have been proved *in vitro* and *in vivo*, the molecular mechanism of action is not clear. Here, we report on the effect and possible mechanisms of *Lycium* extract-mediated protection of Aβ-induced paralysis in *Caenorhabditis elegans*. *Lycium* extracts effectively reduced Aβ accumulation and delayed Aβ-induced paralysis in a transgenic C. *elegans* (CL2006) model that expresses human Aβ1–42. By evaluating the expression of genes related to the proteostasis network, we found that the expression of UPR^{mt}, UPR^{ER} and autophagy-related genes was induced by *Lycium* extracts in CL2006 transgenic strains but not in the wild-type stains. Further RNAi experiments demonstrated that knock down of the UPR^{mt}-related genes could reduce levels of down-regulation induced by *Lycium* extracts, suggesting that UPR^{mt} is necessary for *Lycium* to prevent Aβ aggregation and maintain protein stabilization. Therefore, our studies provide more insights into the action and molecular mechanism of *Lycium barbarum* as a potential neuroprotective agent.

Abbreviations: Aβ: β Amyloid; **AD**: Alzheimer's disease; **LBP**: *Lycium Barbarum* Polysaccharide; **UPR^{mt}**: Mitochondrial Unfolded Protein Response; **UPR^{ER}**: Endoplasmic Reticulum Unfolded Protein Response; **PQ**: Paraquat; **TG**: Thapsigargin

INTRODUCTION

Lycium barbarum berries are a traditional Chinese herbal medicine. Many functional components in *L. barbarum* fruits, including flavonoids, carotenoids, polysaccharides, glycolipids, glycopeptides, anthocyanins, essential oils, organic acids and trace minerals, are responsible for many health-related activities of this plant. In addition to China, the medicinal value of *Lycium barbarum* berries has been widely recognized in many Asian and Arabian countries. For many years, Chinese and foreign scientists have carried out extensive research on the biological function of *Lycium barbarum* berries, mainly analyzing the effect of *Lycium barbarum* extracts in normal physiological conditions or disease models. They found that extracts from *Lycium* species possess a range of biological activities, such as nourishing the liver and kidney, improving eyesight, delaying aging, improving immunity, decreasing blood-glucose and blood-lipids, and acting as an anti-tumor and anti-fatigue factor [1]. Its nerve protection effects also have been proved *in vitro* and *in vivo*. For example, *Lycium barbarum* polysaccharide (LBP) can inhibit 6-hydroxy dopamine (6-OHDA)-induced apoptosis in PC12 cells [2], and the extracts of Lycium barbarum have a protective effect in Aβ1-42- and Aβ25-35-induced neuron injury [3,4]. In a whole animal model, LBP can protect middle cerebral artery occlusion (MCAO)-induced nerve injuries in mice [5,6] and improve the learning and memory abilities in scopolamine-induced brain damage in Sprague-Dawley (SD) rats [7].

Alzheimer's disease (AD) is widely recognized as a common and devastating neurodegenerative disorder characterized by progressive impairment in memory, cognitive function and personality [8,9]. As the most common form of irreversible dementia, AD has become one of the major diseases to harm the health of the aged population, and AD exerts a great influence on families and society [9-11].The formation of the intracellular neurofibrillary tangle (NFT) and extracellular plaques are

two major neuropathological features used for the diagnosis of AD [12]. AD is thought to be caused by the production and deposition of neurotoxic Aβ-peptide in the brain, leading to many consequences such as the formation of neurofibrillary tangles, oxidative stress, and neuronal cell death. Therefore, the focus of research in toxic Aβ production and clearance in the brain of AD patients is one approach for treatment of AD [13]. It has been reported the *Lycium* extracts can dramatically improve the Morris maze learning ability in the APP/PS1 double transgenic mouse model of Alzheimer's disease [14]. Elevated homocysteine levels in the serum will increase the risk of AD, and it is found that *Lycium barbarum* polysaccharides can also inhibit apoptosis in homocysteine-induced neuronal injury [15]. Although increasing data confirm that *Lycium barbarum* can be used to treat AD in animal models, the molecular mechanism is not clear and requires further study.

