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Original Article

3β-Hydroxy-urs-12-en-28-oic Acid Modulates Dietary Restriction Mediated Longevity and Ameliorates Toxic Protein Aggregation in *C. elegans*

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Abstract

Species from lower invertebrates to a spectrum of mammals show antiaging health benefits of phytochemical(s). Here, we explored the pro-longevity effects of a natural triterpenoid, ursolic acid (3β-hydroxy-urs-12-en-28-oic acid; UA) in *Caenorhabditis elegans* with maximal life span being evident at 25 μM UA. Similar to *eat-2* mutants, UA uptake by worm results in reduced fat storage and attenuation of reactive oxygen species (ROS), independent of superoxide dismutase(s) activation. The genetic requirements for UA-mediated longevity are quite similar to dietary restriction (DR) achieved through SKN-1/NRF-2 exhibiting upregulation of downstream target genes *gcs-1* and *daf-9*. Longevity mechanism was independent of PHA-4/FOXA and attributed to partial dependence on *sir-2.1*. Altogether, our study suggests differential use of UA-elicited signaling cascades in nutrient sensing for longevity. Both the redox state and the proteostasis of an organism play critical role in aging and disease resistance. Interestingly, we observed a reduction of toxic protein aggregation in transgenic polyglutamine (polyQ) *C. elegans* model and UA-mediated JNK-1 (c-Jun-NH2-terminal kinase) activation in wild-type animals. Thus, our study demonstrates a small extent of prevention against proteotoxic stress by UA coupled with positive aspects of DR-mediated longevity.

Keywords: Healthy aging, Ursolic acid, Polyglutamine, ROS

Aging is marked by physical decline hence affected by the nutritional state of an organism and its ability to restore redox imbalance. Consistent evidence for the pro-longevity effects of phytochemical(s) has been shown to delay age-linked maladies in various model systems tested with safer toxicity profile (1). So far, diverse pharmacological interventions have been documented to modulate gerontogene(s) taking part in the biological course of aging such as pharmacological suppression of the GH/IGF-1 axis and TOR-S6K pathway, restrictive protein intake, activators of conserved protein deacetylase SIR2, and the use of spermidine, metformin, and other epigenetic modulators (2).

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid; UA) is a pentacyclic ursane type of triterpene usually present in the stem bark, leaves, or fruit peel of plants in the form of free acid or aglycones. It exhibits a wide range of pharmaceutical properties, including anti-inflammatory, hepatoprotection, antitumor, anti-HIV, antiangiogenic, and antibacterial activity (3). In our earlier studies, we have identified UA as an antiaging phytochemical, with enhanced health parameters in *C. elegans* (4). UA has also proved to have broad-spectrum antitumor effects tested in various cancer models (5–7), with a lead in preclinical and clinical studies where liposomal ursolic acid was used to determine the toxicity and pharmacokinetics in healthy adult human volunteers and in patients with advanced solid tumors (8, 9). Moreover, a unified model integrating cancer to aging has been documented in several studies (10,11). This study sought to understand healthy aging; therefore, UA was used to delineate the molecular profile for longevity as healthy aging, prevents many age-associated malignancies and cancer is one of the major risk factors for aging.

Aging studies in mammals are challenging owing to relatively long life span and a limited set of genetic tools that are capable of assessing the genome-wide changes associated with disease. By contrast, *C. elegans* has a shorter life span of only 2 to 3weeks, easily propagated in culture, facilitating rapid genetic analyses. Genetic

resources for *C. elegans* include hundreds of publicly available mutant strains; whole-genome RNA interference (RNAi) libraries etc. Interestingly, there are substantially more protein similarities between *C. elegans* and *Homo sapiens* than in any other cross-species comparison.

Since aging and disease shoot from common mechanisms (12), delaying disease by delaying aging is the rationale behind the ongoing research. To this end, we used transgenic polyglutamine (polyQ) *C. elegans* model typically associated with Huntington's disease (HD). The mutation prone for HD has been recognized as a CAG expansion located at the 5' end of the gene *htt*, translated into a polyQ stretch at protein level (13). Opportunities remain in understanding how therapeutic potential of phytochemical(s) accomplish healthy aging with disease resistance. Henceforth, this study has been conducted taking the safer toxicity profile of UA, exploring its role in nutrient-response pathways and implication in age-associated pathologies.

