The health benefits of cruciferous vegetables as an antioxidant: Sulforaphane improves oxidative stress response in *Caenorhabditis elegans* via SKN-1

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Abstract

Sulforaphane (SFN) is a compound within the isothiocyanate group of organosulfur compounds and found in cruciferous vegetables, such as broccoli, Brussels sprouts, and cabbages. Many health benefits of SFN, such as anti-inflammatory effects, anti-carcinogenic effects and stress resistance have been reported; however, limited research has been conducted on the effects of SFN on aging, obesity, and oxidative stress resistance. Therefore, this study aimed to investigate the effect of SFN on aging and obesity, as well as its effects on stress responses using the animal model *Caenorhabditis elegans* (*C. elegans*). The results showed that 200 μM SFN delayed growth and development in *C. elegans* ($P = 0.0462$) and decreased length by 12% ($P < 0.0001$). SFN provided oxidative resistance ($P < 0.0001$) and promoted the nuclear translocation of SKN-1 in the intestines ($P < 0.01$) . However, both 100 μM and 200 μM SFN did not extend the lifespan of *C. elegans* and did not reduce fat accumulation. SFN reduced oxidative stress in *C. elegans*, and post-translational regulation of SKN-1 may be involved in this activity.

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Introduction

Sulforaphane (SFN) is from the isothiocyanate group of organosulfur compounds and is found in cruciferous vegetables such as broccoli, Brussels sprouts, and cabbages. SFN's cytoprotective effects are known to be associated with the upregulation of Nuclear factor E2-related factor 2 (Nrf2).[1] Nrf2 falls under the ortholog of SKN-1, which is a transcription factor and aids in oxidative resistance. The majority of previous research with SFN has focused on its anticarcinogenic effects: SFN has protective effects in normal human cell cultures, while it diminishes their development and promotes apoptosis in cancer cells by increasing the production of reactive oxygen species (ROS).[1] Nrf2, a proto-oncogene, promotes cell proliferation and blocks apoptosis, but in some carcinomas Nrf2 does not take on the role of an oncogene due to SFN's anti-carcinogenic properties; however, in others SFN may induce pharmacological resistance by supporting Nrf2.[1] Additional studies have shown that SFN can induce Nrf2 nuclear translocation and reduce the translocation of pro-inflammatory proteins in mice, and reduce ROS production (which lightens oxidative stress).[2]

The effects of SFN on metabolism have been also extensively studied in rodents: SFN improved lipid accumulation and insulin levels to mitigate some of the effects of diabetes[3]; SFN improved glucose metabolism[4] and helped prevent the rise of mean arterial blood pressure (lowered hypertension).[5] SFN has also been found to have neuroprotective effects by increasing neural stem cell proliferation,[1] have positive effects in experimental Huntington disease because it can cross the blood-brain barrier,[6] prevent the quinolinic acid-induced mitochondrial degradation[6] and protect the brain of Wistar rats against neuroinflammation.[7] In addition, Whitman et al. found that there is a positive correlation between SFN-induced Nrf2 activation and PGC-1α, which has been shown to be a key regulator of energy and muscle metabolism.[8] In order to test SFN's effects on other biological functions, the animal model *Caenorhabditis elegans (C. elegans)* was used.

C. elegans is a free-living nematode found in temperate soil environments. It is a commonly used animal model for research involving aging, obesity and neurodegenerative diseases. Wild-type worms have a lifespan of about 21 days and reach adulthood within 48 hours after hatching at 25℃. Their large brood size of 300 progeny per hermaphrodite allows for

efficient experimentation of bioactive compounds in *C. elegans*. The animal model can grow in both liquid and solid medium and uses non-pathogenic *Escherichia coli* (*E. coli*) OP50 as food. It was the first animal model to have its full genome sequenced, and over 65% of its genes are associated with human diseases. Research involving *C. elegans* does not necessitate approval by the Institutional Animal Care and Use Committees. The use of *C. elegans* is encouraged for research purposes by the National Institutes of Health, which funds the Caenorhabditis Genetics Center at the University of Minnesota.[9] We used this animal model to determine the role of SFN in aging, obesity and oxidative stress resistance.

Aging is commonly attributed to the accumulation of detrimental changes occurring in cells and tissues that are responsible for the increased risk of disease and death.[10] Aging contributes to the development of many chronic diseases, such as neurodegenerative and metabolic syndromes, cancer and cardiovascular diseases.[11] Aging has also been shown to contribute to the development of obesity.[12] Furthermore, oxidative stress has been reported to increase in elderly subjects, potentially arising from the uncontrolled production of free radicals by aging mitochondria and decreased antioxidant defenses.[13,14] For these reasons, the effect of the dietary supplementation with antioxidants on aging has a growing interest.

Obesity is often associated with the excessive growth of adipose tissue due to an imbalance between energy intake and expenditure. It is associated with the development of chronic diseases and cardiovascular diseases. A variety of factors can lead to obesity, such as genetic traits, the environment or lifestyle.[11] Previous studies have shown that obesity is linked to aging because they share similar cellular and metabolic dysregulation.[15] Therefore, to examine the effect of SFN on obesity in the *C. elegans* model, the triglyceride level in the nematodes was measured because obesity arises when energy intake, mainly stored as triglycerides, exceeds energy expenditure.

