Folic acid supplementation at lower doses increases oxidative stress resistance and longevity in *Caenorhabditis elegans*

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Abstract Folic acid (FA) is an essential nutrient that the human body needs but cannot be synthesized on its own. Fortified foods and plant food sources such as green leafy vegetables, beans, fruits, and juices are good sources of FA to meet the daily requirements of the body. The aim was to evaluate the effect of dietary FA levels on the longevity of well-known experimental aging model *Caenorhabditis elegans*. Here, we show for first time that FA extends organism life span and causes a delay in aging. We observed that FA inhibits mechanistic target of rapamycin (mTOR) and insulin/insulin growth factor 1 (IGF-1) signaling pathways to control both oxidative stress levels and life span. The expression levels of stress- and life span-relevant gerontogenes, viz. *daf-16*, *skn-1*, and *sir-2.1*, and oxidative enzymes, such as glutathione *S*-transferase 4 (GST-4) and superoxide dismutase 3 (SOD-3), were also found to be highly enhanced to attenuate the intracellular reactive oxygen species (ROS) damage and to delay the aging process. Our study promotes the use of FA to mitigate abiotic stresses and other aging-related ailments.

Keywords Folic acid · Oxidative stress · Dietary restriction · *Caenorhabditis elegans* · Aging

Introduction

Long life and healthy aging depend on dynamic interactions among biological, psychological, and environmental factors. Aging is a major risk factor for various diseases, such as diabetes, cancer, and several degenerative diseases, including Alzheimer's, Huntington's, and Parkinson's diseases, and ultimately, all these changes render organisms progressively more likely to die (de Magalhães 2013; Manayi et al. 2014). A large number of nutritional supplements including vitamins enhance organism health span and increase longevity (Alavez and Lithgow 2012; Ernst et al. 2013; Lucanic et al. 2013; Messing et al. 2013).

Vitamins that are considered as vital nutrients cannot be synthesized by the body in sufficient amounts and, therefore, must be taken from the diet (Mahmood 2014). They are very much beneficial for cellular growth, development, health, and delayed aging, mostly because they modulate reactive oxygen species (ROS) levels (Lee et al. 2014). Folic acid (FA), a synthetic form of water-soluble B vitamin folate, is the requisite of the body for the synthesis, repair, and methylation of DNA (Mahmood 2014). FA has also been reported to have anticancer, cardiovascular, and neuroprotective effects (Moens et al. 2008; Tian and Ingwall 2008; Czeizel 2009; Blencowe et al. 2010; Sie et al. 2011). It is found in supplements and fortified foods and also in some

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other food sources like green leafy vegetables, asparagus, broccoli, strawberries, liver, organ meats, and legumes (Bacalini et al. 2014).

Caenorhabditis elegans is a promising model to study the effects of diet on the life history traits such as health span, life span, developmental rate, and fertility (Watson et al. 2014; Watson and Walhout 2014). The feeding behaviors in *C. elegans* have been well characterized, and interestingly, it has been found that mammalian genetic counterparts that control food intake are at least partly conserved in *C. elegans* (Song and Avery 2012). The simple anatomy and rich genetics of this tiny animal model organism *C. elegans* make it a powerful platform to assess the health benefits of nutraceuticals (Collins et al. 2006; Olsen et al. 2006; Pandey et al. 2013; Lee et al. 2014). We used this nematode *C. elegans* as a model system to examine the effects of FA on life span.

Materials and methods

C. elegans: strains and maintenance

C. elegans strains, viz. N2 Bristol (wild type), TK22 (*mev-1(kn1)*), DA1116 (*eat-2(ad1116)*), GR1307 (*daf-16(mgDf50)*), VC-199 (*sir-2.1(ok434)*), EU31 (*skn-1(zu135)*), CL2166 (*dvIs19(gst-4p::GFP)*), and CF1553 (*mulS84(sod-3p::GFP+rol-6)*), and *Escherichia coli* OP50 were procured from the *Caenorhabditis* Genetics Centre (CGC), University of Minnesota. The strains were cultured and maintained at 20 °C on a nematode growth medium (NGM) seeded with *E. coli* OP50 bacteria. A synchronized culture was obtained by sodium hypochlorite treatment (Stiernagle 2006).