Results and Discussion

UA Considerably Affects Life span and Health Span

We observed a significant increase ~31.3% in the mean life span of wild-type (WT) N2 worms treated with UA [MLS ± SEM on 0 µM UA is 17.74 ± 0.01 days, while on 25 µM UA is 23.3 ± 0.03 days, $p < .0001$; Figure 1A, Supplementary Table 1]. UA has poor water solubility. As we move from concentration 25 µM to 50 µM and above, the solution was not homogenous showing cloudiness hence poor bioavailability. There might be chances that it could not be assimilated by *C. elegans*. Therefore, we observed normal life span at 50 µM and 100 µM UA. We did not observe any undesirable effects on all life-span endpoint parameters for these concentration(s). UA treatment also enhanced health parameters; it lowered as well as delayed accumulation of age pigment lipofuscin to a significant extent (47.07%, $p = .0018$; Figure 1C). We also observed a noteworthy protection against heat shock (31.1%, $p = .0006$; Supplementary Figure 1A), healthier chemotaxis index and mobility impairment (Supplementary Figure 2), when compared to control worms. Taken together, UA positively influences life span with health assurance in *C. elegans*.

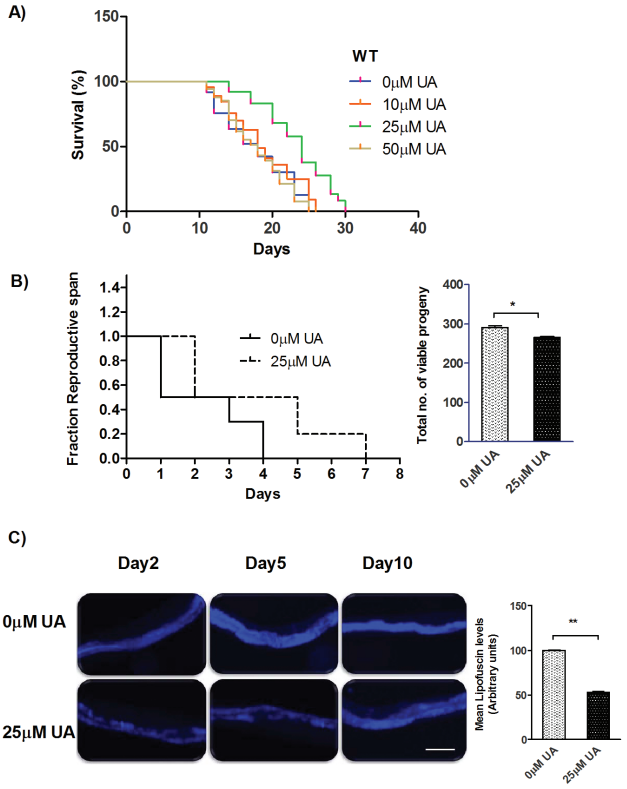

Figure 1. Life span of wild-type (WT) *C. elegans* strain exposed to various conc. of UA. The 25 µM test concentration showed the most significant increase in life span (31.3%, $p < .001$). DMSO (0.05%) was taken as control (0 µM UA) for all the experiments. Life span assay was performed at 20°C. Survival plots were drawn by Kaplan–Meier survival assay. (**B**) UA treatment resulted in longer reproductive span (MRS) on control is 2.3 ± 0.44 days on 25 µM UA is 3.9 ± 0.67 days; $p = .0247$ with small brood size (inset; brood size for 0 µM UA is 290.5 ± 2.5 while for 25 µM UA is 266.0 ± 2.0; $p = 0.0381$) (**C**)The lower and delayed accumulation of lipofuscin in the intestine indicates better health span; scale bars, 100 µm. Right panel represents quantification of the mean fluorescence (47.07%, $p = 0.0018$ in adult day 5 animals) using imageJ.

