Sugar-derived AGEs accelerate pharyngeal pumping rate and increase the lifespan of *Caenorhabditis elegans*

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ABSTRACT

All living organisms are normally undergoing aging. Dietary habits constitute the main environmental factor that may accelerate or decelerate this process. Advanced glycation end products (AGEs) are constituents of dietary products that are consumed daily, such as bread and milk. Although AGEs have been widely regarded as toxic agents, recent studies seem to contradict this view: they either find no adverse effects of AGEs or even attribute beneficial properties to them. The aim of our study was to investigate the effects of sugar-derived AGEs on organismal lifespan using as a model the nematode *Caenorhabditis elegans*. Exposure to sugar-derived AGEs prolonged the lifespan of wild type animals; this lifespan extension was accompanied by an enhanced pharyngeal pumping rate. We demonstrate that elevation of the pharyngeal pumping rate depends on *W06A7.4* and *eat-4* expression, as well as on *daf-16*, which encodes a FOXO family transcription factor. Our results suggest that sugar-derived AGEs modulate the lifespan of *C. elegans* at least in part through transcriptional regulation of pharyngeal pumping throughout the animals’ lifespan.

INTRODUCTION

Aging is a naturally complex process occurring in most organisms. Progressively, the aged individuals lose their normal function and homeostatic balance thus, becoming more susceptible to diseases. Proteostasis, DNA damage repair, and nutrient-sensing are such pathways that are deregulated during aging progression and their dysfunction constitutes a hallmark of aging.

Protein glycation is a nonenzymatic protein modification that, if occurring extensively, promotes disruption of proteostasis. Advanced glycation end products (AGEs) constitute an heterogeneous group of protein glycation products formed through Maillard’s browning reaction [1], which is initiated by interaction between the carbonyl moiety of the open form of a saccharide and a protein amino group, both in *vivo* (as aging progresses) and *in vitro* in food and tobacco industry. The *in vivo* production of AGEs is a long-term process preferably affecting long-lived proteins, such as collagen and elastin [2,3]. AGE modification of proteins alters their structure thereby rendering them more resistant to degradation. AGE-modified proteins may then form cross-links resulting in aggregations that contribute to the hardening of the extracellular matrix (ECM) eventually leading to tissue dysfunction [4]. AGEs have been categorised as toxic or nontoxic. Nontoxic AGEs include pyrraline, Nɛ-carboxymethyllysine (CML) or Nɛ-carboxyethyllysine (CEL). On the other hand, glyceraldehyde- or glycolaldehyde-derived AGEs are usually toxic [5], as described in diabetic patients [6]. Despite their potential harmful effects, AGEs are widely used in food industry and during food preservation to improve the taste and bioavailability of food and to ensure longer preservation periods. The formation of AGEs during (industrial) food production is a very fast procedure, due to high temperatures and elevated sugar concentrations that are used. Powdered milk, dry heated meat, chicken or fish, cereal and bakery products are dietary products that may contribute large...
quantities of consumable AGEs [7–9]. Daily dietary intake of AGEs ranges from 25 to 75 mg/day with bakery products and milk contributing the most [10].

The nematode Caenorhabditis elegans is a model organism very frequently used to study aging. Its pharynx is one of the most essential organs; it is contracted through a coordinated process to perform food intake and distribute it to the animal’s digestive tract [11]. Aging has been associated with pumping rate alterations and it has been suggested that the pumping rate can be used as a predictor of lifespan, as prolonged lifespan of the nematode C. elegans has been associated with an increased pumping rate [12]. Several genes regulate pharynx contraction duration and coordination. For example, loss of the gene encoding the vesicular glutamate:sodium symporter eat-4, can prolong pharyngeal pumping duration leading to decreased pumping rates [13]. W06A7.4, which encodes a carbohydrate transmembrane transporter, is another paradigm of a gene that has been shown to be regulated selectively upon treatment with specific compounds and to affect pharyngeal pumping; W06A7.4 up-regulation coincides with both methyl 3,4-dihydroxybenzoate (MDBH)-induced lifespan extension and increased pharyngeal pumping rate [14].