Test compound and life span analysis

FA was dissolved in water to prepare 10, 25, and 50 µM stock solutions. All selected doses of FA were assessed for their toxicity on *C. elegans*. *E. coli* OP50 was spotted to form a circular lawn on the center of each NGM plate, and different concentrations of FA including water as a control were placed uniformly over the OP50 food. Eggs were allowed to hatch on these NGM plates, and worms were transferred to fresh plates every 2–3 days. Progeny development was controlled using 50 µM FUdR (Sigma-Aldrich), and touch-provoked method was used for survival analysis. The experiment

was repeated three times independently, each time in triplicate.

Reproduction assay, measurement of body size, and pharyngeal pumping rate

For fecundity assay, ten worms were placed on fresh NGM plates with or without FA (25 µM) and allowed to lay eggs until the end of the reproductive period. Quantification of infertile eggs and hatched nematodes was performed after 48 h of progeny incubation at 20 °C.

L1 worms were raised similar to life span analysis. Ten day 2, day 5, and day 10 worms were randomly picked for measurement of body size and pharyngeal pumping rate per 20 s using the Leica Application suite V3 software (version 3.40).

Chemotaxis assay

The chemotaxis assay was performed as described (Shukla et al. 2012). Briefly, water agar plates were prepared and 10 µl of attractant (1 M sodium acetate) was placed on one side and 10 µl of water on the opposite side. Ten microliters of 1 M sodium azide was placed at the gradient peak to immobilize the worms. Day 2 worms (both control as well as FA treated) were washed with M9 buffer and placed in the center of plate. The worms were scored after 90 min to calculate chemotaxis index (CI) using the following formula:

$$CI = \frac{A-B}{A+B}$$

A and *B* represent the number of worms at the attractant and control locations, respectively. *A+B* is the total number of worms in the plate.

Oxidative stress resistance and measurement of intracellular ROS

About 90 gravid, well-fed, and pretreated with FA (25 µM) adult day 2 worms were exposed to 250 µM juglone, a pro-oxidative stressor, and assayed for survival after 6 h of continuous exposure.

For measurement of intracellular total reactive oxygen species (ROS), 100 day 2 age-synchronized worms were collected in 300 µl of 0.1 % PBST buffer and then washed, homogenized, and sonicated (Pant et al. 2014). The samples were then treated with 15 ml of 10 mM H2DCF-DA, and fluorescent readings were taken every

20 min for 2 h at 37 °C using an Agilent Technologies microplate reader at an excitation/emission wavelength of 485 and 530 nm.

Fluorescence visualization and quantification

Fluorescent measurements were carried out by mounting the day 2 worms ($n=20$) onto a 3 % agarose pad on a glass slide anesthetized with 2 % sodium azide and sealed with a cover slip. For lipofuscin assay, day 10 worms were used. Photomicrographs were taken with a DAPI filter (with excitation at 340–380 nm and emission at 435–485 nm) using a DMI 3000 B (Leica, 205 Wetzlar, Germany) fluorescence microscope at $\times 10$. The fluorescence levels were quantified using the ImageJ software (NIH).

Quantitative RT-PCR

Total RNA was isolated from day 2 worms using an RNazol reagent (Invitrogen Inc., Carlsbad, CA, USA), reversely transcribed into complementary DNA (cDNA) using a cDNA synthesis kit (Invitrogen) and quantified with a real-time PCR system (Applied Biosystems 7900HT) using an SYBR green detection method. An average value of mRNA levels of β -actin (*act-1*) was used for normalization, and the data was analyzed using the Ct method described in the manufacturer's manual.

Data analysis

For life span assay, survival of worms was plotted using the Kaplan–Meier survival assay in the MedCalc software version 12.7.7.0 and the survival curves resulting in P values of ≤ 0.05 (calculated by log-rank significance test) relative to control were considered statistically significant. Other statistical data analyses were carried out using ANOVA (Duncan's test) in the Assistat 7.6 beta statistical assistance software.

Results

FA extends the life span of wild-type *C. elegans*

To examine whether FA has any effect on the life span of *C. elegans*, we treated nearly 100 N2 wild-type *C. elegans* with three different concentrations of FA

(10, 25, and 50 μ M) at 20 °C. Though both the doses (10 and 25 μ M) of FA significantly increased (17.14 and 26.57 %; $P \leq 0.0001$) the life span of *C. elegans*, we observed the maximum increase of life span at 25 μ M, 55 % relatively higher than the 10 μ M dosage of FA (Fig. 1a, Table 1). However, we did not find any significant life span extension at 50 μ M FA. We conducted additional life span assays with heat-killed *E. coli* OP50 (30 min at 65 °C) to evaluate whether the effect is solely from FA or is the result of a secondary metabolite elicited by bacterial action on this compound. We noticed that the life span extension effect of FA was still observable under dead bacterial conditions as 25 μ M FA treatment significantly enhanced the mean life span of heat-killed *E. coli* OP50-fed worms by 14.97 % ($P \leq 0.0001$) (Fig. 1b, Table. 1).