UA Works Independently of Insulin/IGF-1 (Insulin-Like Growth Factor 1) Signaling

To elucidate the mechanistic basis for UA-dependent life-span extension, we tested some of the pathway mutant(s) starting from the canonical GH/IGF-1 axis. In this pathway, a mutation in *daf-2* [insulin/IGF-1 (insulin-like growth factor 1) signaling (IIS) receptor] results in life-span extension under the control of conserved FOXO transcription factor DAF-16 and heat shock transcription factor (HSF)-1 (14). When *daf-2 (e1370)* worms were supplemented with UA, we observed a significant life-span extension compared to control worms [MLS ± SEM on 0 µM UA is 25 ± 0.11 days, while on 25 µM UA is 32.35 ± 0.09 days; $p < .0001$; Supplementary Figure 3A, Supplementary Table 1] suggesting that UA may act through a parallel pathway. In addition, the UA-mediated life-span extension requires neither *daf-16* nor *hsf-1* [MLS ± SEM of *daf-16* (mgDf50) on 0 µM UA is 12.77 ± 0.11 days, on 25 µM UA is 16.35 ± 0.07 days, $p < .0001$; Supplementary Figure 3B]. *hsf-1(sy441)* mutants have an impaired protein-folding capacity and we investigated whether UA could rescue the short life span by reducing proteotoxic stress. UA failed to extend life span of *hsf-1(sy441)* mutant worms compared to control worms at 20°C [*hsf-1(sy441)* on 0 µM UA is 12.20 ± 0.37 days and on 25 µM UA is 12.57 ± 0.56 days, $p = .066$; Supplementary Figure 3C, Supplementary Table 1]. Higher temperature is unfavorable for *hsf-1(sy441)* so, we further tested its life span at 15°C to support our hypothesis. We observed an extended life span of UA-treated worms over control worms [*hsf-1(sy441)* on 0 µM UA is 14.78 ± 0.42 days and on 25 µM UA is 20.27 ± 0.21 days, $p < .0001$; Supplementary Figure 3D, Supplementary Table 1]. Altogether, this genetic data show that UA influence longevity independent of the IIS pathway possibly using a different mechanism.

UA Supplementation Mimics a dietary restriction-Like State

Since UA acts independently of the IIS pathway for longevity, we next examined its role in other longevity pathways. The *eat-2* mutants have been used as a genetic surrogate of the dietary restriction (DR); characterized by long life span and reduced fat storage (15,16). Accordingly, we quantified the levels of stored fat in UA-supplemented worms using Oil Red O (17). We observed significantly reduced fat storage in the intestine (31.52%, $p = .0006$; Figure 2A) compared to control worms. Consequently, we assessed genetic interactions of UA with *eat-2*. We found that UA failed to increase life span of