Previous research on SFN's anticarcinogenic and positive metabolic effects make it an intriguing compound to investigate in the field of food science. However, no research in *C. elegans* has been conducted on the effects of SFN on aging, obesity, and stress resistance, specifically oxidative stress. Therefore, the purpose of this study was to examine SFN's effect on these parameters in the *C. elegans* model.

Materials and Methods

Materials

Chemicals used were from Fisher Scientific (Pittsburgh, PA, USA) unless said otherwise. 2'-Deoxy-5-fluorouridine (FUDR) was from TCI America (Portland, OR, USA), carbenicillin was from Fisher Bioreagents (Pittsburgh, PA, USA), ampicillin was from Sigma-Aldrich Co. (St. Louis, MO, USA). All the nematode strains: N2 wild-type, TJ356 (zls356 IV) and LD1 [ldIs7] were obtained from the Caenorhabditis Genetics Center, University of Minnesota, USA.

C. elegans culture

Nematode growth medium (NMG) plates, S Medium and M9 were prepared according to Wormbook instructions.[16] Synchronized populations of N2 wild-type were obtained by isolating eggs and growing in 25℃, except *skn-1* mutant which was grown at 20℃.

Lifespan and oxidative stress assay

For the lifespan analysis, L4 synchronized N2 wild-type nematodes were put in 96 well plates with 6 mg/mL of *E. coli* OP50, 100 μg/mL of ampicillin, 50 μg/mL of carbenicillin and 120 μM of FUDR. Worms were treated with 2 μL of 0.1% dimethyl sulfoxide (DMSO) as control or SFN (100 μM or 200 μM). To determine SFN's effect on oxidative stress resistance, L4 synchronized N2 wild-type *C. elegans* were treated with 5 mM paraquat and either 0.1% DMSO or SFN (100 μM or 200 μM). Nematode survival was recorded every day until all the worms died, with day one being two days post-L4 treatment with SFN. The medium was changed weekly for the lifespan assay, and not changed at all for the oxidative stress assay since the predicted lifespan was short; by not changing the media in the oxidative stress assay, the added variability of drying out/adding new media on the health of the *C. elegans* was reduced.

Triglyceride and protein assay

C. elegans samples were washed with 0.05% Tween®20 and sonicated on ice for 2 minutes at 50% amplitude. Infinity Triglycerides Reagent from Thermo Fisher Scientific Inc. (Pittsburgh, PA) was used to determine fat accumulation. BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) was used to determine the protein content in nematodes so the triglyceride data could be normalized. Photo colorimetric assays were performed using SoftMax Pro 6.5 in a microplate reader to determine triglyceride and protein levels.

Development and growth assay

To analyze the effect of SFN on development in *C. elegans,* synchronized L1 worms were cultured on NGM plates at 20 °C for 48 h. Then, sodium azide (NaN_3) at a final concentration of 10 mM was added to nematodes. A subjective analysis was conducted to determine the number of worms at each developmental stage (most usually around L4 stage), with each result expressed as % of worms at each stage. To determine SFN's effect on growth (post-48 hour treatment), body size was analyzed using the WormLab tracking system (MBF Bioscience). An illuminator and digital camera set were used with the WormLab tracking system. Nematodes were recorded on low-peptone NGM plates that were seeded with drops of OP50 bacteria 10 minutes before tracking. Dead *E. coli* OP50 was used to eliminate the variable of the *E. coli* metabolizing the compound.[17] Worms were added to the plate and allowed to acclimate to the light 20 minutes before tracking. A 1-min recording (7.99 frames per second) was captured. Then, WormLab software was used to track the moving behavior of 45-55 animals per treatment. After that, the average worm size (length and width) were analyzed using the software (Fig. 1).

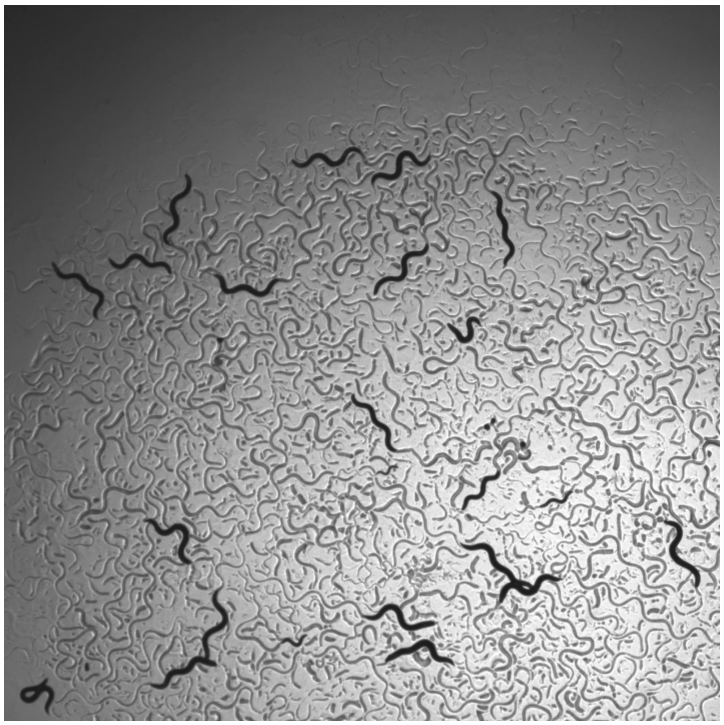


Fig. 1: Image captured by the WormLab tracking system of *C. elegans* on low-peptone NGM plate.