In this study, we sought to investigate the effects of fructose- and glucose-derived artificial AGEs on the lifespan and healthspan of C. elegans. We demonstrate that fructose- and glucose-derived AGEs can promote lifespan extension in a daf-16-dependent manner. This extension is accompanied by an increased activity of cathepsins and a daf-16-dependent acceleration of the pharyngeal pumping rate. Transcriptional analysis shows that elevation of W06A7.4 and eat-4 transcript levels is daf-16-dependent; both W06A7.4 and eat-4 encode transmembrane transporters that are related to rhythmic pharyngeal contractions.

Materials and methods

Nematode strains and growth conditions

Standard procedures were followed for C. elegans strain growth and maintenance. All strains were maintained at 20 °C. The following strains were used: N2 [wild type Bristol isolate], DR26 [daf-16(m26) I], YD3[xzEx3[Punc-54::UbiG76V::Dendra2]] [15], YD27 [xzEx27[Pvha-6::UbiG76V::Dendra2]] [16], and polyubiquitin reporter strain: N2 [Pvha-6::UIM2-ZsProSensor] [17].

Compound treatment

1.25 moles of d-glucose or d-fructose were incubated with 15 μM (1 mg/mL) bovine serum albumin (BSA) in PBS (pH 7.4), sterile filtered and stored for 6 weeks at 37 °C. After this incubation time, the solution was dialysed for 24 h against PBS to remove unbound sugars and again sterile filtered. Following dialysis, Fehling’s test for reducing sugars was performed to verify the absence of unbound/unreacted sugars. Protein content was measured with the Bradford assay before and after the incubation to verify the existing BSA concentration. Consequently, stock preparations contained 1 mg/mL fructose- or glucose-treated BSA. Fluorescence measurement (ex/em: 360 nm/460 nm) was used to verify the generation of AGEs following our incubations as compared to nontreated 1 mg/mL BSA stock. Nematodes were exposed to the final indicated concentrations per nematode growth medium (NGM) agar plate after diluting the original stock. Final concentrations per plate were 10, 20, 40, and 60 μg/mL fructose- or glucose-treated BSA (named hereafter as fructose- or glucose-derived AGEs). Stock solutions of all compounds were stored at −20 °C. The appropriate amount of each compound or M9 (control) was added on top of UV-irradiated OP50 bacteria lawn. Different preparation batches (fructose- or glucose-treated BSA) were produced and similar results in biological assays were obtained.

Lifespan assay

Synchronised L4 or young adult animals (110–130 animals per condition) were transferred to fresh NGM agar plates containing the relative concentration of control (M9) or fructose- or glucose-derived AGEs and day 1 of adulthood was set as t = 0. Fresh OP50 was replenished every two days. Alive animals were verified following provoked movement and pharyngeal pumping. The Kaplan and Meier survival curves were produced by plotting the percentage of living nematodes against days of adulthood. The log-rank (Mantel–Cox) test was used to evaluate differences and to determine p values for all independent data. N indicates the number of animals that died over the total number of animals used (animals that died and animals that were excluded due to internally extruded gonads, hatched eggs or desiccation due to crawling off the plates). Each assay was performed at least three times. Median lifespan values are expressed as mean of medians ± standard error of the mean of medians (SEM).

RNA isolation and quantitative reverse-transcription/polymerase chain reaction (qRT-PCR) analysis

RNA was extracted from synchronised animals after exposure to fructose-, glucose-derived AGEs or M9.
(control) on their first and sixth day of adulthood and analysed by standard methods. Primers are summarised in Table 1. Data were analysed using the comparative $2^{-\Delta\Delta Ct}$ method and are presented as the $x$-fold difference in mRNA transcript abundance in fructose- or glucose-derived AGEs-treated animals, normalised to the endogenous $cdc-42$ gene, relative to control-treated animals. Experiments were performed twice.