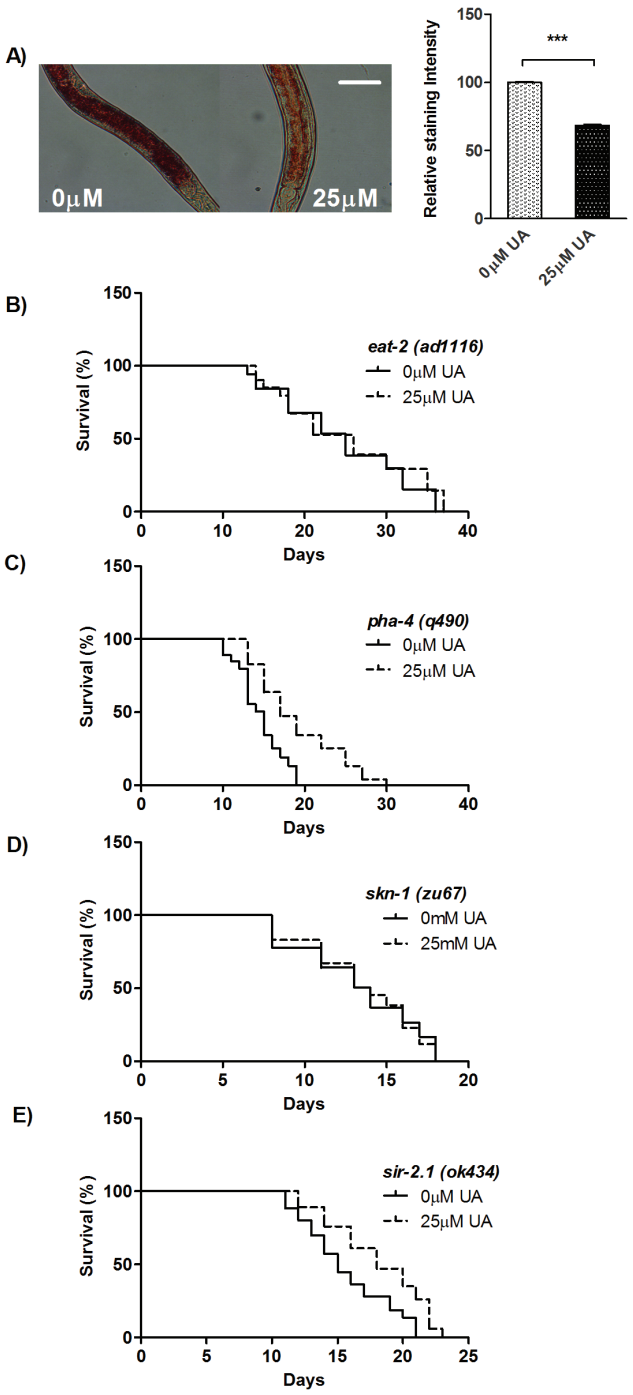

Figure 2. UA regulates longevity in response to dietary restriction. **A**) Oil Red staining of fixed WT worms grown on Control (0 µM UA) or 25 µM UA shows significant decrease in fat storage by UA (31.52%, $p = .0006$), right panel represents quantification of staining. **B**) UA failed to extend life span *eat-2 (ad1116)* to a significant extent whereas it increased the life span of **C**) *pha-4 (q490),* suggesting no role for it. It also failed to increase life span of **D**) *skn-1(zu67),* to a significant extent. **E**) UA-mediated longevity was attributed to partial dependence on *sir-2.1(ok434).* Life-span assay were performed at 20°C. ***$p < .0001$.

eat-2 (ad1116), compared to the control [MLS ± SEM on 0 µM UA is 24.80 ± 0.17 days, while on 25 µM UA is 25.41 ± 0.06 days; $p = 0.119$; Figure 2B, Supplementary Table 1], indicating that they may function in similar way for life-span modulation. Quite similar to *eat-2* mutants (18,19), UA-treated worms have small brood size [inset; brood size on 0 µM UA is 290.5 ± 2.5, while on 25 µM UA is 266.0 ± 2.0, $p = .0381$] and longer reproductive span (mean reproductive span on 0 µM UA is 2.3 ± 0.44 days, while on 25 µM UA is 3.9 ± 0.67 days; $p = .0247$; Figure 1B) compared to control worms. One of the characteristics of DR is low pharyngeal pumping rate and small body size compared to well-fed animals (15). Interestingly, we found UA-fed worms were having regular body size and feeding rate (Supplementary Figures 4A and B).

FOXA transcription factor, PHA-4 is specifically required for genetic and nongenetic models of DR (20) for life-span extension and it does not have any requirement in the longevity governed by IIS pathway. Subsequently, next, we asked whether UA-mediated longevity mechanism requires *pha-4*. We evaluated whether UA supplementation can increase life span in the absence of *pha-4*. Under this condition, UA significantly increased the life span [MLS ± SEM on 0 µM UA is 14.51 ± 0.23 days, while on 25 µM UA is 19.05 ± 0.08 days; $p < .0001$; Figure 2C, Supplementary Table 1]. Hence, unlike *eat-2* mutant UA does not require PHA-4 for longevity. The stress-protective transcription factor SKN-1/NRF-2 regulate DR-induced longevity in the ASI neurons, whereas, in the intestine, it acts downstream of the IIS pathway to modulate oxidative stress tolerance (21–23). We asked whether UA-mediated longevity requires SKN-1. In UA-supplemented plate, it failed to increase the life span [MLS ± SEM on 0 µM UA is 13.29 ± 0.03 days, while on 25 µM UA is 13.49 ± 0.13 days; $p = .77$; Figure 2D, Supplementary Table 1] to a significant extent over control worms. Thus, UA-mediated longevity depends on *skn-1*, in a way similar to *eat-2* mutant (23). Altogether, multiple lines of evidence suggest that UA supplementation extends longevity and initiate a DR-like state.

