BIOSCIENCE JOURNAL

EZETIMIBE INCREASES RESISTANCE TO OXIDATIVE STRESS AND EXTENDS LIFESPAN MIMICKING DIETARY RESTRICTION IN *Caenorhabditis elegans*

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How to cite: PARK, S., PARK, J.S. and PARK S.K. Ezetimibe increases resistance to oxidative stress and extends lifespan mimicking dietary restriction in *Caenorhabditis elegans*. *Bioscience Journal*. 2023, **39**, e39027. https://doi.org/10.14393/BJ-v39n0a2023-65305

Abstract

Ezetimibe is an approved drug for lowering plasma LDL (low-density lipoprotein) level via inhibition of cholesterol absorption. Derivatives of ezetimibe reduce inflammatory response and oxidative stress. In the present study, we investigated the effect of dietary supplementation with ezetimibe in response to environmental stressors and found that ezetimibe increases resistance to oxidative stress and ultraviolet irradiation. Ezetimibe also significantly extended lifespan accompanying reduced fertility, which is a common trade-off for longevity in *C. elegans*. Cellular level of reactive oxygen species was increased and the expression of stress-responsive genes, *hsp-16.2* and *sod-3*, was induced by dietary supplementation with ezetimibe, suggesting a hormetic effect on oxidative stress response and lifespan. Ezetimibe also significantly prevented amyloid beta-induced toxicity and completely reversed increased mortality by high-glucose diet. Nuclear localization of DAF-16 required for the prevention of amyloid beta-induced toxicity was enhanced by ezetimibe supplementation. Lifespan assay using known long-lived mutants, *age-1*, *clk-1*, and *eat-2*, revealed that lifespan extension by ezetimibe specifically overlapped with that of *eat-2* mutants, which are genetic models of dietary restriction. Effect of ezetimibe on lifespan of worms fed with diluted bacteria suggested that ezetimibe mimics the effect of dietary restriction on lifespan. These findings suggest that ezetimibe exhibits anti-oxidative and anti-aging effects through hormesis and works as a dietary-restriction mimetic on lifespan extension.

Keywords: *Caenorhabditis elegans*. Ezetimibe. Dietary Restriction. Lifespan. Stress Response.

1. Introduction

Aging is one of the most complex biological pathways and is characterized by universal, progressive, and irreversible decline in biological function and eventual death of an organism. Several theories explaining aging process and causes of aging have been suggested so far, but the mechanisms underlying aging have not fully understood yet. The free radical theory of aging, proposed first by Dr. Harman in 1956, suggests that age-related accumulation of free radicals generated as a byproduct of cellular metabolism results in oxidative damage, which is a major causative factor in aging (Harman 1956). According to the genetic control theory of aging, the genome of each individual organism determines aging process and lifespan. Genetic analyses with model organisms revealed several genetic pathways regulating the rate of aging, which includes insulin/IGF-1-like signaling and TOR signaling (Tatar et al. 2003; Robida-Stubbs et al. 2012). The telomere theory of aging suggests that the rate of telomere attrition with cell replication

determines the rate of aging (Fossel 1998). The membrane theory of aging focuses on age-related decrease in the integrity of plasma membrane (Pathath 2017).

Based on theories of aging, people investigated the role of dietary interventions that can extend lifespan and retard age-related pathophysiological changes. The most widely-studied interventions include dietary supplementation with anti-oxidants. Resveratrol, a compound found abundant in red wine, exhibited a lifespan-extending effect in various model organisms, including *Caenorhabditis elegans* and *Drosophila melanogaster* (Howitz et al. 2003; Gruber et al. 2007; Long et al. 2009). Curcumin, a yellow ingredient used in Indian curry, conferred longevity phenotype in *Drosophila melanogaster* and mice, and delayed ovarian aging (Azami et al. 2020). Anti-aging effects of anti-oxidant super foods were also investigated using model organisms. Both green tea extract and black tea extract increased resistance to oxidative stress and lifespan in *Drosophila melanogaster* (Li et al. 2007; Peng et al. 2009). Green tea polyphenols reduced amyloid beta (Aβ) accumulation in patients with Alzheimer's disease (AD) (Mancini et al. 2017). Among nutritional interventions proposed to retard aging, the most successful intervention is dietary restriction (DR), which is defined as reduced food intake without malnutrition. Since the first reported lifespan-extending effect of DR in rats, it has been demonstrated in yeast, nematode, fruit fly, fish, mice and primates (Fontana et al. 2010). DR also improves health span, retarding age-related functional decline in various organs, including muscle, brain, and heart, and reducing incidence of many age-related diseases, including cancer, AD, and cataracts (Fontana et al. 2010). Due to the difficulty of routine implementation in daily life in humans, studies are exploring dietary interventions that mimic the effects of DR. Metformin, a widely-used drug for type Ⅱ diabetes, increases both mean and maximum lifespan and prevented development of tumors in mice (Anisimov 2010). Recent studies showed that dietary supplementation with cysteine derivatives, N-acetyl-L-cysteine and selenocysteine, extended lifespan mimicking DR response and showed preventive effects against age-related disorders in *Caenorhabditis elegans* (Oh et al. 2017).