In the present study, we used the Aβ-expressing nematode model strain CL2006 to investigate the molecular mechanism of *Lycium* function. This transgenic nematode strain expresses the human 42 amino acid sequence of Aβ under the control of the muscle-specific unc-54 promoter/enhancer of *C. elegans* and responds to Aβ expression with increased paralysis [16]. It has been reported that neuromuscular synaptic transmission is specifically impaired by Aβ in this model [17]. Because of its short lifespan and its ease of culturing, the nematode is an advantageous animal model. Therefore, the strain CL2006 provides a good model for important insight into the mechanisms of Aβ toxicity.

Our studies demonstrated that the extracts of *Lycium* could protect the pathological behaviors in CL2006 transgenic worms by reducing the Aβ level. The *Lycium* extracts promoted Aβ degradation through UPR^{mt}.

MATERIALS AND METHODS

Caenorhabditis elegans strains and culture conditions

The *C. elegans* strains used in this study are Bristol N2, CL2006, *hsp6pr::gfp*, *hsp4pr::gfp* and *sqst-1::gfp*. The Bristol N2 strain was obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota, USA. The Cl2006 and *sqst-1::gfp* strains are gifts from Hong Zhang's lab at the Chinese Academy of Sciences. The *hsp4pr::gfp* [18] and *hsp6pr::gfp* [19] strains are gifts from Ying Liu's lab at Peking University with the permission of Professor Cole M. Haynes. All strains were maintained at 20°C on nematode growth medium (NGM) seeded with the *Escherichia coli* OP50 feeding strain.

Lycium extract preparation and treatment

Lycium barbarum berries were kindly provided by the Hospital of Traditional Chinese Medicine in Zhongning County, Yinchuan, Ningxia, China. The dried berries (100 g) were soaked in water (1 L) at room temperature after being washed five times. The soaked berries were decocted with neutral water (2 L) at a boiling temperature twice, and decocting times were 2.0 h and 1.5 h. The combined concentrated decoctions were filtered by a hollow fiber membrane. The above filtrates were merged and evaporated under a vacuum (1 KPa) at 45°C to remove water and obtain the concentrate. The constant volume of the resulting concentrate was 100 mL, and it was used for the following experiments and stored at -20 °C. The *Lycium* extracts include mainly water-soluble *Lycium barbarum* polysaccharides, flavonoids, carotenoids, anthocyanins, referenced the published papers which used the similar protocol to extract *Lycium barbarum* [20,21].

Lycium extracts were mixed into the OP50 bacteria according to an indicated dilution ratio. The transgenic worms were given the treatment from the L4 stage and the treatment was lasted for 5 days. In particular, to assay the function of *Lycium barbarum* extracts at different age stages, the worms were given *Lycium* at different stages.

Worm synchronization

Worm synchronization was implemented by alkaline hypochlorite treatment of gravid adults. Worms were first washed with M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 mL of 1 mol/L $MgSO_4$, in H_2O to 1 L) and pelleted by centrifugation (2000 g). Then, the worms were incubated in hypochlorite solution (1 mL of 2 N NaOH, 800 μL of sodium hypochlorite solution, 2.2 mL of dH2O) for 3-5 min to homogenize the large worm particles. Eggs were pelleted by centrifugation and washed at least three times with M9 buffer; then, they were incubated in M9 buffer and allowed to hatch overnight at 20°C. The synchronized L1-stage worms were put on standard NGM plates coated with OP50 at 20°C.

Paralysis assay

Transgenic *C. elegans* strain CL2006 was egg-synchronized and transferred onto the culture plates containing OP50 with or without *Lycium* extracts at the L4 stage. To identify paralysis, each worm was gently touched with a platinum loop. The worm was considered paralyzed if it did not move or moved only its head after being touched. The worms were tested for paralysis every day.

Body bends

The control or *Lycium* extract-treated adult worms were placed on unseeded NGM plates and allowed to acclimatize for 5–10 min. The number of body bends was counted for 20 s. A complete body bend was defined as the bending of the head region across the central-line of the animal [22].

Pumping assay

Pumping assays were operated on NGM plates with bacterial lawns and *Lycium*-bacteria mixed lawns at a 20°C temperature. To assay for pumping rate, we measured the time required to complete 20 pumps. Four to six measurements were recorded for every animal, and 16 animals were tested per experiment. Three independent experiments were performed.