Higher concentrations of FA are toxic to *C. elegans*

Since 50 μ M concentration of FA failed to enhance the life span of *C. elegans*, we were interested to check if concentrations higher than 50 μ M could have deleterious effects on the worms. We exposed *C. elegans* to various concentrations of FA (10 μ M to 1 mM), and we found the lethality rate that was significantly reduced by 10 and 25 μ M doses of FA, compared to control, started to decline with concentrations higher than 50 μ M. It was observed that following exposure to higher concentrations of FA (250 μ M, 500 μ M, and 1 mM), the life span of *C. elegans* gets drastically reduced (Fig. 2).

FA declines the pharyngeal pumping rate of *C. elegans*

Evaluation of pharynx pumping rate is one measure of aging in *C. elegans* (Bolanowski et al. 1981; Huang et al. 2004), and it has been observed that a decline in pumping rate can induce dietary restriction (DR)-like effects in *C. elegans* (Huang et al. 2004). We tested whether FA has any effect on the pharyngeal pumping rates of *C. elegans* and found that FA treatment significantly decreases the pumping rates of adult day 2, day 5, and day 10 worms compared to control group in a dose-dependent manner (Fig. 2b). These findings suggest that intake of FA affects the feeding behavior of worms and induces DR-like effects in *C. elegans*.

Since the maximum life span extension of the wild-type nematodes was achieved at 25 μ M concentration of FA, therefore, all other experiments were performed using the 25 μ M dosage of FA.

Fig. 1 Effect of FA on the life span of N2 wild-type *C. elegans* at 20 °C. **a** The curves shown represent changes in percentage survival of worms at different times (days) after treatments with each individual supplement (water (control) and 10, 25, and 50 µM concentrations of FA). Significant increase of life span by 17.14 % ($P \leq 0.0001$) and 26.57 % ($P \leq 0.0001$) was observed at

10 and 25 µM concentrations of FA. However, FA failed to augment the life span of *C. elegans* at 50 µM. **b** Life span assay conducted with heat-killed *E. coli* OP50 (30 min at 65 °C). Twenty-five micromolars of FA treatment significantly enhanced the mean life span (14.97 %; $P \leq 0.0001$) of worms fed with heat-killed *E. coli* OP50

FA increased the chemotaxis index of worms

Chemotaxis behavior declines with aging in *C. elegans* (Glenn et al. 2004). Therefore, to investigate the effects of FA on responses to sensory stimuli, we examined the CI of day 2 and day 5 worms. In FA-treated worms, chemotaxis was significantly improved (Fig. 3a).

FA slows down the fecundity rate and increases the body size of *C. elegans*

Life span enhancement and stress resistance are often associated with reduction in fecundity and growth (Kirkwood 1977; Partridge et al. 2005). It has also been reported that in comparison to well-fed worms, DR results in an

Table 1 Life span data: effects of folic acid (FA) treatment on wild-type and mutant *C. elegans*

Strains	Treatment (µM)	Mean life span (±SE)	No. of worms (N)	% change	P value	Max. mean life span (±SE)
N2	Control	16.56±0.26	100			20.66±0.33
	10 µM	19.40±0.55	105	17.14	<0.0001	26.33±0.33
	25 µM	20.96±0.48	111	26.57	<0.0001	30.66±0.66
	50 µM	16.52±0.15	120	0.24	0.2371	18.66±0.33
N2	Control (live)	16.24±0.05	129			25±1
	Control (dead OP50)	19.37±0.56	135			30.66±0.66
	FA (dead OP50)	22.27±0.57	132	14.97	<0.0001	35±0.57
<i>mev-1(kn-1)</i>	Control	12.42±0.38	83			18.33±0.67
	25 µM	13.71±0.26	98	13.29	0.0071	21.33±0.67
<i>daf-16(mgDf50)</i>	Control	14.19±0.72	89			19.66±1.66
	25 µM	14.33±0.74	70	1.03	0.2443	22±1
<i>sir-2.1(ok434)</i>	Control	17.80±0.70	101			23.66±0.33
	25 µM	17.74±0.86	98	-0.32	0.7928	22.66±1.33
<i>skn-1(zu135)</i>	Control	19.21±0.71	80			28.66±0.33
	25 µM	19.95±0.67	92	3.85	0.5578	29.66±0.66
<i>eat-2(ad1116)</i>	Control	14.98±0.47	106			18.66±0.33
	25 µM	15.10±0.55	103	0.83	0.5223	18.66±0.33