### RNA interference

Synchronised young adult animals (100–120 animals per condition) were transferred to NGM agar plates containing 2 mM isopropyl-$\beta$-D-1-thiogalactopyranoside (IPTG; Invitrogen, Carlsbad, CA) to induce dsRNA expression and seeded with HT115 (DE3) bacteria transformed with either the $eat-4$ RNAi-encoding construct or the empty pL4440 vector plus sugar-derived AGEs or control (M9). Experiments were performed twice.

### Phenotypic analysis

For all assays, N2 animals were allowed to lay eggs for 15–20 min on NGM agar plates containing fructose-, glucose-derived AGEs, or M9 (control).

### Developmental timing

The progeny were frequently observed to record the needed time to reach the L4 larval stage from egg hatching. Experiments were performed three times.

### Pharyngeal pumping

Pharyngeal pumping rate at days 1 and 6 of adulthood was assessed for 15 s (60 s rate is plotted). At least 60 animals per condition were examined. Pharyngeal pumping was also tested in $daf-16$ mutants.

### Defecation assay

Defecation rate (period in seconds from defecation to defecation) was measured at day 1 of adulthood. At least 60 animals per condition were examined.

### Fecundity assay

Single wt L4 larvae were transferred on NGM agar plates containing fructose-, glucose-derived AGEs, or M9. Each animal was transferred daily to a fresh NGM agar plate containing fructose-, glucose-derived AGEs, or M9. Progeny of each animal were scored at L2–L3 larval stage. At least 10 animals per condition were examined.

### Cathepsin activity assay

Synchronised wt N2 animals were exposed to control and sugar-derived AGEs from egg, collected in 1 mM dithiothreitol (DTT) on day 1 of adulthood and sonicated. Lysates were centrifuged at 12,000 $\times g$ for 10 min and the supernatants were used for determination of activity of cathepsins following protein content determination using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). More specifically, 20 $\mu$g of total protein were incubated in the reaction buffer (50 mM sodium acetate, 8 mM cysteine hydrochloride, 1 mM EDTA, pH 4.0) containing the substrate z-FR-AMC (Enzo Life Sciences GmbH, Lorrach, Germany) for cathepsins L, B, and C or the substrate MCA-GKPILFFRLK(Dnp)-D-R-NH$_2$ for cathepsins D and E (Enzo Life Sciences GmbH, Lorrach, Germany) for 30 min at 37°C. The fluorescence was measured with a fluorescence microplate reader Infinite 200 Pro (Tecan, Grödig, Austria) at $\lambda_{ex} = 350$ nm, $\lambda_{em} = 440$ nm. Results are calculated as percentage (%) and control-treated animals were set to 100%.

### Proteasome peptidase assay

Synchronised wt N2 animals were exposed to control and sugar-derived AGEs from egg, collected in lysis buffer (250 mM sucrose, 25 mM HEPES, 10 mM MgCl$_2$, 1 mM EDTA, and 1.7 mM DTT) on day 1 of adulthood and sonicated. Lysates were centrifuged at 12,000 $\times g$ for 10 min and the supernatants were used for determination of chymotrypsin-like (CT-L) proteasome activity. Samples of the supernatants were incubated in 225 mM tris buffer (pH 7.8), 45 mM KCl, 7.5 mM magnesium acetate, 7.5 mM MgCl$_2$, 1 mM DTT, and 100 $\mu$M ATP. CT-L activity was determined in the presence of the fluorogenic peptide Suc-LLVY-AMC (G$_{final} = 200 \mu$M) (G1101, UBP-Bio, Aurora, CO). Methyl coumarin liberation was measured every five minutes with a fluorescence microplate reader Infinite 200 Pro (Tecan, Grödig, Austria) at $\lambda_{ex} = 360$ nm, $\lambda_{em} = 485$ nm for one hour in total. Results are calculated as percentage (%) and CT-L activity in control-treated animals was set to 100%.