Ezetimibe is a potent cholesterol absorption inhibitor, lowering LDL cholesterol in serum and commonly used to treat hypercholesterolemia. Combined treatment with statin, an inhibitor of cholesterol synthesis, and ezetimibe increased its efficacy against hypercholesterolemia compared with statin alone (Pearson et al. 2005). Clinical trials revealed that ezetimibe reduced LDL and total cholesterol as a result of decreased absorption of cholesterol and increased cholesterol synthesis in humans (Sudhop et al. 2002). Recent studies suggest that anti-oxidant activity of ezetimibe was a mechanism underlying the effects of ezetimibe in various diseases. Ezetimibe treatment increased glutathione level, one of the biomarkers of oxidative stress, in liver subjected to ischemia/reperfusion (Trocha et al. 2014).

In the present study, we investigated the effects of dietary supplementation with ezetimibe on stress response and aging in *C. elegans*. Survival of worms under oxidative stress and ultraviolet (UV) irradiation was monitored. Lifespan and fertility of ezetimibe-treated animals were compared with those of untreated controls. Effect of ezetimibe on age-related diseases was determined using genetic and nutritional disease model. We also investigated the possible mechanisms underlying the effects of ezetimibe. The results of this study identified novel bioactivities of ezetimibe and provide a therapeutic rationale for ezetimibe.

2. Material and Methods

Worm culture

N2 strain was used as a wild-type control for all experiments. Worms were cultures at 20℃ on Nematode Growth Media (NGM) agar plates (25 mM NaCl, 2.5 mg/mL peptone, 50 mM KH_2PO_4 (pH 6.0), 5 μg/mL cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 1.7% agar) spotted with *Escherichia coli* OP50 as a food source.

Survival under environmental stress

Three-day-old age-synchronized worms were treated with ezetimibe for 24 h. To induce oxidative stress, 30 worms were transferred to 96-well plate containing 2 mM hydrogen peroxide (H_2O_2) in S-basal medium without cholesterol (5.85 g sodium chloride, 1 g potassium phosphate dibasic, and 6 g potassium phosphate monobasic in 1 L sterilized distilled-water). Survival was recorded after 6 h of incubation. For UV irradiation, 60 age-synchronized worms pre-treated with ezetimibe for 24 h were irradiated with 20 J/cm^2/min of UV for 1 min in a UV crosslinker (BLX-254, VILBER Lourmat Co., Torcy, France).

Lifespan assay

Sixty age-synchronized worms were grown on NGM plates containing ezetimibe and 12.5 mg/L of 5-Fluoro-2'-deoxyruridine. Live and dead worms were counted every day. The log-rank test was used for statistical analysis (Peto and Peto 1972).

Fertility assay

After age-synchronization, 12 worms were transferred to fresh NGM plates daily and allowed to lay eggs from day 2 after hatching. Progeny hatched from eggs on each day were counted after 48 h of incubation at 20℃.

Measurement of ROS level

Seven-day-old worms (n = 20) were transferred to 195 µL of PBST solution in a 96-well black plate and 5 µL of H_2DCF-DA (Sigma-Aldrich, St. Louis, USA) was added to each well. Fluorescence intensity of each worm was measured after 3 h of incubation using a fluorescence multi-reader (Infinite F200, Tecan, Grodig, Austria).

Expression of stress-responsive genes

CL2070 (dvIs70 [*Phsp-6.2::GFP, rol-6*]) and CF1553 (muIs84 [*Psod-3::GFP, rol-6*]) were used to monitor the expression of *hsp-16.2* and *sod-3*, respectively. Age-synchronized 7-day-old worms (n=20) were anesthetized with 1 M sodium azide on a slide glass coated with 2% agarose. Fluorescence intensity was determined with a fluorescence multi-reader (Infinite F200, Tecan, Grodig, Austria).

Paralysis induced by Aβ transgene

CL4176 (dvIs27 [*myo-3/Aβ$_{1-42}$/let UTR, rol-6*]) worms containing a muscle-specific human Aβ_{1-42} transgene were transferred to fresh NGM plates and allowed to lay eggs for 2 h at 15℃. After 24 h, sixty worms were incubated at 25℃ for 24 h to induce muscle-specific expression of human Aβ gene. Paralyzed worms were recorded every hour after induction.

Survival under high-glucose diet (HGD)

Sixty age-synchronized worms were fed with HGD; 100 µL of 40 mM glucose was spread on an NGM plate. Survival of worms was recorded every day until all worms were dead.

Intracellular localization of DAF-16

After anesthetizing TJ356 (zIs356 IV [*daf-16p::daf-16a/b::GFP, rol-6*]) worms with 1 M sodium azide on a slide glass, cellular distribution of GFP was monitored using a fluorescence microscope (n = 60).

DR

Two different concentrations of bacterial solution were prepared using OP50 overnight culture: 5 × 10^9 bacteria/mL for AL (*ad libitum*) group and 5 × 10^8 bacteria/mL for DR group. A 200 µL aliquot of each bacterial solution was spotted onto solid NGM plates containing 5-fluoro-2'-deoxyuridine and ampicillin. Survival was monitored using a previously mentioned lifespan assay.