a

Fig. 2 Toxicity and pharyngeal pumping of *C. elegans* exposed to different concentrations of FA. **a** Higher concentrations of FA (>250 μ M) are toxic to worms and greatly reduces their life

extended period of egg laying with an overall reduction in progeny production (Hughes et al. 2007). We treated the worms with FA and allowed them to lay eggs until the end of the reproductive period. We found that untreated worms stopped progeny production after the 10th day of adulthood whereas FA-treated worms produced progeny in 12-day-old adults (Fig. 3b). After careful examination of the effect of FA treatment on the progeny production profile, we observed that FA reduces total progeny production. Total progeny production was 342.7 ± 4.8 progeny/animal (mean $\pm$ SEM) for the control ($n=10$) and 284.6 ± 9.5 progeny/animal for 25 μ M FA ($n=10$; $P<0.01$). These results indicate that FA extends the reproductive span of N2 worms by maintaining self-fertile reproduction at a slower rate, with an overall reduction in total progeny production.

We were also interested to check the effect of FA on the body size (length and width) of *C. elegans*, and we observed significant increase in the body size of day 5 ($P<0.001$) and day 10 ($P<0.001$) worms (Fig. 3c, d).

FA enhances oxidative but not thermal stress resistance in *C. elegans*

Numerous studies showed that an increased life span is closely associated with enhanced survival under conditions of oxidative or heat stress (Lithgow et al. 1995; Kenyon 2010; Zhang et al. 2013). Therefore, to study whether FA affects stress resistance in *C. elegans*, wild-type N2 worms pretreated with FA were exposed to oxidative and thermal stresses. In the oxidative stress assay, synchronized L1 larvae were treated with FA for

b

span **b** N2 nematodes show decline in pharyngeal pumping up on FA exposure. Mean pumping rates (pumps per 20 s) are shown for each time point ($n=10$ animals aged 2, 5, and 10 days)

72 h at 20 $^{\circ}$ C before being exposed to juglone (250 μ M). Juglone is an organic compound that generates intracellular ROS, thereby causing oxidative stress. The worms were incubated for 5 h at 20 $^{\circ}$ C and then assayed for survival (de Castro et al. 2004). We observed that FA pretreatment results in an increased survival rate of worms by 12.22 % ($P<0.01$) (Fig. 4a), signifying that FA increases the oxidative stress resistance in *C. elegans*. To gain further insights into the potential effects of FA under oxidative stress, we evaluated the intracellular ROS generation in adult day 2 nematodes. The total ROS levels were significantly ($P<0.01$) reduced (46.8 %) in FA-treated worms compared to untreated controls (Fig. 4b), indicating that FA acts as a potent ROS scavenger in *C. elegans*.

For thermal stress assay, worms were raised similar to oxidative stress assay and adult day 2 wild-type worms were exposed to thermal stress at 35 $^{\circ}$ C for 3 h. The survival of worms was examined similar to life span assay. We did not find any significant difference among FA-treated and untreated worms (data not shown).

FA reduces the intestinal lipofuscin fluorescence, a marker for aging and oxidative damage

Lipofuscin is an autofluorescent aging pigment that primarily consists of lipid peroxidation products and oxidized proteins (Yin 1996). Accumulation of lipofuscin gradually increases throughout *C. elegans* adulthood in secondary lysosomes, and such autofluorescent granules can be detected by fluorescence microscopy (Koenig 1963; Clokey and Jacobson

Fig. 3 Effect of FA on the aging end point parameters in *C. elegans* **a** Chemotaxis index. FA treatment improves the chemotaxis behavior of N2 worms. **b** FA extends the reproductive span of *C. elegans* and also reduces the total progeny production of

worms. **c, d** FA increases the body size (length and width) of N2 wild-type *C. elegans*. Body size of day 2 worms was unaffected by FA pretreatment. ** $P < 0.01$, significant changes

1986; Brunk and Terman 2002). We noticed the significant reduction of lipofuscin levels in FA-treated day 10 worms by approximately 23.84 % ($P \leq 0.001^*$) (Fig. 5).

Genetic requirements for FA-mediated life span extension in *C. elegans*

Many genes and pathways are known to be involved in *C. elegans* aging (Wolff and Dillin 2006; Smith et al.