### Table 1. Gene specific primer sequences.

| Gene | Primer sequence | Product size (bp) |
|------|----------------|------------------|
| $cdc-42$ | F 5'-CTGCTGACAGGAAGATTACG-3' | 111 |
| | R 5'-CTGGGACATTCTCGAATGAAG-3' | |
| W06A7.4 | F 5'-TGCAATGTTGGTGAGGAGG-3' | 169 |
| | R 5'-ATGGAAGCGGCAAGG-3' | |
| $eat-4$ | F 5'-TCCACCAAACAAACTATTACGG-3' | 136 |
| | R 5'-AACTCCTGAAACAGGCCCT-3' | |
Proteasome activity in intestine-specific polyubiquitin reporter animals and in intestine-specific and muscle-specific UbG76V-Dendra2 animals

Synchronised transgenic L4 larvae expressing polyubiquitin reporter in the intestine, or UbG76V-Dendra2 in the intestine or in the muscles were exposed to control or sugar-derived AGEs until day 1 of adulthood. At indicated time points, animals were mounted on a 2% agarose pad on glass slides and immobilised using 0.1 mM levamisole. Imaging was performed using a Zeiss AxioImager.Z1 upright epifluorescence microscope (Carl Zeiss Microscopy GmbH, Stockholm, Sweden). Images of whole worms were acquired with 10×0.45 NA.

Statistical analysis

Statistical analysis and graphs were produced using GraphPad Prism (GraphPad Software Inc, La Jolla, CA). Data in all assays are depicted as the average of at least three independent experiments, unless otherwise indicated. Error bars denote ± SEM. Student’s t-test was used for comparisons. Asterisks denote p values as follows: *p<.05, **p<.01, ***p<.001.

Results

Treatment with sugar-derived AGEs promotes lifespan extension in C. elegans

We investigated whether treatment with sugar-derived AGEs affects the lifespan of wt C. elegans. Sugar-derived AGEs were administered to wt animals (N2) throughout their adult lifespan in different concentrations (10, 20, 40, and 60 μg/mL; Figure 1). All concentrations above 20 μg/mL of both fructose- and glucose-derived AGEs prolonged wt C. elegans lifespan, with the 20 μg/mL concentration being the lowest one yielding statistically significant results (Figure 1). We therefore continued our analysis with this concentration.

Treatment with sugar-derived AGEs induces activity of cathepsins

AGEs appear in aggregated formations due to the crosslinking of glycated proteins. We therefore hypothesised that the cellular proteolytic mechanisms might be up-regulated in C. elegans, thus leading to the degradation of toxic forms of AGEs, resulting in lifespan extension. We examined proteasome and cathepsin activities of AGE-treated animals; CT-L proteasome activity was found decreased in AGE-treated animals as compared to control animals as shown in in vitro and in vivo assays (Figure 2(A,B)). In contrast, activity of cathepsins was up-regulated. Upon treatment with fructose-derived AGES, the activities of all tested cathepsins (B, C, D, E, and L) were enhanced (Figure 2(Cii)), whereas only cathepsin D and E activity was slightly increased following treatment with glucose-derived AGES (Figure 2(Cii)). In total, enhanced activity of cathepsins is observed in animals exposed to sugar-derived AGEs.