3. Results

Ezetimibe increases resistance to oxidative stress and UV irradiation

After 6 h of incubation with H_2O_2, 61.1 ± 4.44% (mean ± standard error (SE)) of worms survived in the untreated control. Among different concentrations of ezetimibe tested, 100 µM of ezetimibe resulted in a significant increase (80.0 ± 5.09%) in survival (p = 0.049). In the repeated experiment, a significant increase in survival was observed in worms pre-treated with 10 µM of ezetimibe. Mean survival was 91.1 ± 1.11 and 96.7 ± 0.00% in untreated control and 10 µM ezetimibe-treated group, respectively (p = 0.007) (Figure 1A). The survival of 50% of worms after UV irradiation was also increased from 6.4 d in untreated control to 7.8 d in 10 µM ezetimibe-treated group (p = 0.011) (Figure 1B).

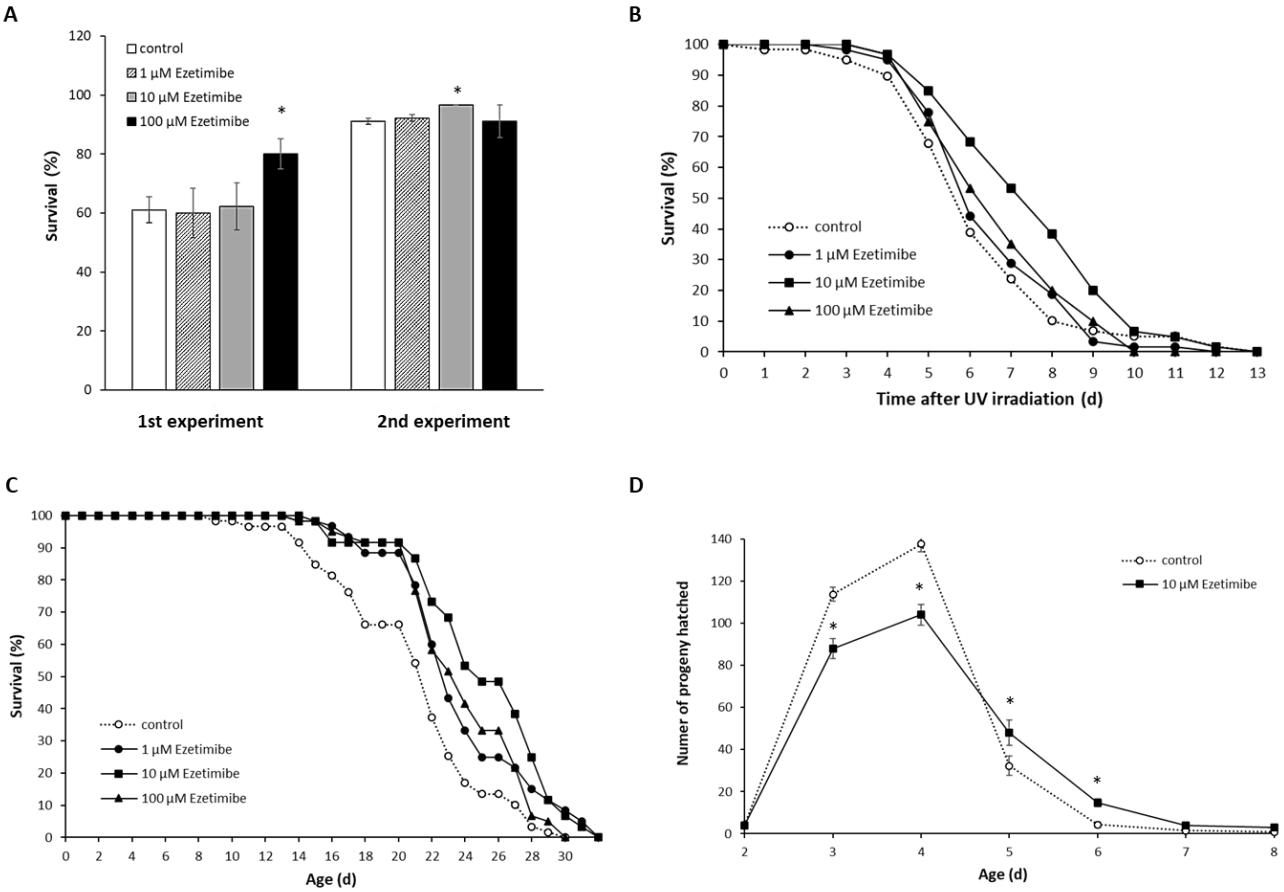

Figure 1. Effect of ezetimibe on stress response and lifespan of *C. elegans*. A - Survival under oxidative stress (n = 30) was determined in worms pre-treated with different concentrations of ezetimibe: 1, 10, and 100 µM of ezetimibe. B – Survival of worms after UV irradiation (n = 60) was monitored with or without ezetimibe treatment. C - Lifespan of worms supplemented with different concentrations of ezetimibe was compared with that of untreated control (n = 60). D - Effect of ezetimibe on progeny production was examined on each day during a gravid period. Values are averages of each group (n=12). Error bars indicate standard error. *, statistically significant (p < 0.05).

Ezetimibe confers longevity phenotype accompanying reduced fertility

Dietary supplementation with ezetimibe significantly increased lifespan in *Caenorhabditis elegans*. The mean lifespan of wild-type control was 20.9 d and that of 1 µM ezetimibe-treated groups was 23.8 d (p = 0.004), 25.2 d in 10 µM ezetimibe-treated group (p < 0.001), and 23.9 d in 100 µM ezetimibe-treated group (p = 0.008) (Figure 1C and Table 1).