DR may have cross-talk with other genetic pathways (24). Therefore, we further asked if *sir-2.1* (encodes a protein deacetylase of Sirtuin family) has a role in the pathway underlying the effect of UA. The role of *sir-2.1* in longevity course has been fiercely debated; in some of the studies, it is shown to have life-span–promoting effects (25,26) but not in others (27). We assessed life span of null mutant *sir-2.1(ok434)* and observed significant increase over control worms [MLS ± SEM on 0 µM UA is 15.65 ± 0.30 days, while on 25 µM UA is 18.13 ± 0.41 days; $p < .0001$; Figure 2E, Supplementary Table 1] but not to the same extent as WT control worms, symptomatic of partial dependent on *sir-2.1*. Altogether, our findings show different ways of life span extension by UA acting either independently or in nonlinear genetic pathways in congruence with the earlier reports (28).

UA Regulates Expression of a Subset of SKN-1 Target Genes

Genes downstream of SKN-1 coordinately affect the rate of aging in all the cells in the body. In the qRT-PCR expression analysis (Supplementary Figure 5), we observed a significant upregulation of *gcs-1* (1.52-fold, $p = .02$) for conferring oxidative stress resistance along with the upregulation of *daf-9* (encodes for cytochrome P450; 1.36-fold, $p = .049$) involved in xenobiotic detoxification. In addition to this, we also observed increase in mRNA transcript levels of *sir-2.1* (1.34-fold, $p = .043$) confirming its positive role in pro-longevity.

UA Directly Mitigates Reactive Oxygen Species and Depends on the Activity of Mitochondrial Complex II

The most probable underlying cause of aging is damage to cellular macromolecules rendered by potent oxidizing agents (29,30).

Reduced reactive oxygen species (ROS) levels have always been a remarkable feature of DR. In line with this supposition, we quantified the total intracellular ROS in UA-supplemented worms and found significantly lower ROS levels over control worms (51.15%, $p \leq 0.001$; Supplementary Figure 6A). This was supported by gene expression profiling of UA-fed worms in qRT–PCR analysis as we observed no significant upregulation in superoxide dismutase(s) genes (Supplementary Figure 5). Also, in the biochemical analysis, the total cellular superoxide dismutase activity along with core antioxidant enzymes glutathione (GST) and catalase (CAT) activities remained unchanged (Supplementary Figure 7). We propose that antioxidant potential of UA directly mitigates oxidative damage, thus reducing the need for the activation of intrinsic antioxidant defense. This reduced expression of key antioxidants in UA-mediated longevity, suggests evolutionarily conserved effects of DR. Also, in UA-fed worms, we observed greater stress resistance against paraquat that induces severe oxidative stress (18.33%, $p = .003$; Supplementary Figure 1B). To correlate these phenotypic manifestations of redox status with molecular response, we assessed life span of short-lived *mev-1* mutants in which a mutation in cytochrome b of the mitochondrial Complex II results in overproduction of superoxide and increased oxidative stress. UA failed to rescue the short life span of these animals significantly [MLS ± SEM on 0 µM UA is 10.87 ± 0.18 days, while on 25 µM UA is 10.46 ± 0.16 days; $p = .327$; Supplementary Figure 6B, Supplementary Table 1]. This genetic analysis suggests that UA reduces oxidative stress and requires an endogenous detoxification pathway to support life-span extension. However, recent human studies have documented that antioxidant supplementation failed to show any betterment in health span; provoking debates on the oxidative stress theory of aging (31). Moreover, other relevant evidence have reported a beneficial role for ROS in life span under stress conditions which in turn, induce protective responses that slow aging (32–37).