Quantification of Aβ

The Aβ aggregation was determined using a thioflavin T (ThT) fluorescence assay. The nematodes were harvested with M9 buffer and washed three times, then treated with lysis buffer (HEPES 50 mM, NaCl 150 mM, EDTA 5 mM, DTT 2 mM) and repeatedly frozen and thawed to extract proteins. Subsequently, the concentration of extracted proteins was determined by performing a Bradford Protein Assay (CW biotech). Proteins were incubated at room temperature with ThT (final concentration: 20 μM; Sigma) in PBS buffer. The fluorescence intensity was measured using an excitation wavelength of 440 nm and an emission wavelength of 482 nm in an automatic microplate reader (Thermo).

Feeding RNA interference

For the feeding RNA interference experiment, the RNAi bacterial clones used were from previously published libraries [23]. Each RNAi colony was grown in LB medium with carbenicillin (50 μg/mL) overnight and then, 1 mmol/L isopropylthiogalactoside (IPTG) was added to induce dsRNA expression for 1 h. A volume of 200 μL of the bacterial was applied to a 60-mm plate, to which approximately 500 synchronized L1 larvae were added. Exceptionally, the RNAi interference of *lmp-2* was given to the worms at L4 stage, because interference at L1 stage will inhibit the growth of the worms. The *Lycium* extract treatment was given to the worms from the L4 stage.

Real-time qPCR

Total RNA was extracted using TRIzol reagent according to the manufacturer's (Invitrogen) protocol.RNA samples were then reverse transcribed using M-MuLV reverse transcriptase (Promega), and the mRNA levels were measured by RT-qPCR using a 7500 real time PCR system (Applied Biosystems), as described previously [24].

Fluorescence microscopy

The strains *hsp6pr::gfp, hsp4pr::gfp* and *sqst-1::gfp* were used to analyze the effect of *Lycium* extracts on UPR^{mt}, UPR^{ER} and autophagy by detecting the intracellular expression of *hsp6, hsp4* and *sqst-1* in living nematodes. Paraquat (PQ, 100μM) or thapsigargin (TG, 1μM) treatment was used as a positive control for UPR^{mt} and UPR^{ER}, respectively. After treatment with *Lycium* extracts for 24 h, approximately 20 worms were placed onto 3% agarose on a glass slide. Fluorescence images were taken using a confocal laserscanning microscope (LSM750) (Carl Zeiss).

The proteasome activity

For protein extraction, the worms were treated with lysis buffer (HEPES 50 mM, NaCl 150 mM, EDTA 5 mM, DTT2 mM); the concentration of proteins was determined by the Bradford Protein Assay (CW biotech). Quantification of proteasome activity was accomplished using a fluorogenic peptide substrate assay. The proteins were incubated with Suc-LLVY-AMC (final concentration100 μM; Sigma) in proteasome activity assay buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, and 5 mM ATP) at room temperature. The fluorescence intensity was measured in triplicate over 1h every 10 min with excitation at 355 nm and emission at 460 nm using an automatic microplate reader (Thermo).

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM) of at least 3 independent experiments. The statistical significance of the difference between two means was calculated using Student's t-test. For all analyses, $p<0.05$ was considered statistically significant.

RESULTS

Lycium extracts suppress the pathological behaviors in CL2006 by down-regulating Aβ

To investigate whether *Lycium* extracts specifically protect against Aβ-induced toxicity *in vivo*, the transgenic *C. elegans* strain CL2006 was used as an AD model. In this transgenic *C. elegans* model, human Aβ42 protein was expressed and aggregated in the body wall muscle, leading to progressive paralysis [25].The worms were treated with *Lycium* extracts at dilution ratios of 1:100, 1:50 and 1:20 for 5 days. *Lycium* extracts at dilution ratios of 1:50 and 1:20 significantly delayed Aβ-induced paralysis in this transgenic worm (Figure 1A). In particular, the rates of paralysis at Day 13 decreased by 20% at the 1:100 dilution and decreased by 30% at the 1:50 and 1:20 dilutions (Figure 1B). To further confirm the protective effects of *Lycium* extracts on Aβ-induced toxicity, the number of body bends over 20 s was counted in control and *Lycium* extract (1:50 dilution)-treated groups. *Lycium* extracts also significantly improved the frequency of body bends (Figure 1C). To exclude the effect of food preference of *C. elegans*, pumping rates of the worms fed with or without *Lycium* extracts were assessed, and there was no significant difference (Figure 1D). Therefore, Aβ-induced pathological behaviors in the transgenic *C. elegans* was alleviated by *Lycium* extracts.