Table 1. Effect of ezetimibe on lifespan in *C. elegans.*

	Ezetimibe (µM)	Mean lifespan (d)	*P* value[1)]	% effect[2)]
1st experiment	0	20.9		
	10	25.2	< 0.001	20.3
2nd experiment	0	19.7		
	10	22.6	< 0.001	14.6
3rd experiment	0	21.6		
	10	24.0	< 0.001	11.2

[1)] *P* value was calculated using the log-rank test by comparing the survival of untreated control group (0 µM ezetimibe) to that of ezetimibe-treated group (10 µM ezetimibe). [2)] % effects were calculated by (*C*-*E*)/*C**100, where *E* is the mean lifespan of ezetimibe-treated group and *C* is the mean lifespan of untreated control group.

Our findings based on environmental stressors and lifespan assay prompted the use of 10 µ M of ezetimibe in subsequent experiments. Many lifespan-extending interventions entail reduced reproduction as a trade-off (Johnson 1990; Gruber et al. 2007). The daily distribution of progeny during a gravid period was also affected by dietary supplementation with ezetimibe. Number of progenies decreased from 113.7 ± 3.29 in untreated control to 87.8 ± 4.81 in worms treated with ezetimibe on day 3. A similar significant reduction in fertility was observed in 4-day-old worms: 137.6 ± 3.78 in untreated control and 103.9 ± 4.98 in ezetimibe-treated group (p < 0.001). An opposite effect of ezetimibe was found in 5- and 6-day-old worms. In wild-type control, the reproductive ability decreased rapidly after day 4: the number of progenies was 32.3 ± 4.48 and 4.4 ± 1.28 on days 5 and 6, respectively. However, supplementation with ezetimibe slowed down this rapid decrease in fertility. In ezetimibe-treated worms, 48.0 ± 5.98 on day 5 and 14.7 ± 2.00 progenies were produced on day 6, which was a significant decrease compared with age-matched wild-type control (p < 0.05) (Figure 1D).

Ezetimibe increased cellular ROS and expression of stress-responsive genes

Cellular ROS levels were analyzed to verify whether the observed increase in survival under oxidative stress conditions was due to a decrease of ROS generation. Surprisingly, the cellular ROS level was increased by supplementation with ezetimibe. The relative ROS level compared with untreated wild-type control (100.0 ± 10.98) was 181.8 ± 9.05 in ezetimibe-treated worms (p < 0.001) (Figure 2A). Dietary supplementation with ezetimibe significantly increased the expression of both *hsp-16.2* and *sod-3*. There was a 1.5-fold increase in expression of *hsp-16.2* following supplementation with ezetimibe; the relative expression in ezetimibe-treated worms was 154.3 ± 6.29 compared with untreated control (100.0 ± 4.21) (p < 0.001). Expression of *sod-3* was also increased from 100.0 ± 3.69 in untreated control to 127.9 ± 4.51 (p < 0.001) (Figure 2B).

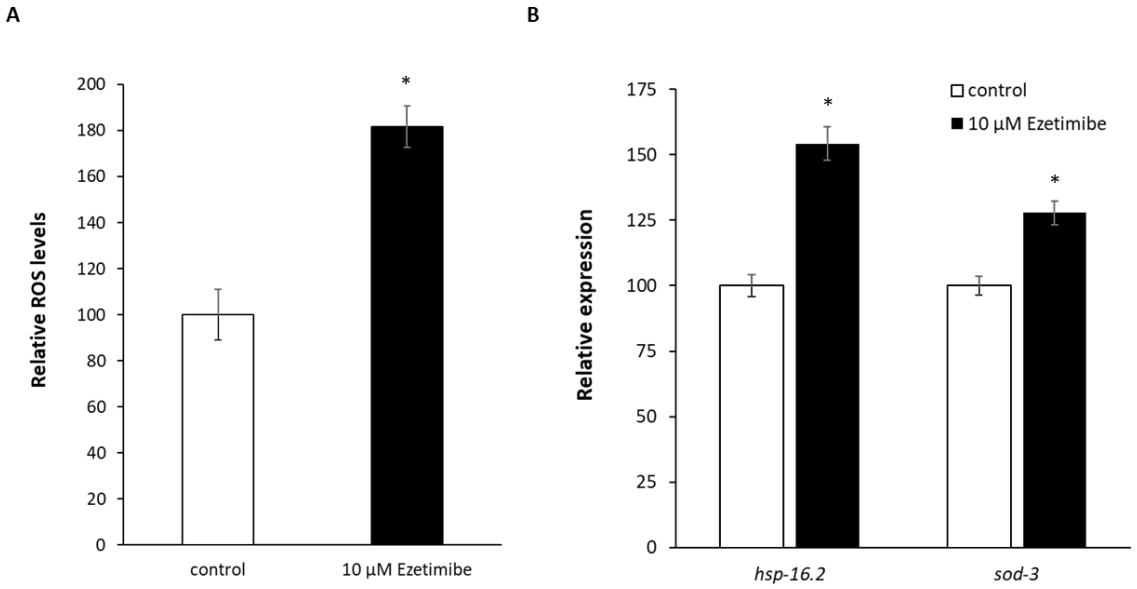

Figure 2. Altered cellular ROS levels and expression of stress-responsive genes by ezetimibe. A - Fluorescence intensity reflected cellular ROS levels was compared between untreated control and worms treated with 10 µM of ezetimibe in 7-day-old worms (n = 20). B - Expression of Green fluorescence protein (GFP) induced by *hsp-16.2* or *sod-3* promoter was monitored in 7-day-old worms treated with or without 10 µM of ezetimibe (n = 20). Values are averages of each group and error bars indicate standard error. *, statistically significant ($p < 0.05$).