2007; Kennedy 2008; Kenyon 2010). In our study, we used a series of mutant strains and transcriptional profiling experiments to examine the FA-targeted gerontogenes and/or longevity pathways in *C. elegans*. Dietary restriction that extends life span in many species, from yeast to primates including *C. elegans* (Colman et al. 2009), is actively regulated by nutrient sensing pathways like kinase mechanistic target of rapamycin (mTOR) (Kapahi et al. 2004; Kaeblerlein

Fig. 4 Effect of FA on the oxidative stress sensitivity. **a** Pretreatment (72 h of worm exposure) of FA significantly (** $P < 0.01$) promotes oxidative stress resistance in *C. elegans*. Oxidative stress was induced by continuous exposure of worms to juglone for 6 h.

b Relative formation of reactive oxygen species (ROS) after 72 h of exposure to 25 μ M concentration of FA, depicting reduction of ROS level by 53 % in FA-treated worms

et al. 2005; Hansen et al. 2007), insulin/insulin-like growth factor 1 (IGF-1) (Honjoh et al. 2009; Arum et al. 2009), and sirtuins (Rogina and Helfand 2004; Li et al. 2008).

eat-2 mutations are reported to increase life span by means of mTOR inhibition, and PHA-4 transcription factor is required for mTOR inhibition (Honjoh et al. 2009). We therefore tested the effect of FA on *eat-2(ad1116)* mutants by examining the life span of *eat-2* worms after FA supplementation. No life span extension in *eat-2* worms was found after FA treatment (Fig. 6a, Table 1), showing that *eat-2* is involved in FA-mediated life span extension in *C. elegans*. We next checked the effect of FA on *pha-4* gene expression by real-time PCR, and we observed upregulation of *pha-4* by 2.3-fold (Fig. 9). Our results signify that FA targets the mTOR pathway to extend life span in *C. elegans*.

FA may likely extend life span by inhibiting insulin/IGF-1 signaling that is largely dependent on DAF-16 transcription factor. Therefore, we examined the effect of FA on the *daf-16(mgDf50)* mutant. We did not notice any significant increase in the life span of *daf-16* mutants after supplementation with FA (Fig. 6b, Table 1). FA was found to enhance the expression of *daf-16* gene significantly by 2.5-fold (Fig. 9). In *C. elegans*, overexpression of the sirtuin gene *sir-2.1* extends life span by activating DAF-16/FOXO (Kenyon 2005; Berdichevsky et al. 2006). The impact of FA on *sir-2.1* was analyzed using the *C. elegans sir-2.1(ok434)* mutant. Similar results as of *daf-16* were observed for the *sir-2.1* mutant, where we found no increase in the life span (Fig. 6c, Table 1) and also its expression enhancement by 4.7-fold (Fig. 9). *C. elegans* manganese superoxide dismutase 3 (SOD-3) is an antioxidative enzyme

Fig. 5 Effect of FA on the accumulation of lipofuscin autofluorescence. FA significantly (* $P < 0.05$) reduces lipofuscin levels of *C. elegans*. Representative images of *A* untreated and *B* FA-treated N2 animals ($n = 20$) are shown. Images were quantified using the ImageJ software

Fig. 6 FA extends the life span of *C. elegans* via *eat-2*, *daf-16*, *sir-2.1*, and *skn-1*-dependent mechanism. Survival curves of mutants **a** *eat-2(ad1116)*, **b** *daf-16(mgDf50)*, **c** *sir-2.1(ok434)*, and **d** *skn-*

1(zu135) grown on plates containing water control (blue) or 25 μ M of FA (red) are shown

that is tightly regulated by DAF-16, and its expression is elevated in many long-lived *C. elegans* mutants (Honda and Honda 1999; Kenyon 2005). We evaluated the effect of FA on *sod-3* and found its expression doubled by FA (Fig. 9). Moreover, we also analyzed the expression of *sod-3* in the green fluorescent protein (GFP) expressing transgenic strain CF1553 (SOD-3::GFP). FA-treated worms showed significantly higher intensity (34.49 %) of SOD-3::GFP expression (Fig. 7). SKN-1 transcription factor that is also a member of insulin/IGF-1 signaling pathway extends life span in response to dietary restriction (Tullet et al. 2008). We were also interested to check the effect of FA on this transcription factor. We used the *skn-1(zu135)* mutant for the experiment, and FA was found to have no effect on the life span of the *skn-1* mutant (Fig. 6d, Table 1); also, gene expression results showed its upregulation by 2.3-fold

(Fig. 9). Such results prompted us to examine the expression of *gst-4*. Glutathione *S*-transferase (GST-4) is a phase II detoxification enzyme and works downstream of *skn-1*. FA treatment upregulated (2.8-fold) the expression of *gst-4* and also significantly enhanced its induction in CL2166 (GST-4::GFP) transgenic strain by 23.52 % (Fig. 8). The upregulation of *daf-16*, *sod-3*, *skn-1*, and *gst-4* implies the involvement of insulin/IGF-1 signaling (ILS) in the FA-mediated life span extension in *C. elegans*.