Because the paralysis behavior of the transgenic stain CL2006 is the result of over-expression and aggregation of Aβ, to investigate how *Lycium* functions, we first checked the Aβ level in N2 and CL2006 nematodes with or without *Lycium* treatment, using EGCG as a positive control. EGCG, Epigallocatechin gallate, is a major component of green tea polyphenols. It was reported that EGCG could reduce beta amyloid (Abeta) deposits and inhibited Abeta oligomerization in transgenic *C. elegans* (CL2006) [26]. CL2006 showed a 6-fold increase in aggregated proteins versus N2 wild-type nematodes, and the amount of aggregated protein was dose-dependently reduced by *Lycium*, to a similar to that of the positive control EGCG (Figure 1E). Moreover, the level of Aβ was reduced by half at the 1:50 and 1:20 dilutions (Figure 1E), which was associated with a concomitant reduction of paralysis in the nematodes. Due to Aβ aggregation increasing with age, we also detected the different effects of *Lycium* at different age stages. At the 1:50 dilution, Aβ was reduced by half if *Lycium* was given at the L1 stage, and the reduction decreased gradually as the treatment time was delayed (Figure 1F). After Day6, *Lycium* treatment no longer had a significant effect (Figure 1F). In conclusion, *Lycium* extracts inhibited paralysis by reducing Aβ levels in CL2006. Given that enhanced aggregation of Aβ might

be the result of impaired quality control of protein homoeostasis [27], we focused on determining the mechanism by which *Lycium* extracts might induce some pathways for degrading damage proteins.

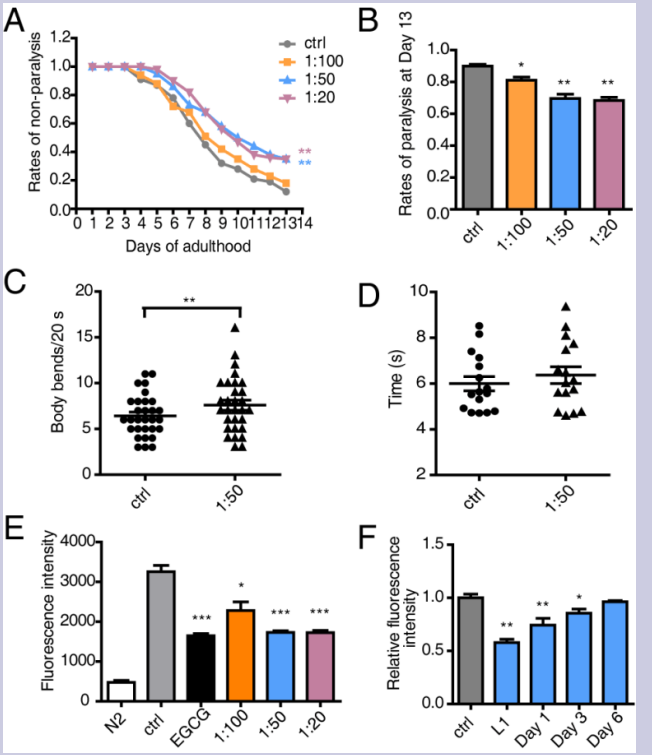

Figure 1. *Lycium* extracts protect against the Aβ-induced pathological behaviors in *C. elegans* strain CL2006. (A) Paralysis was analyzed in worms treated with *Lycium* at dilution ratios of 1:100, 1:50 and 1:20. (B) The rates of paralysis on Day 13are displayed separately. (C) The number of body bends over 20 s was counted. (D) Pumping rates of the worms were assessed. (E) The Aβ aggregation was determined using a thioflavin T fluorescence assay (EGCG was used as a positive control). (F) The different effects of *Lycium* on Aβ aggregation were measured at different stages of worms (L1, Day 1, Day 3 and Day 6). Error bars represent means ± SEMs (*P<0.05, **P<0.01, ***P<0.001).

Lycium extracts induce UPR^{mt}- and UPR^{ER}-related gene expression in CL2006

The maintenance of protein homeostasis is essential to preserve cell function. The major players in the maintenance of proteostasis include the mitochondrial unfolded protein response (UPR^{mt}), the endoplasmic reticulum unfolded protein response (UPR^{ER}) and two proteolytic systems, the ubiquitin-proteasome and the autophagy systems.