Toxicity induced by Aβ and HGD was ameliorated by ezetimibe

Paralysis induced by accumulation of Aβ transgene was significantly delayed by ezetimibe treatment. The results showed a 33.9% increase in time required for the survival of 50% of worms paralyzed by Aβ accumulation from 7.3 h in untreated control to 9.8 h in ezetimibe-treated group ($p < 0.001$) (Figure 3A and Table 2).

Table 2. Effect of ezetimibe on Aβ-induced toxicity in *C. elegans.*

	Ezetimibe (µM)	Time when 50% of worms were paralyzed (h)	*P* value[1)]	% effect[2)]
1st experiment	0	7.3		
	10	9.8	< 0.001	33.9
2nd experiment	0	7.0		
	10	9.1	0.002	29.8
3rd experiment	0	13.0		
	10	14.2	0.03	9.4

[1)] *P* value was calculated using the log-rank test by comparing the rate of paralysis in untreated control group (0 µM ezetimibe) to that of ezetimibe-treated group (10 µM ezetimibe). [2)] % effects were calculated by (*C*-*E*)/*C**100, where *E* is the time when 50% of worms were paralyzed in ezetimibe-treated group and *C* is the time when 50% of worms were paralyzed in untreated control group.

HGD resulted in premature death as expected, as the mean survival time was 19.4 d in untreated control, which was reduced to 16.7 d by HGD treatment ($p < 0.001$). Simultaneous supplementation with HGD and ezetimibe completely prevented early death caused by HGD. Mean survival time of worms treated with both HGD and ezetimibe was restored to 19.3 d, which was significantly different from that of worms treated with HGD alone ($p < 0.001$) (Figure 3B and Table 3).

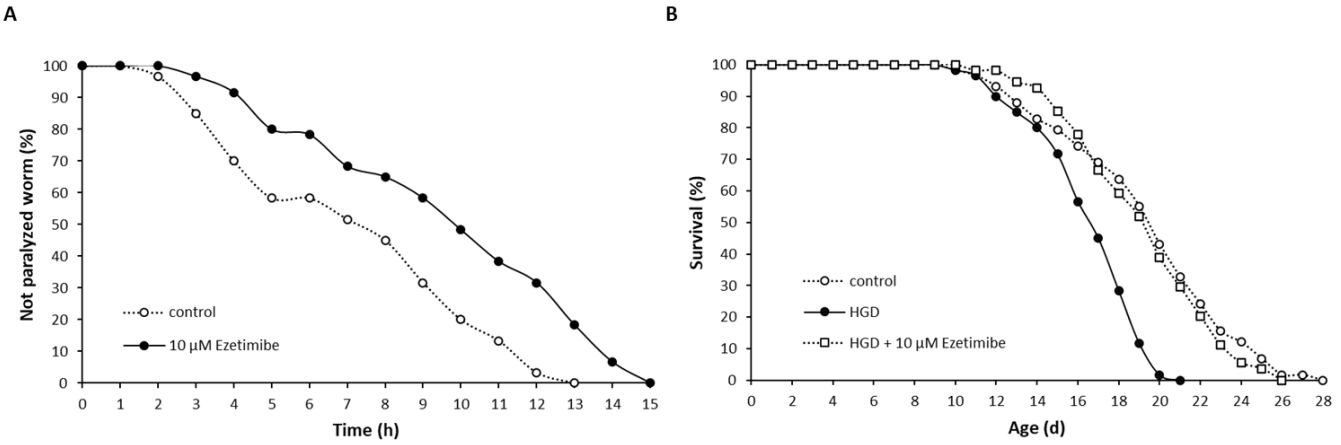

Figure 3. Reduced toxicity of Aβ transgene or HGD by ezetimibe. A - Paralysis occurred by muscle-specific induction of human Aβ transgene was compared between untreated control and worms treated with 10 μM of ezetimibe (n = 60). B - Survival curves of untreated control, HGD only-treated, and both HGD and 10 μM ezetimibe-treated worms were monitored until all worms were dead (n = 60).

Ezetimibe induced nuclear localization of DAF-16

A previous study showed that DAF-16 is required for prevention of Aβ-induced toxicity in *C. elegans* (Cohen and Dillin 2008). As shown in Figure 4, ezetimibe changed subcellular distribution of DAF-16. On day 5, more worms showed intermediate and nuclear localization and fewer worms showed cytosolic distribution with ezetimibe treatment. The percent of worms showing intermediate localization were 16.1 ± 6.96 and 45.0 ± 1.67 in untreated control and ezetimibe-treated worms (p = 0.016), respectively, which followed a decrease in the proportion of worms showing cytosolic distribution from 77.2 ± 13.62 in untreated control to 42.8 ± 8.07 in ezetimibe-treated worms (p = 0.095). A similar rapid nuclear localization of DAF-16 with ezetimibe treatment was observed in 7- and 9-day-old worms (Figure 4 and Table 4).