We cannot purge all stress. And in fact, that is not desirable because with moderate stress comes survival behavior, motivation, and positive striving dealing with various aspects of aging on a population level (33). However, it cannot be ruled out that specific targets (proteins, lipids, or nucleic acids) accumulate damage over time and accelerate aging rate. Therefore, the stringent molecular details are required to regulate ROS and validate the latest aging theories postulating a linkage between ROS and aging. To this end, we have further measured toxic protein aggregation (a new aging biomarker) at organismal level in transgenic polyglutamine (polyQ) *C. elegans* model for HD. The preclinical and clinical studies have indicated a link between oxidative stress and toxic protein aggregation. The use of antioxidants including essential fatty acids, coenzyme Q10, and creatine, has been reported as potential therapeutic strategies for such detrimental effects (38–41).

UA Modulates JNK-1 to Prevent Polyglutamine Protein Aggregation

DR has the remarkable ability to protect against multiple diseases including diabetes, cancer, cardiovascular, and neurodegenerative disorders (42). One of the hallmarks of aging is the gradual loss of proteostasis over time, and multiple neurological disorders exhibit symptoms of impaired proteostasis. In a study, it has been documented that aging process itself, in the absence of disease, leads to the insolubilization and increased aggregation of toxic proteins (43). In addition, it was reported that higher levels of inherent protein aggregation aggravated toxicity in a *C. elegans* HD model and genetic pathway regulating longevity can alter the time course of age-associated polyglutamine-mediated phenotypes (44). Taking UA as a lead antiaging phytochemical, we next evaluated whether UA improves protein homeostasis. We quantified aging-induced polyQ protein aggregation in unc-54p:Q40::YFP transgenic worms (45). These transgenic animals show a soluble Q40::YFP distribution in body wall muscle cells immediately after hatching and reach adulthood with an entirely Q40::YFP-aggregated phenotype. We observed that the UA-fed Day 1 Q40::YFP animals had approximately less than half as many aggregates as control animals (No. of aggregates in control = 17.5 ± 0.5 and in 25 µM UA is 9.5 ± 0.5; $p = .0035$; Figure 3A). Although the average number of aggregates increased with age, the UA treatment had no effect on Q40::YFP aggregation in Day 5 and Day 7 adults in which number of aggregates were increased along with diffused fluorescence for polyQ foci. This result suggests that UA treatment prevents the aggregation at the young adult stage and preserves the impaired proteostasis.

To further strengthen our hypothesis, the α-synuclein model for Parkinsons' disease was examined employing transgenic *C. elegans* strain NL5901 strain, constitutively expressing YFP-fused human α-syn protein in the body wall. As it is well-established that the over-expression of α-synuclein correlates with increased ROS levels and UA mitigates ROS levels, it might also be able to reduce the toxicity of α-synuclein (46). The α-syn aggregation in the head area was comparable between the tested treatment groups. A significant reduction in the accumulation of α-syn in UA-fed Day 1 worms was observed by up to 36.52%; $p < .0001$; Supplementary Figure 8. The UA treatment had no effect on α-syn levels in Day 5 and Day 7 adult animals, suggesting efficacy of UA treatment at early stages of age-associated neurological disorder(s).

Research conducted on the cellular and animal models of HD have shown that expanded polyQ proteins induce a stress response associated with the c-Jun-NH2-terminal kinase (JNK) pathway (47). In order to assess the importance of this pathway in UA-mediated longevity, we examined life span of the mutants for JNK pathway, including, *jnk-1*/JNK and upstream kinase *jkk-1* (48). In both