Besides serving in the regulation of aging, both DAF-16 and SKN-1 control *C. elegans* tolerance to various types of stresses (Murphy et al. 2003; Wolff and Dillin 2006). Also, SIR-2.1 activates DAF-16 directly in response to oxidative stress and many long-lived mutants are resistant to oxidative stress (Kenyon 2005). To explore whether FA has any effect on oxidative stress

Fig. 7 FA treatment significantly ($*P<0.05$) enhanced GFP expression of *sod-3::gfp* mutants. **a** Day 2 adult worms ($n=20$) were exposed to water (control) and **b** 25 μM concentration of FA for 72 h, and quantification of GFP was performed using the ImageJ software

resistance, we analyzed the effect of FA on *mev-1(kn1)* mutants. *mev-1*, a mutant strain of *C. elegans*, is hypersensitive to oxidative stress and has a reduced life span due to the overproduction of mitochondrial ROS (Ishii et al. 1998). We tested this mutagenic strain and found significant increase in the mean survival time of worms by 13.29 % (Fig. 10), indicating that FA is also capable of improving survival during mild to severe oxidative stress.

Discussion

Vitamins are well-known topics of extensive research on nutrition, and their potential benefits on human health have been intensely investigated (Mora et al. 2008; Barry 2008; Dolaru et al. 2012; Dror et al. 2014; Jensen

2014). FA, a vitamin of the B group, is essential for the production and maintenance of new cells, prevention of neural tube defects, improved neuropsychological development in children, and prevention of some other congenital anomalies and low birth weight as well as chronic diseases such as cardiovascular disease, cerebral stroke, cancer of various sites, depression, dementia, and osteoporosis (Eichholzer et al. 2006; De Wals et al. 2007; Shelke and Keith 2011; Valera-Gran et al. 2014). Superparamagnetic iron oxide nanoparticles conjugated with FA (FA-PEI- Fe_3O_4) were also recently successfully prepared and reported as a promising tumor-targeting agent as well as a T2-negative contrast agent in MR imaging applications (Li et al. 2015). With the aforementioned points in mind, it would be interesting to see if FA has any effect on the longevity. We implemented a well-known experimental aging model

Fig. 8 FA induces expression of the oxidative stress response gene *gst-4*. **A** Control *gst-4::gfp*. **B** FA-treated *gst-4::gfp* worms ($n=20$). The data was statistically analyzed using ANOVA (Duncan's test) in the Assistat 7.6 beta statistical assistance

software, and images were quantified using the ImageJ software. Differences between the data were considered statistically significant at $*P<0.05$

Fig. 9 FA exposure (25 μ M) effect on the life span and stress-related gene expression in *C. elegans*. Expression levels were determined by real-time PCR using actin-1 as an internal control. Bars represent means $\pm$ SE. ** $P < 0.01$

organism *C. elegans* to examine the effects of FA on life span. Earlier, it has been observed that *C. elegans* contain a folate carrier (FOLT-1) whose expression is adaptively regulated during development and by substrate levels in the culture medium (Balamurugan et al. 2007). Also, similar observations have been reported with mammalian-reduced folate carrier in the intestine on changing extracellular folate levels (Said et al. 2000; Subramanian et al. 2003). We observed significant life span increase of 17.14 and 26.57 % at 10 and 25 μ M concentrations of FA. However, we did not find any significant change in life span at 50 μ M FA (Fig. 1a). Further, toxicity assays showed that higher doses of FA (≥ 250 μ M) are toxic to *C. elegans* as the treatment of worms with these doses (250 μ M to 1 mM) shortened life span relative to control. This is not specific with FA,

Fig. 10 FA extends life span under stress conditions. The mean survival of oxidative stress-prone mutant *mev-1(kn1)* was enhanced by 13.24 % (shown as dotted blue survival curve) compared to control (red)

as many and perhaps all compounds are toxic at sufficiently high doses. Even in the case of vitamins, many studies have shown that vitamins such as vitamin E at higher concentrations are often non-effective and even show toxic effect on worms (Ernst et al. 2013). We do not know the exact reason, but from the continuous decline in pumping rates with increase in FA concentrations, we speculate that the impaired pharyngeal pumping might be the probable reason for toxic effect (shortening of life span) of FA where starvation-induced excessive autophagy in pharyngeal muscles could decrease the survival of worms. Taken together, our findings show that FA like other vitamins at higher concentrations loses its life span-extending beneficial effects and also becomes toxic to worms. Similar findings, where sulfamethoxazole (a sulfonamide drug that blocks folate synthesis by competing with PABA for the enzyme dihydropteroate synthase) was observed to act through inhibition of bacterial folate synthesis to increase *C. elegans* life span, were recently reported by Virk and co-workers (2012).