Table 3. Effect of high-glucose-diet and ezetimibe on lifespan of N2.

		Mean lifespan (d)	*P* value
1st experiment	N2	19.4	
	N2 + HGD	16.7	< 0.001[1)]
	N2 + HGD + Ezetimibe	19.3	< 0.001[2]
2nd experiment	N2	22.1	
	N2 + HGD	20.3	0.001[1)]
	N2 + HGD + Ezetimibe	21.3	0.031[2]
3rd experiment	N2	22.4	
	N2 + HGD	19.5	< 0.001[1)]
	N2 + HGD + Ezetimibe	23.6	< 0.001[2]

[1)] *P* value was calculated using the log-rank test by comparing to the survival of N2. [2)] *P* value was calculated using the log-rank test by comparing to the survival of N2 + HGD. HGD, high glucose diet (40 mM glucose); Ezetimibe, 10 μM of ezetimibe.

Table 4. Effect of ezetimibe on subcellular localization of DAF-16.

	Subcellular localization	Relative distribution (%)		
		Control	Ezetimibe	*P* value[1)]
Day 5	cytosolic	77.2 ± 13.62	42.8 ± 8.07	0.095
	intermediate	16.1 ± 6.96	45.0 ± 1.67	0.016
	nuclear	6.67 ± 6.67	12.2 ± 9.73	0.662
Day 7	cytosolic	64.2 ± 15.22	32.5 ± 13.50	0.171
	intermediate	26.3 ± 7.21	48.8 ± 7.12	0.068
	nuclear	9.6 ± 9.58	18.8 ± 12.20	0.576
Day 9	cytosolic	6.7 ± 2.89	8.3 ± 4.19	0.760
	intermediate	40.6 ±2.94	22.2 ± 4.94	0.033
	nuclear	52.8 ± 5.30	69.4 ± 8.68	0.177

[1)] *P* value was calculated using the Student's t-test; Ezetimibe, 10 uM of ezetimibe.

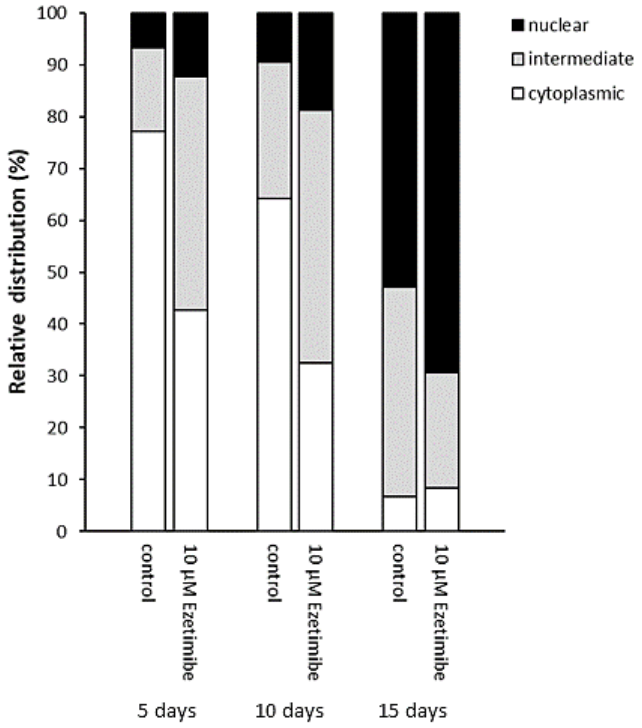

Figure 4. Accelerated nuclear localization of DAF-16 by ezetimibe. Subcellular distribution of DAF-16 was classified according to localization of GFP protein carrying *daf-16* promoter. Nuclear, fluorescence observed only in nucleus; Intermediate, fluorescence was observed both in nucleus and cytosol; Cytoplasmic, fluorescence was spread evenly in cells.

Lifespan-extending effect of ezetimibe specifically overlaps with that of DR

The lifespan of *age-1* was increased from 31.9 d in untreated control to 33.8 d in ezetimibe-treated group (p = 0.025) (Figure 5A). There was an additional lifespan extension following supplementation with ezetimibe in *clk-1* mutants. The mean lifespans were 17.5 and 20.8 d in untreated control and ezetimibe-treated group, respectively (p < 0.001) (Figure 5B). However, the lifespan of *eat-2* was not affected by ezetimibe treatment. The mean lifespan was 24.8 d in untreated control and 23.8 d in ezetimibe-treated group (p = 0.911) (Figure 5C and Table 5).

Table 5. Effect of ezetimibe on lifespan in long-lived mutants.

		Mean lifespan (d)		
		Control	Ezetimibe	*P* value[1)]
age-1 (*hx546*)	1st experiment	31.9	33.8	0.025
	2nd experiment	32.2	34.2	0.009
clk-1 (*e2519*)	1st experiment	17.5	20.8	< 0.001
	2nd experiment	18.2	20.8	< 0.001
eat-2 (*ad465*)	1st experiment	24.8	23.8	0.911
	2nd experiment	24.1	25.4	0.156

[1)] *P* value was calculated using the log-rank test by comparing the survival of untreated control group to that of ezetimibe-treated group. Ezetimibe, 10 μM of ezetimibe.