Treatment with sugar-derived AGES accelerates pharyngeal pumping rate

Similarly to AGEs, humic substances or constituents are produced through the Maillard reaction and have been found both to extend C. elegans lifespan and to alter some of its phenotypic characteristics [18]. Therefore, we tested whether sugar-derived AGES confer any phenotypic differences. Defecation rhythm, fecundity, developmental time and pharyngeal pumping were assayed; pharyngeal pumping rates were found enhanced in AGES-treated animals (Table 2, Figure 3(A), day 1). We therefore further investigated this observation. Since pumping rate has been reported to decrease upon aging progression, we assessed the pumping rate during the first and the sixth day of adulthood. Pharyngeal pumping rates of fructose- and glucose-derived AGES treated animals were maintained significantly higher during the sixth day of adulthood (Figure 3(A)), as compared to the control animals, despite the age-related decline shown in both sugar-derived AGES-treated and control animals (Figure 3(A); compare first and sixth day of the same population). In total, pharyngeal pumping rate is enhanced in the presence of fructose- and glucose-derived AGES and despite its decline during aging, it remains enhanced (day 6) as compared to the relative levels in control populations.

Treatment with sugar-derived AGES leads to increased expression of genes related to pharyngeal pumping rate

Regulation of the pharyngeal pumping rate has so far been linked to several differentially expressed genes, such as W06A7.4 and eat-4 [13,14]. We therefore examined the expression levels of W06A7.4 and eat-4 genes in our animals. Significantly increased mRNA expression levels of W06A7.4 were detected in animals treated with sugar-derived AGES as compared to control wt animals on the sixth day of adulthood (Figure 3(B)). With regard to eat-4 expression, significantly increased mRNA expression levels were detected in animals treated with
sugar-derived AGEs compared to control wt animals on the first (for fructose-derived AGEs) and sixth day of adulthood (for both sugar-derived AGEs; Figure 3(C)). Importantly, silencing of eat-4 abolished the significant differences in the pumping rates of AGEs-treated animals (Figure 3(D)) in contrast to AGEs-treated animals fed with bacteria expressing only the empty vector pL4440, thus confirming the link between the expression of eat-4 gene and the pharyngeal pumping rate. We were not able to achieve effective RNAi for W06A7.4 gene expression. In summary, both W06A7.4 and eat-4 transporters genes seem to be involved in the pharyngeal contractions rate. The increased pharyngeal pumping rates coincide with the decelerated aging phenotype observed in fructose- and glucose-derived AGEs-treated wt animals.

Figure 1. Sugar-derived AGEs promote lifespan extension in wt *C. elegans*. Survival curves of wt *C. elegans* treated with fructose- or glucose-derived AGEs (AGE-Fru and AGE-Glu, respectively) or M9 (control). Control: median lifespan = 15.9 ± 0.2, n = 529/603; AGE-Fru 10 μg/mL: median lifespan = 16.3 ± 0.3, n = 297/339, NS; AGE-Glu 10 μg/mL: median lifespan = 16 ± 0.3, n = 297/354, NS; AGE-Fru 20 μg/mL: median lifespan = 17.9 ± 0.2, n = 458/559, p < .001; AGE-Glu 20 μg/mL: median lifespan = 17.2 ± 0.2, n = 370/439, p < .001; AGE-Fru 40 μg/mL: median lifespan = 16.7 ± 0.2, n = 324/364, p < .05; AGE-Glu 40 μg/mL: median lifespan = 16.8 ± 0.2, n = 319/358, p < .05; AGE-Fru 60 μg/mL: median lifespan = 19.1 ± 0.2, n = 327/372, p < .001; AGE-Glu 60 μg/mL: median lifespan = 17.5 ± 0.2, n = 319/355, p < .001.
Prolonged lifespan and increased pharyngeal pumping rate are daf-16-dependent

Given that DAF-16/FOXO transcription factor is a key factor in *C. elegans* aging, we investigated the daf-16 dependence of the observed lifespan extension. Sugar-derived AGEs did not alter the lifespan of daf-16 mutants (Figure 4(A)). Since daf-16 is also implicated in several processes associated with dietary habits and the