Numerous conditions like oxidative stress (Heidler et al. 2010), heat (Lee and Kenyon 2009), chemosensory signals (Alcedo et al. 2010) and signals from the reproductive system (Kenyon 2005, 2010), and calorie restriction (Ingram et al. 2006; Everitt et al. 2010) can increase the life span of *C. elegans*. Therefore, we examined the effect of FA on all these parameters.

Longevity and stress resistance are an interrelated phenomenon, and response of stress and nutrient sensors to different environmental and physiological signals mediates life span extensions (Lithgow and Walker 2002; Kenyon 2010). The accumulation of reactive oxygen species (ROS) that causes severe oxidative damage to biomolecules arises with age and thereby increases the oxidative stress. There are multiple examples in the literature suggesting that life span-extending interventions are also effective for resisting oxidative stress and many antioxidants confer both increased resistance to oxidative stress and longevity (Onken and Driscoll 2010; Schlernitzauer et al. 2013; Upadhyay et al. 2013; Kim and Park 2013). In contrast, some antioxidants failed to show a life span-extending effect, such as supplementation of coenzyme Q10 or α -lipoic acid in mice (Lee et al. 2004). Moreover, it has also been observed that overexpression of antioxidant enzymes like superoxide dismutase (SOD) and *gst-4* increased resistance to various stressors including oxidative stress

but failed to increase life span (Finkel and Holbrook 2000; Leiers et al. 2003). Thus, the role of oxidative stress in life span determination remains unclear (Muller et al. 2007). Antioxidant activities of FA as reported by several researchers (Bayés et al. 2001; Joshi et al. 2001; Racek et al. 2005) motivated us to study the stress-modulating effects of FA in *C. elegans*. Our findings indicate a positive correlation between life span and oxidative stress tolerance, as FA-treated worms showed increased resistance to juglone-induced oxidative stress. Survival curves showed that FA supplementation enhanced survival of worms under oxidative stress by 12.22 %. Interestingly, we observed that the mean survival of the oxidative stress-hypersensitive mutant, *mev-1(kn1)*, was also extended by 13.29 %. Moreover, we also found reduced ROS levels by 46.8 % in FA-treated adult day 2 nematodes. Such results show that FA acts as a potent ROS scavenger. Lipofuscin, an autofluorescent lipid peroxidation aging pigment, serves as a marker of aging/oxidative damage, and its accumulation in secondary lysosomes gradually increases throughout *C. elegans* adulthood (Brunk and Terman 2002). We observed significant reduction in lipofuscin levels in FA-treated worms, indicating that FA retards aging in *C. elegans*. Some authors also reported correlation of heat resistance with life span (Lithgow et al. 1995; Lee and Kenyon 2009); however, in our case, we did not notice any effect on the thermal tolerance of worms by FA. Worms modify their behavior under adverse environmental conditions and also with age, leading to change in their learning and motor activities (Glenn et al. 2004; Zhang et al. 2005; Murakami and Murakami 2005). FA improved CI in adult day 2 worms, suggesting the ability of FA to improve health span. Correlation of fecundity with life span and stress resistance has been noticed by several authors (Kirkwood 1977; Tissenbaum and Ruvkun 1998; Partridge et al. 2005). In fact, reports show that calorie restriction results in an extended period of egg laying with an overall reduction in progeny production (Hughes et al. 2007). In our study, we also found extended reproductive span and less number of progeny production in FA-treated worms.