To confirm ezetimibe mimicked the effect of DR on lifespan, we analyzed the effect of ezetimibe on another method of DR. Limited food supply with diluted bacteria significantly increased lifespan; 22.9 d with AL diet and 25.1 d with DR diet (p = 0.008). Dietary supplementation with ezetimibe failed to result in additional lifespan extension in diet-restricted worms. The mean lifespan of worms treated with both DR and ezetimibe was 25.0 d, which was not significantly different from that of worms treated with DR alone (p = 0.680) (Figure 5D and Table 6).

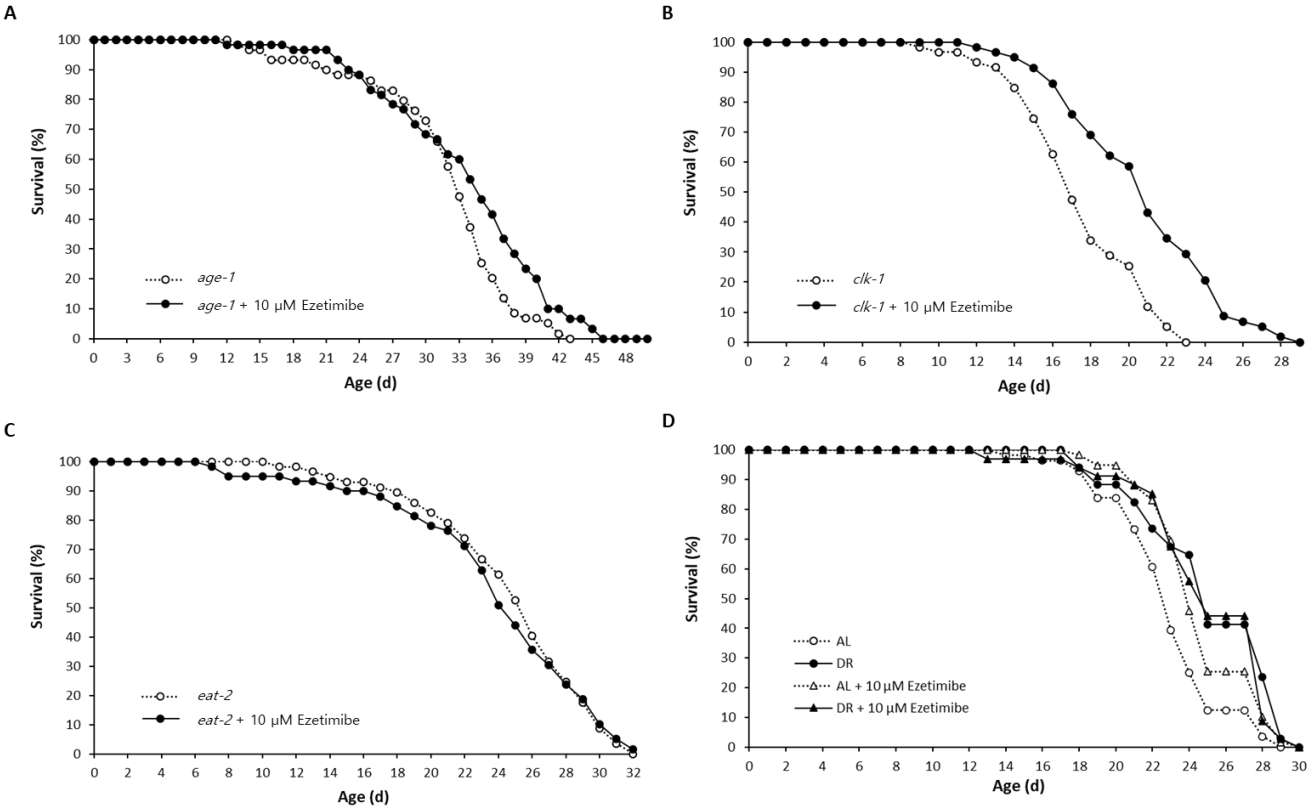

Figure 5. Effect of ezetimibe on long-live genetic mutants and diet-restricted worms. A - Survival of worms treated with or without 10 μM ezetimibe was compared in long-lived *age-1* mutant. B – The lifespan of *clk-1* was determined with or without 10 μM ezetimibe treatment. C – Effect of ezetimibe on the lifespan of *eat-2* mutant was examined. D - Lifespan assay was performed for worms fed with AL (*ad libitum*), DR, AL with 10 μM ezetimibe, and DR with 10 μM ezetimibe. AL, 5×10^9 bacteria/ml; DR, 5×10^8 bacteria/ml.

Table 6. Effect of ezetimibe and DR on lifespan in N2.

	Mean lifespan (d)	*P* value
AL	22.9	
DR	25.1	0.008[1)]
AL + Ezetimibe	24.6	0.025[1)]
DR + Ezetimibe	25.0	0.680[2)]
AL	24.1	
DR	26.3	0.052[1)]
AL + Ezetimibe	26.5	0.019[1)]
DR + Ezetimibe	26.3	0.705[2)]

[1)] *P* value was calculated using the log-rank test by comparing to the survival of AL. [2)] *P* value was calculated using the log-rank test by comparing to the survival of DR; AL, *ad libitum* (5×10^9 bacteria/ml); DR, dietary restriction (5×10^8 bacteria/ml); Ezetimibe, 10 μM ezetimibe.