**Figure 2.** Sugar-derived AGEs induce activity of cathepsins and reduce chymotrypsin-like (CT-L) proteasome activity. (A) Percentage (%) of CT-L proteasome activity in wt *C. elegans* treated with 20 μg/mL fructose- or glucose-derived AGEs (AGE-Fru and AGE-Glu, respectively) or M9 (control). Mean value of CT-L activity in control treated animals set to 100%. (B) Representative fluorescence micrographs of animals expressing UbG76V-Dendra2 in the intestine or in the muscles, or the polyubiquitin reporter (Pvha-6::UIM2-ZsPro-Sensor) in the intestine treated with 20 μg/mL fructose- or glucose-derived AGEs (AGE-Fru and AGE-Glu, respectively) or M9 (control). (C) Percentage (%) of activity of cathepsins (L, B, C; (Ci)) and D, E; (Cii) in wt *C. elegans* treated with 20 μg/mL fructose- or glucose-derived AGEs (AGE-Fru and AGE-Glu, respectively) or M9 (control). Mean value of activity in control treated animals set to 100%. NS: not significant, *p<.05, **p<.01, ***p<.001.
related neuronal behaviour, we sought to investigate the potential daf-16 dependence of the pharyngeal pumping differences. In the presence of mutated daf-16, no statistically significant differences in the pumping rates of the AGEs-treated animals were revealed (Figure 4(B)) in contrast to the observation in wt animals (Figure 3(A)). In accordance, in daf-16 mutants the previously observed up-regulation in the RNA expression of W06A7.4 and eat-4 genes upon sugar-derived AGEs treatment was abrogated (Figure 4(C,D)). In fact, RNA expression of both genes was further decreased in daf-16 mutants upon treatment with sugar-derived AGEs, an observation that needs further investigation. These data reveal a daf-16 dependence of the regulation of eat-4 and W06A7.4 expression and of the resulting pharyngeal pumping rates upon treatment with sugar-derived AGEs.

**Discussion**

The rapid spreading of Western lifestyle that is accompanied by enhanced consumption of processed food and sugar is mirrored in the increased appearance of modified biomolecules such as AGEs in our diet. Food processing and cooking consist the main sources of exogenously generated AGEs. Their involvement in the accelerated development of chronic diseases such as atherosclerosis, vascular dementia, and diabetes [19–22], among others, even in the young populations nominates AGEs as highly interesting targets for further investigation. However, more recently contradictory data appeared regarding their toxic potential [23,24]. On top of the above, the inevitable existence of AGEs in our diet (i.e. bread crust) pushes the research towards the detailed investigation of their effects on cellular and organismal homeostasis. In the present study, artificial sugar-derived AGEs (mimicking AGEs in processed food containing sugars) were found to positively affect *C. elegans* lifespan in a daf-16-dependent manner. This lifespan extension was also accompanied by enhanced pharyngeal pumping rates and altered expression of genes related to pharynx physiology and function.

Sugar-derived AGEs (fructose- and glucose-derived) treatment promoted lifespan extension in wt *C. elegans*. This is in contrast to data from *Drosophila melanogaster* [25] and mice [26], where AGEs treatment accelerated aging or attenuated the beneficial effects of caloric restriction, respectively. Nevertheless, Ravichandran et al. [27] have shown that low doses of MG extend the lifespan of wt *C. elegans* through proteohormesis by mimicking mediators of glycine-C-acetyltransferase impairment, known to promote lifespan extension in *C. elegans*. It is also suggested that MG is highly reactive and may rapidly form AGEs when administered to cells [28]. Consequently, one cannot rule out the possibility that treatment with low doses of MG could result to low doses of AGEs that eventually led to lifespan extension, which would be in consistency with our results. Moreover, antioxidant properties have also been attributed to several Maillard’s reaction products derived from vegetables processing and have been linked to beneficial effects of dietary AGEs [24]. The fact that AGEs treatment is beneficial in lower eukaryotes like *C. elegans* could suggest that this organism might possess a different lifespan-extending pathway responsive to AGEs that was then lost in evolution. Identification of such response would be highly valuable and should be further investigated.