To investigate whether *Lycium* extracts can stimulate the UPR pathways, *hsp6pr::gfp* and *hsp4pr::gfp* transgenic strains were used to evaluate the UPR^{mt} and UPR^{ER}, respectively, because the chaperone homologs HSP6 and HSP4 are considered markers of UPRmt and UPRER, respectively. Compared to the positive control paraquat (PQ) that could induce UPR^{mt} significantly, no GFP expression could be induced in the *Lycium* extract-treated *hsp6pr::gfp* nematodes (Figure 2A), while in the *hsp4pr::gfp* nematodes, *Lycium* extracts could also not induce the UPR^{ER}, in which thapsigargin (TG), a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, was used as a positive control (Figure 2B). These data suggested that *Lycium* extracts did not cause stress leading to the accumulation of unfolded proteins in the normal nematodes without pathological behaviors. To further study whether *Lycium* extracts have functions in the nematode disease model, the expression of UPR-related genes was detected in CL2006 in the presence and absence of *Lycium* extracts. *dve-1*, encoding a transcription factor that binds to the *hsp-6* and *hsp-60* promoters upon mitochondrial stress, was significantly increased by *Lycium* extracts at the 1:20 dilution (Figure 2C). UBL-5, a ubiquitin-like protein homolog, is essential for UPR^{mt}, with increased nuclear levels following induction of the UPR^{mt} to help promote the interaction between DVE-1 and the DNA. The mRNA level of *ubl-5*was up-regulated by *Lycium* extracts in a dose-dependent manner (Figure 2D). To strengthen the argument of the specific role of UPR^{mt} induction related to the antagonistic effect of *Lycium* extracts in *C. elegans*, the expression of some other markers of UPR^{mt}, hsp60, clpp and haf-1 was also detected after *Lycium* extracts (1:50) treatment.

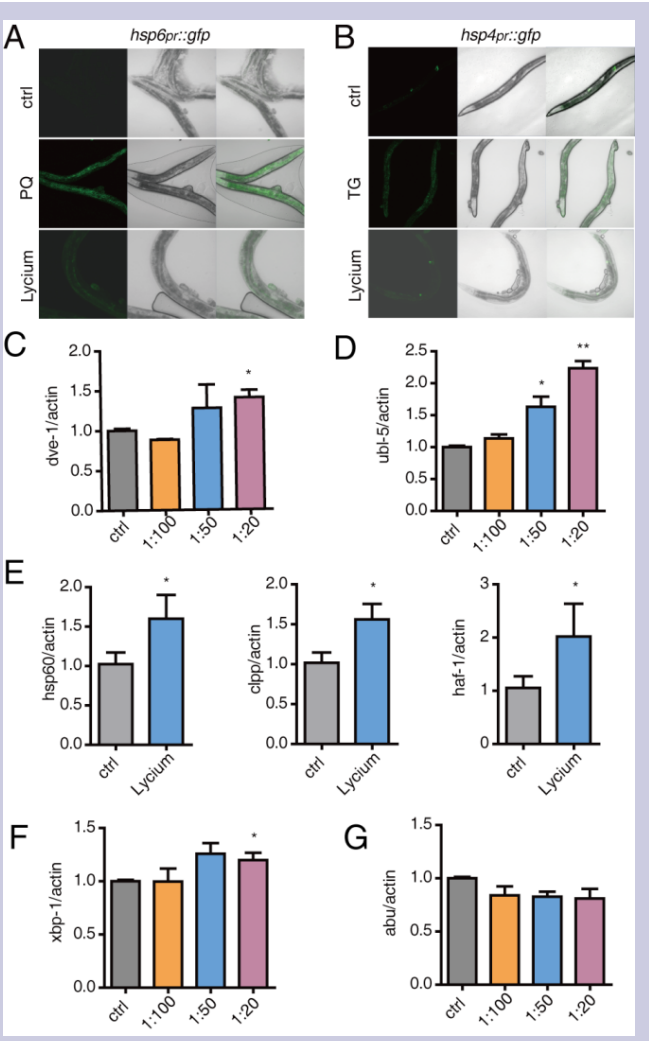

Figure 2. *Lycium* extracts induce UPR^{mt}- and UPR^{ER}-related gene expression in CL2006 strain. (A, B) *hsp6pr::gfp* and *hsp4pr::gfp* transgenic strains were used to evaluate the effect of *Lycium* on UPR^{mt} and UPR^{ER,} with PQ or TG as a positive control, respectively. (C, D) The effect of different dosages of *Lycium* on the expression of*dve-1* and *ubl-5* in CL2006 was detected by qRT-PCR. (E) Expression of hsp60, clpp and haf-1 in CL2006 worms treated with *Lycium* extracts (1:50) was also detected by qRT-PCR. (F, G) The effect of different dosages of *Lycium* on the expression of *xbp-1*and *abu-1* in CL2006 was detected by qRT-PCR. Error bars represent means ± SEMs (*P<0.05, **P<0.01, ***P<0.001).