4. Discussion

The free radical theory of aging suggests that accumulation of oxidative damage caused by ROS plays a pivotal role in aging and cellular anti-oxidant defense mechanisms can modulate detrimental damages (Harman 1956). Dietary interventions with anti-oxidants yielded promising results. Supplementation with resveratrol extended lifespan in yeast, nematode, fruit fly, and fish (Howitz et al. 2003; Gruber et al. 2007; Long et al. 2009). Vitamin E showed an age-dependent effect on the lifespan of *C. elegans* (Ernst et al. 2013). In *Drosophila melanogaster*, the effect of vitamin E on lifespan was dose-dependent, increasing both mean and maximum lifespan up to 15% (Ernst et al. 2013). In the present study, ezetimibe increased resistance to environmental stresses and extended the lifespan of *C. elegans*. The disposable soma theory suggests that cells decide to allocate limited cellular resources to cellular maintenance and reproduction according to their genetic or environmental conditions (Kirkwood 1977). The long-lived *age-1* mutants, exhibiting reduced insulin/IGF-1-like signaling, produced reduced progeny as a trade-off for longevity (Johnson 1990). Lifespan extension by supplementation with resveratrol also

accompanies a decrease in fertility (Gruber et al. 2007). Our data showed that time-course distribution of progeny during a gravid period were significantly affected by ezetimibe. Taken together, these findings suggest that ezetimibe confers longevity phenotype following reduced fertility as a trade-off. Further studies focusing on anti-aging effect of ezetimibe in other higher organisms are necessary in near future.

Hormesis is defined as beneficial effects of sub-lethal doses of substances, which are detrimental at higher doses (Calabrese et al. 1999). The lifespan of *C. elegans* was extended by 2 h of heat stress at 35°C or repeated exposure to mild heat stress throughout life (Cypser et al. 2006). Low dose of radiation induced longevity phenotype in fruit flies, mice, and rats (Rattan 2008). Dietary supplementation with ezetimibe increased cellular ROS levels and expression of *hsp-16.2* and *sod-3*. These findings suggest that increased resistance to oxidative stress and lifespan conferred by ezetimibe may be attributed to its hormetic effect. Many dietary interventions showing increased stress response and lifespan, such as resveratrol, curcumin, coenzyme Q_{10}, and alpha lipoic acid, exhibit dose-dependent hormetic effect (Rattan 2008). The underlying biological mechanisms involved in hormesis are not fully understood. However, dietary interventions extending lifespan via hormesis carry enormous potential in developing novel anti-aging nutriceuticals and therapies.

Lifespan assay revealed that the lifespan-extending effect of ezetimibe overlaps with that of *eat-2* mutation and there was no additional lifespan extension when DR and ezetimibe were administered concomitantly. These findings indicate that dietary supplementation with ezetimibe can mimic the effects of DR on lifespan without strict regulation of food intake. In addition, ezetimibe showed preventive effects in age-related disease models, as previously reported with DR. Dietary supplementation with resveratrol increased both mean and maximum, which requires activation of sirtuin, a mediator of DR response (Howitz et al. 2003; Wood et al. 2004). A transcriptional profiling study revealed that resveratrol mimics the effect of DR in mouse liver (Barger et al. 2008). Metformin, a common drug used to manage type 2 diabetes, significantly increased lifespan in *C. elegans*, via a mechanism overlapping with that of DR (Onken and Driscoll 2010). Lifespan extension by metformin requires SKN-1, a transcription factor promoting DR-induced longevity and oxidative stress response (Onken and Driscoll 2010). In mice, metformin induced alterations in transcriptional profile similar to long-term DR (Dhahbi et al. 2005). Since the effect of DR mimetic is not universal, additional studies focusing on the effect of ezetimibe on lifespan and age-related pathophysiological changes in other model organisms are needed in the near future. In addition, the elucidation of the underlying mechanisms involved in DR-mimicking effects of ezetimibe is necessary for the development of an efficient and safe DR mimetic.

5. Conclusions

Dietary supplementation with ezetimibe survivals after oxidative stress or UV irradiation and significantly extended lifespan in C. elegans. The increased longevity phenotype conferred by ezetimibe accompanied reduced fertility as a trade-off. Expression of stress responsive genes was up-regulated while cellular ROS levels were increased with ezetimibe treatment. Ezetimibe showed beneficial effects in disease model of AD and diabetes. Genetic analysis revealed that the effect of ezetimibe on lifespan specifically overlapped with that of DR. In conclusions, ezetimibe exhibits anti-oxidative and anti-aging effects through hormesis and works as a dietary-restriction mimetic on lifespan extension.

Authors' Contributions: PARK, S.K.: conception and design and drafting the article; PARK, S.: acquisition of data and analysis and interpretation of data; PARK J.S.: acquisition of data and analysis and interpretation of data. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

Acknowledgments: This work was supported by the Soonchunhyang University Research Fund and the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (2021R1F1A105671911).

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Received: 4 April 2022 | **Accepted:** 20 September 2022 | **Published:** 24 February 2023