Enhanced elimination of AGEs could be a possible way through which the nematodes might have ended up in exhibiting extended lifespan. Nevertheless, the proteasome activity was found to be inhibited upon treatment with AGEs in agreement with what has been already shown in *D. melanogaster* [25]. The interplay between the proteasome and AGEs has been shown before to alter proteasome assembly favouring the assembly of immunoproteasome that exists however before to alter proteasome assembly promoting the degradation of AGEs [33]. In agreement with those findings, activity of cathepsins was demonstrated to play a significant role in the intracellular degradation of AGEs [33]. In agreement with those findings, activity of cathepsins was found significantly enhanced in our model (Figure 2(C)); similar results were also found in *D. melanogaster* following treatment with AGEs [25]. Likewise, AGE overloads have been shown to be dissolved in proximal tubular epithelial cells through enhanced biogenesis and function of lysosomes suggesting their autophagy-mediated degradation [34]. Similarly, the endo-lysosomal apparatus has been

|                    | Defecation rhythm (s) | Fecundity (eggs) | Developmental time (h) |
|--------------------|-----------------------|------------------|------------------------|
| Control            | 44.2                  | 294              | 52.7                   |
| AGE-Fru            | 41.5                  | 311              | 53.4                   |
| AGE-Glu            | 40.5                  | 334              | 53.2                   |

AGE-Fru: 20 μg/mL fructose-derived AGEs; AGE-Glu: 20 μg/mL glucose-derived AGEs; NS: not significantly different from control. All assays were performed at 20 °C unless noted otherwise.

Discussion

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**Table 2.** Phenotypic characteristics of wt *C. elegans* treated with sugar-derived AGEs.

|                | Defecation rhythm (s) | Fecundity (eggs) | Developmental time (h) |
|----------------|-----------------------|------------------|------------------------|
| Control        | 44.2                  | 294              | 52.7                   |
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shown to be responsible for the disposal of AGE-peptides that can be found in the urine of diabetic rats and patients [35]. The activation of cathepsins could also be, at least partially, the result of the observed proteasome inhibition. It has been shown that when the proteasome is inhibited, autophagy gets activated to compensate for the reduced proteasome activity [36–42]. Therefore, it is possible that accumulation of AGEs results to proteasome inhibition that in turn induces autophagy that could possibly eliminate AGEs. Apart from the possible enhanced elimination of AGEs, repair of the glycated products could also result in the absence of toxic effects upon treatment with AGEs. Nevertheless, only one enzyme with deglycase activity has been identified so far, namely DJ1 from E. coli [43] but nothing yet in eukaryotes. Moreover, measurement of AGEs in human faecal samples has indicated that colonic bacteria may metabolise a vast majority of AGEs leading to beneficial health outcomes [23]. Therefore, further investigation is needed.

Pharyngeal pumping is a predictor of lifespan in C. elegans; the higher the levels of pharyngeal pumping rate, the longer the animal will probably live [12]. Our results indicate that treatment with sugar-derived AGEs enhanced pharyngeal pumping rate as judged by measurements from day 1 to day 6 of adulthood. This is obviously due to the AGE modifications of the carbohydrates since it has been shown before that treatment with glucose or fructose does not alter the pharyngeal pumping rate in wt animals [44]. Our results are also in agreement with findings showing enhanced pumping rate upon treatment with MDHB, a small molecule that belongs to the humic substances group [14]. Humic substances are products of Maillard’s reaction, similarly to the exogenous artificial AGEs used in the current study. Treatment with MDHB was shown to up-regulate the expression of various genes linked to feeding behaviour such as W06A7.4 [14]. Treatment with sugar-derived AGEs resulted to similar W06A7.4 transcription profile. Up-regulation of eat-4 has been linked to accelerated pharyngeal contractions and to higher rates of food intake [13,45]. Given that W06A7.4 was found

Figure 3. (Continued) were arbitrarily set to 1 in control treated animals, cdc-42 gene expression was used as normaliser. (D) Number of pharyngeal pumps per 60 s in wt animals treated with 20 μg/mL fructose- or glucose-derived AGEs (AGE-Fru and AGE-Glu, respectively) or M9 (control) on the first and sixth day of their adulthood on plates seeded with eat-4 RNAi bacteria or bacteria with the empty vector (pL4440). *p < .05, **p < .01, ***p < .001.
enhanced at day 6 while eat-4 at both days (1 and 6), a timely concerted regulation of genes related to pharyngeal pumping and food intake can be assumed.