The data suggested that *Lycium barbarum* extracts could induce hsp60, clpp and haf-1 expression in CL2006 (Figure 2E), providing further evidence that UPR^{mt} is involved. xbp-1, encoding a transcription factor involved in ER stress, was also increased after *Lycium* extracts treatment (Figure 2F). abu-1 is a gene activated when UPR^{ER} is blocked to compensate for ER stress, so it was down-regulated by *Lycium* extracts (Figure 2G). In conclusion, *Lycium* extracts increase UPR*mt*- and UPR^{ER}-related gene expression in the transgenic stain CL2006. It is possible that *Lycium* extracts reduce the Aβ level through these pathways.

Lycium extracts up-regulate autophagy-related genes but have no effect on proteasome activity in CL2006 strain

Autophagy and the ubiquitin-proteasome are two major proteolytic systems responsible for cytosolic protein degradation. To monitor whether autophagy is involved in *Lycium* extract-induced Aβ down-regulation, the *sqst-1::gfp* transgenic strain was employed. Sqst-1 (the homolog of human p62) is a substrate that is degraded during autophagy. GFP was highly expressed in the control nematodes and dramatically degraded by PQ stress-induced autophagy (Figure 3A, 3B). The high level of GFP expressed in the *Lycium* extract-treated nematodes suggested that autophagy was not induced by *Lycium* extracts in this strain without pathological behavior (Figure 3B). Similarly, the expression of *bec-1*, which is indispensable for the formation of autophagosomes, was detected in CL2006 with or without *Lycium* extract treatment. The dose-dependent up-regulation of *bec-1* expression suggested that *Lycium* extracts might also activate the autophagy pathway in CL2006 (Figure 3C). In order to investigate whether mitophagy also occurs, two mitophagy markers, *pink-1* and *pdr-1*, were measured (Figure 3D). We found that *Lycium* extracts could not up-regulate the expression of these two genes. On the other side, *Lycium* extracts did not change the level of *uba-1*, which is the ubiquitin-activating enzyme in *C. elegans* (Figure 3E). Further measuring the proteasome activity in CL2006, we found that there was no significant difference after *Lycium* extract treatment (Figure 3F). Therefore, autophagy rather than the proteasome may be involved in *Lycium* extract-mediated Aβ down-regulation.

Lycium extracts reduce Aβ aggregation through UPR^{mt}

Given the induction of UPR^{mt}, UPR^{ER} and autophagy in CL2006 nematodes treated with *Lycium* extracts, to determine their function in the prevention of Aβ aggregation, RNA-interference (RNAi) was used, and the knockdown efficiency for each gene was detected (Figure 4A). In the vector control (L4440), *Lycium* extracts could reduce the Aβ level by 60% compared to the control (Figure 4B). When *dve-1* and *atfs-1*, the key transcriptional factors in UPR^{mt}, were knocked down by RNAi, the Aβ level could not be reduced by *Lycium* extracts (Figure 4B). When *hsp6* and *hsp60* were knocked down by RNAi, although *Lycium* extracts could slightly reduce the Aβ level, there was no significant difference. These results indicate that UPR^{mt} is necessary to prevent Aβ aggregation and maintain protein stabilization. To estimate the influence of UPR^{ER} on Aβ-induced toxicity, *xbp-1* was knocked down, and in this condition, Aβ levels could still be decreased by 50% by *Lycium* extracts. Thus, RNAi for *xbp-1* did not prevent the reduction of Aβ aggregation by *Lycium* extracts. Similar to the knockdown of *xbp-1* for UPR^{ER}, RNAi for *bec-1* also did not prevent the decrease in the Aβ level by the *Lycium* extracts. To further confirm this result, *lmp-2*, encoding a lysosomal receptor homolog, was also knocked down. Aconsistent result was obtained, suggesting that the autophagy pathway is not a target of the *Lycium* extracts (Figure 4B). In conclusion, only UPRmt is involved in *Lycium* extract-induced Aβ down-regulation.