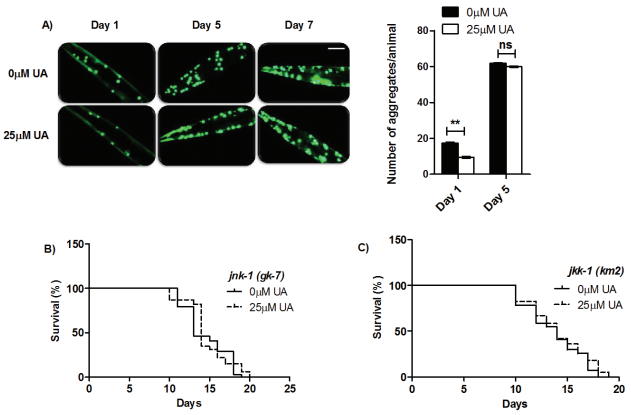

Figure 3. UA reduces toxic protein aggregates in *C. elegans* transgenic polyQ model. **A**) Fluorescence micrographs of transgenic animals expressing Q40::YFP in body wall muscle cells with or without UA treatment. Scale bars, 100 µm. Right panel shows quantification of Q40::YFP aggregates. Results are expressed as mean + SEM numbers of aggregates/animal from three biological repeats. **B**) UA failed to extend life span of JNK-1 deletion mutant *jnk-1(gk7)* and **B**) JNK-1 upstream kinase JKK-1 deletion mutant *jkk-1(km2)*, to a significant extent, suggesting a positive interaction with UA.

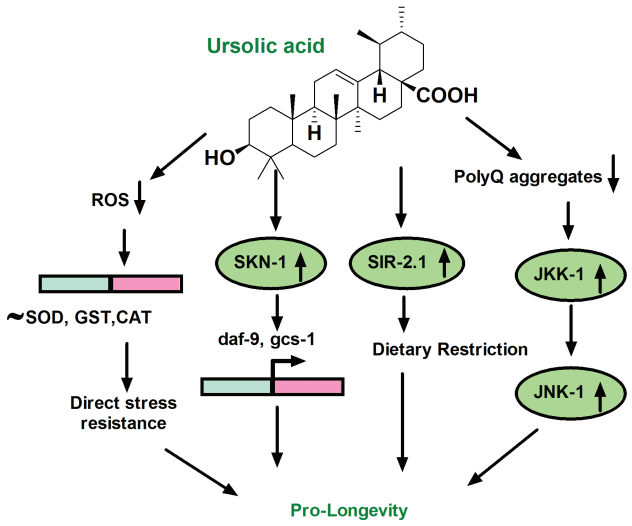

Figure 4. UA in dietary restriction (DR) mediated longevity. Owing to its own antioxidant potential UA attenuates ROS directly independent of intrinsic antioxidant enzymatic system. As observed in DR worms, SKN-1 and *eat-2* mediating longevity in partial dependence of *sir-2.1*. Moreover, this natural triterpenoid restores proteostasis in transgenic *C. elegans* polyQ model and mediates JNK-1 activation in wild-type N2 worms. It makes UA a much favored life-span–extending intervention which may have substantial implications in aging and associated pathologies.

the mutants, UA treatment failed to considerably increase the life span; *jnk-1 (gk7)* [MLS ± SEM on 0 µM UA is 14.53 ± 0.17 days, while on 25 µM UA is 14.69 ± 0.76 days; p = . 2182; Figure 3B, Supplementary Table 1] and in the upstream kinase *jkk-1(km2)* [MLS ± SEM on 0 µM UA is 13.74 ± 0.09 days, while on 25 µM UA is 14.18 ± 0.21 days; p = .114; Figure 3C, Supplementary Table 1]. In addition, we also observed increased mRNA transcript levels of *jnk-1* (3.14-fold, p = .048; Supplementary Figure 5) in qRT-PCR gene expression profiling. Altogether, these results suggest that UA mediates JNK-1 activation to elicit its positive effects and are congruent with our earlier findings (4).

In summary, we present results where UA regulates the longevity of *C. elegans* in DR-dependent manner (Figure 4) and prevents toxic protein aggregation by inducing JNK-1 in WT animals. HD is more amenable to early pharmacological interventions because of its monogenic nature making it fully penetrant compared to other more prevalent neurodegenerative disease(s), including Alzheimer's disease and Parkinson's disease, which share such features as impaired proteostasis, selective neuronal vulnerability, and delayed onset (49). Understanding the shared genetic network of aging and associated diseases implies in harnessing the full benefits of phytochemical(s) on life span and health span. It remains interesting to study whether the positive effects of UA can delay onset of age-related morbidities in mammals.

Supplementary Material

Supplementary data is available at *Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

Experimental Procedures

Detailed experimental procedures and associated references are provided as supplementary information.

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Conflict of Interest

None declared.

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