The conserved insulin-like signalling pathway (IIS) is a key regulator of lifespan in *C. elegans* [46]; activation of its downstream effector, namely DAF-16 transcription factor leads to elongated lifespan [47]. We herein show that treatment with sugar-derived AGEs leads to a DAF-16-dependent lifespan extension. The link between cellular redox status, DAF-16 and lifespan has been revealed before. More specifically, reactive oxygen species (ROS) have been shown to stabilise the active form of DAF-16 that gets translocated to the nucleus to perform its action [48]. Moreover, it has been shown that glycation reactions lead to ROS production [49] while proteasome inhibition (that also occurs in our system) has been also shown to promote ROS accumulation [50]. Therefore, our results could suggest that AGEs administration may induce a mild oxidative stress accompanied by elevated ROS levels that might in turn activate DAF-16 and eventually lead to extended lifespan. Additionally, the link between DAF-16 and AGEs has been shown before. For example, reduced generation of endogenous AGEs following treatment with rifampicin has been shown to lead to lifespan extension in a DAF-16-dependent manner [51]. Analogous reduction of endogenous AGEs production was also shown upon GLOD-4 overexpression [52] that was also suggested to rely on DAF-16 [53]. Although it is unclear how exogenous AGEs treatment affects IIS pathway in *C. elegans*, AGEs administration in human cells has been shown to inhibit insulin-mediated AKT activation [54], thus lowering the IIS pathway eventually leading to lifespan elongation and increased stress resistance. We have also revealed a DAF-16 dependence of the enhanced mRNA expression of eat-4 and *W06A7.4* transcripts; the former has been already shown before. More specifically, IIS pathway has been suggested to be upstream of glutamatergic signalling and DAF-16 has been shown to function cell-autonomously to control glutamatergic neurotransmission [55].
Collectively, our findings reveal that dietary treatment with AGEs can be beneficial leading to extended lifespan of the nematode *C. elegans* in contrast to the outcomes in higher eukaryotes. Possible changes in the neurotransmission rate may lead to shorter pharyngeal contraction periods resulting to an accelerated pharyngeal pumping rate that in turn constitutes a predictor of prolonged lifespan. How and why exogenous AGEs contribute to the accelerated pharyngeal pumping still remains unclear. Similarly, it remains to be investigated why this beneficial effect was lost upon evolution. Unravelling the molecular mechanisms behind this beneficial outcome may indicate pathways in higher eukaryotes that need to be induced to compensate for the potential detrimental effects that exogenous AGEs overload may have.

**Acknowledgements**

Nematode strains used in this study were provided by the Caenorhabditis Genetics Center, supported by the NIH National Center for Research Resources (NCRR).

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

Research from N.C. Lab is currently cofinanced by the European Union and Greek National Funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH – CREATE – INNOVATE [project code: T1EDK-00353 and T1EDK-01610] as well by the Project “STHENOS-b” [MIS 5002398], which is funded by the Operational Program “Competitiveness, Entrepreneurship and Innovation” [NSRF 2014-2020] and cofinanced by Greece and the EU (European Regional Development Fund). N.P. receives a PhD fellowship from Empirikon Foundation. JAT is funded by Instituto de Salud Carlos III [CIBERobn CB12/03/30038]. This article is based upon work from COST Action NutRedOx-CA16112 supported by COST (European Cooperation in Science and Technology).

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