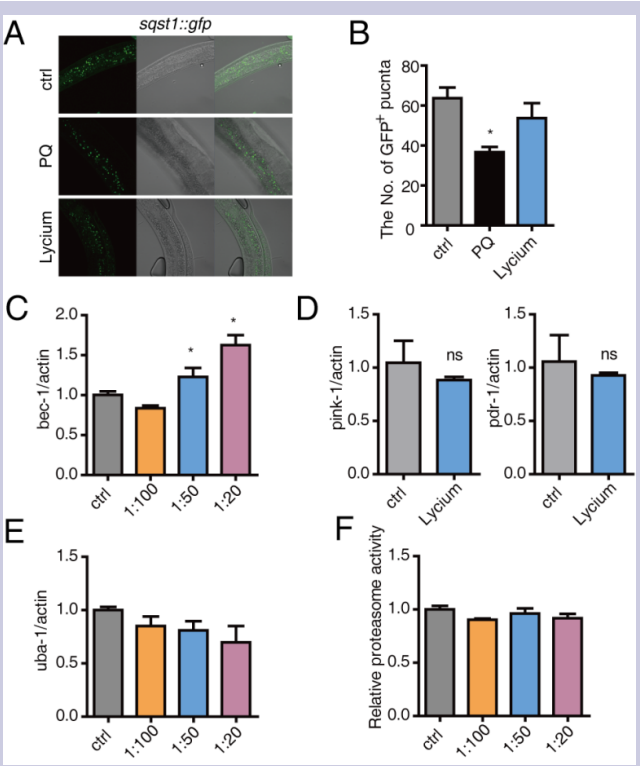

Figure 3. *Lycium* extracts up-regulate autophagy-related genes but have no effect on proteasome activity in CL2006 strain. (A) The *sqst-1::gfp* transgenic strain was used to evaluate the effect of *Lycium* on autophagy, with PQ as a positive control. (B) The number of the GFP+ puncta was counted to estimate the sqst-1 expression level. (C) The effect of different dosages of *Lycium* on the expression of*bec-1* in CL2006 was detected by qRT-PCR. (D) The expression of pink-1 and pdr-1 in CL2006 treated with or without *Lycium* was detected by qRT-PCR. (E) The effect of different dosages of *Lycium* on the expression of*uba-1* in CL2006 was detected by qRT-PCR. (F) The proteasome activity in CL2006 treated with different dosages of *Lycium* was measured using a fluorogenic peptidesubstrate assay. Error bars represent means ± SEMs (*P<0.05, **P<0.01, ***P<0.001).

DISCUSSION

In this study, we used the transgenic strain CL2006 to monitor the function of *Lycium barbarum* in AD. We found that the amount of aggregated Aβ and paralysis were dose-dependently reduced by the *Lycium* extracts. To investigate whether *Lycium* extracts impact the proteostasis network, UPR^{mt}, UPR^{ER}, autophagy and the proteasome system were evaluated in the wild-type and CL2006 transgenic strains treated with or without *Lycium* extracts. The expression of UPR^{mt}, UPR^{ER} and autophagy-related genes was induced by *Lycium* extracts in the CL2006 transgenic strains but not in the wild-type stains. Furthermore, to identify which member of the proteostasis network is required for the *Lycium* extract-induced Aβ down-regulation, RNAi was exploited. Knock down of UPR^{mt}-related genes could prevent *Lycium* extract-

induced Aβ down-regulation, suggesting that UPR^{mt} is necessary to prevent Aβ aggregation and maintain protein stabilization.