Dietary restriction (DR) signals also increase resistance to oxidative stress and extend life span in many species (Houthoofd et al. 2005; Colman et al. 2009). The decline in pharyngeal pumping rate with age causes reduction in food intake and induces DR-like effects on *C. elegans* (Powolny et al. 2011). FA-treated worms

showed reduced rates of pharyngeal pumping, signifying that FA may stimulate DR-like effects on *C. elegans*. Genetic studies of aging in *C. elegans* have provided remarkable information about the molecular mechanisms that modulate aging through DR signals, and several signaling pathways like kinase mechanistic target of rapamycin (mTOR), insulin/insulin-like growth factor (IGF-1), sirtuins, etc., in life span determination of *C. elegans* through DR have been proposed (Kenyon 2010; Lapierre and Hansen 2012). In fact, most of these pathways such as insulin/IGF-1-like signaling are evolutionarily conserved from yeast to humans. mTOR is also now referred to as the mechanistic target of rapamycin in all organisms except yeast. Similarly, increased expression of the sirtuin gene has been reported to promote longevity in yeast, flies, worms, and mice (Tissenbaum and Guarente 2001; Rogina and Helfand 2004; Herranz et al. 2010; Kanfi et al. 2012), although the role of sirtuins in life span regulation is controversial (Burnett et al. 2011). These findings make it possible to dissect the impact of exogenous molecules on such complex pathways while making use of simple model organisms like *C. elegans*.

C. elegans eat-2 mutants have reduced pharyngeal pumping rate due to defects in the pharyngeal function and serve as a best model for DR (Lakowski and Hekimi 1998). Such mutations (*eat-2*) are likely to increase life span by means of mTOR inhibition, and PHA-4 transcription factor is required for mTOR inhibition (Panowski et al. 2007). FA failed to augment the mean life span of *eat-2* mutants and also upregulated the expression of the *pha-4* gene, showing the involvement of DR-mediated mTOR pathway.

Life span extension in *C. elegans* through DR requires sir-2.1, and its increased dosage extends life span and stress resistance (Narasimhan et al. 2009; Kenyon 2010; Rizki et al. 2011; Viswanathan and Guarente 2011). We administered FA to *sir-2.1(ok434)* mutants, and did not notice any life span extension; also in real-time quantification experiments, 4.7-fold (Fig. 9) increase of *sir-2.1* was recorded. These results show the involvement of *sir-2.1* in the observed FA-mediated life span extension in *C. elegans*. SIR-2.1 activates DAF-16, a major transcription factor regulating a number of longevity and stress tolerance-associated genes (Murphy et al. 2003; Gami and Wolkow 2006; Kenyon 2010). Furthermore, inhibition of insulin/IGF-1 signaling

increases life span through DAF-16, a FOXO transcription factor, and SKN-1, a Nrf-like xenobiotic response factor (Kenyon 2005; Tullet et al. 2008). Our observations were also consistent with these findings, as FA supplementation increased the expression of *daf-16* by 2.5-fold (Fig. 9). The results were further supported by failure of FA to extend the life span of *daf-16(mgDf50)* mutants. SKN-1 transcription factor also mediates longevity through DR and by modulating oxidative stress response in worms (An and Blackwell 2003; Bishop and Guarente 2007; Park et al. 2009). In our DR regimen, we found the upregulation of *skn-1* by 2.3-fold (Fig. 9) and no life span extension in *skn-1(zu135)* mutants after dietary intake of FA supplement. Several compounds could act by activating the *skn-1* (Nrf2) pathway in an ILS-independent manner in *C. elegans* (Onken and Driscoll 2010; Alavez et al. 2011; Powolny et al. 2011). However, the participation of DAF-16 in FA-mediated longevity shows the involvement of ILS pathway. The activation of these transcription factors (DAF-16 and SKN-1), in turn, upregulates or downregulates diverse genes to control life span in *C. elegans* (Kenyon 2010). DAF-16 and SKN-1 are known to mediate stress-induced transcription of *sod-3* (Honda and Honda 1999; Kenyon 2005) and *gst-4* (Kell et al. 2007; Choe et al. 2009; Landis and Murphy 2010). As expected, FA significantly upregulated the expression levels of these genes. Moreover, we also observed higher intensity of GFP in *C. elegans* transgenic strains (CF1553 and CL2166) expressing SOD-3::GFP and GST-4::GFP proteins. Upregulation of these genes (*sod-3* and *gst-4*) explains how FA significantly promotes the longevity and stress resistance of *C. elegans* through insulin-like signaling pathway.

We conclude that FA with good antioxidant effects extends life span in worms by acting as a calorie restriction mimetic. Other studies also report the well-known CR mimetics such as resveratrol to have potent antioxidant effects (de la Lastra and Villegas 2007; Gülçin 2010). Such results imply that FA may prolong life span via several mechanisms that include its potent antioxidant effects (Figs. 4 and 10) and its ability to influence gene expression in a manner similar to calorie restriction.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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