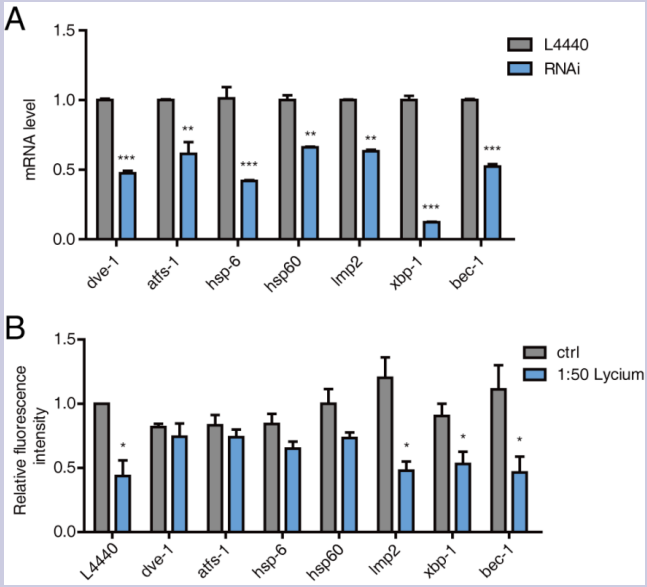

Figure 4. *Lycium* extracts reduce Aβ aggregation through UPR^{mt}. (A) The knockdown efficiency for each gene was checked by qRT-PCR. (B) Aβ aggregation levels were assessed in the conditions where UPR^{mt}, UPR^{ER} and autophagy-related gene were knocked down by RNAi. Error bars represent means ± SEMs (*P<0.05, **P<0.01, ***P<0.001).

It is very interesting that *Lycium* extracts could induce UPR^{mt}, UPR^{ER} and autophagy in the CL2006 transgenic strains that have aggregated Aβ but that do not exert effects in the wild-type strains. CL2006 exhibited a 6-fold increase in aggregated proteins compared with N2 wild-type nematodes; therefore, *Lycium* extracts may function by enhancing the aggregated protein-mediated activation of UPR^{mt}, UPR^{ER} or autophagy but without inducing these processes directly. Thus, *Lycium* extracts have no influence on proteostasis under normal physiological conditions. Although the genes related to UPR^{mt}, UPR^{ER} and autophagy were increased by *Lycium* extracts in CL2006, only UPR^{mt} is required for *Lycium* extract-induced Aβ down-regulation according to the RNAi experiments. Therefore, UPR^{ER} and autophagy are only concomitant results, not causes. UPR^{mt}, which functions through the sensing of mitochondrial stress to coordinate the appropriate response, plays a significant role in *Lycium*-mediated proteostasis maintenance. It is known that UPR^{mt} decreases with age [28], which is consistent with our result that *Lycium* extracts have more significant effects at the early stage in CL2006. After Day6, *Lycium* treatment no longer had a significant effect, which may be due to decreased UPR^{mt}.

According to the published paper, *Lycium* extracts have high anti-oxidative activity, and we also found that *Lycium* extracts could reduce the ROS level in both wild-type and transgenic strains (Data is not shown). So was it possible that the restorative effect of the extracts was due to anti-oxidative property? If Aβ first disrupted the redox balance in the mitochondria that in turn affected UPR^{mt} induction and *Lycium* extracts function through their anti-oxidative property, the result would be decreased ROS level and reduced UPR^{mt} after *Lycium* extract treatment. However, in our results, *Lycium* extracts significantly increased UPR^{mt}, and induced UPR^{mt} is necessary for the restorative effect of the extracts. Therefore, the anti-oxidative ability of the extracts was not necessary for decreased Aβ aggregation. This new mechanism is very important for explaining the function of *Lycium barbarum* as a potential neuroprotective agent.

CONCLUSION

Our studies provide evidence that *Lycium* extracts reduce Aβ-induced toxicity and protect from pathological behavior in *C. elegans* through regulating UPR^{mt}, and this effect is more significant at an early stage. Although the protective effect of *Lycium* on Aβ-induced cytotoxicity has been reported *in vitro* [3,4], the mechanism has not been explained. Since intracellular Aβ is cytotoxic and an early causative event in the development of AD, inhibition of Aβ aggregation is one approach for treatment of AD. Moreover, many studies indicate that diverse neurodegenerative diseases might have a common cause and pathological mechanism - the misfolding and aggregation of proteins in the brain, resulting in neuronal apoptosis. Impaired proteostasis is one of the main features of all amyloid diseases. *Lycium* extract-induced Aβ down-regulation is mediated by UPR^{mt}, which plays an important role in the proteostasis network. Thus, our studies provide more insights into the action of *Lycium barbarum* as a potential neuroprotective agent.

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DISCLOSURE

Jiao Meng, Zhenyu Lv, Xiaopeng Li, Chuanxin Sun, Zhengguo Jiang, Wanchang Zhang and Chang Chen declare that they have no conflict of interest.

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