Locomotive behavior and pumping rate assay

To determine the pumping rate, the number of pharyngeal contractions of 10 randomly chosen nematodes was counted under the microscope for 30 seconds. Locomotive behavior was analyzed using the WormLab tracking system as described in the "development and growth assay," except the average worm speed and maximum amplitude (largest curve in their body while moving) were analyzed. These assays were double-blind; during this experiment, it was not known which nematodes were treated with SFN and which were in the control group to minimize bias and variability.

Nuclear translocation of SKN-1::GFP

Nuclear translocation is a process in which cytoplasmic proteins are transported into the cell nucleus. The transgenic strain LD1 [ldIs7 (skn-1b/c::GFP;rol-6] was used to determine the

intracellular translocation of SKN-1::GFP. Adult nematodes were pre-treated one day before experimentation with 200 μM SFN. Worms were placed on an agarose pad on microscope pads, treated with 50 mM sodium azide (10 mM) and capped with a coverslip. Using the Fluorescence Microscope (Nikon Eclipse Ti-U), 15 pictures were taken per treatment and analyzed using a chi-squared test. Through subjective analysis, the worms exhibited either low, medium or high nuclear translocation in SKN-1::GFP worms.

Polymerase Chain Reaction (PCR)

RNA was extracted using trizol, chloroform and isopropanol. cDNA was obtained using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.), and Quantitative Real-Time PCR (qrt-PCR) was carried out using StepOnePlus™ Real-Time PCR system (Applied Biosystems). The internal control was *ama-1* (Ce02462726-m1), and the target gene was *skn-1* (Ce02407444-m1). The results were analyzed using the comparative Ct (fold change) method.[18]

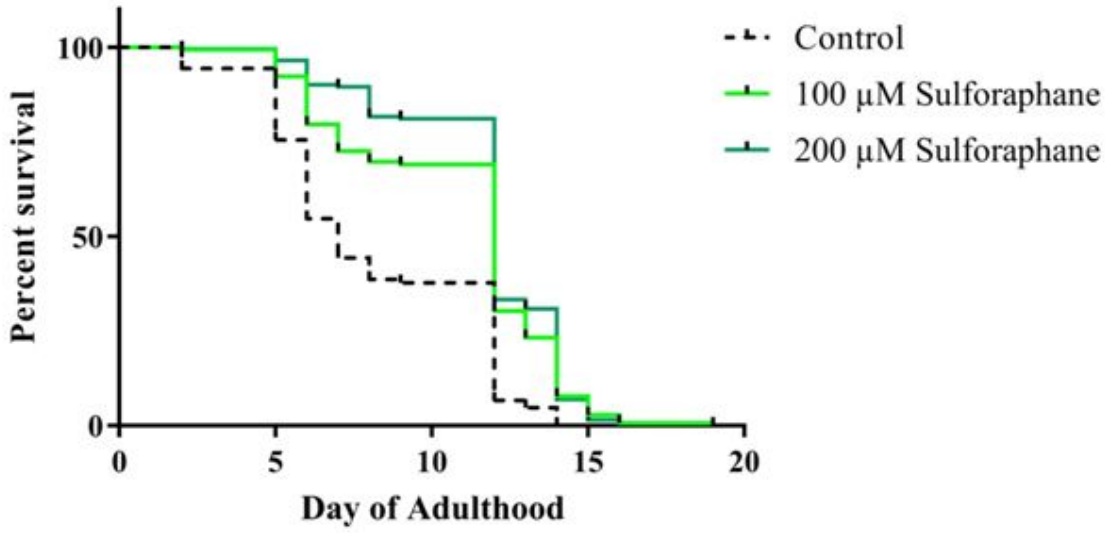
Statistical Analysis

Using the Statistical Analysis System (SAS®) software, the data were expressed as the mean ± standard error (S.E.). Significance between groups was determined with a one-way ANOVA, and defined if $P < 0.05$. Logrank tests in GraphPad Prism version 7.4 were performed to analyze the survival curves.

Results

Sulforaphane provides oxidative stress resistance in *C. elegans*

The worms were treated with both 100 µM and 200 µM SFN when given paraquat to induce oxidative stress. Both concentrations significantly improved oxidative stress response, which was visible through their increased lifespan, compared to the control group ($P < 0.0001$ by the logrank test; Fig. 3). There was no significant difference found between 100 µM and 200 µM SFN. Both these concentrations caused a ≅36% increase in median lifespan compared to the control.



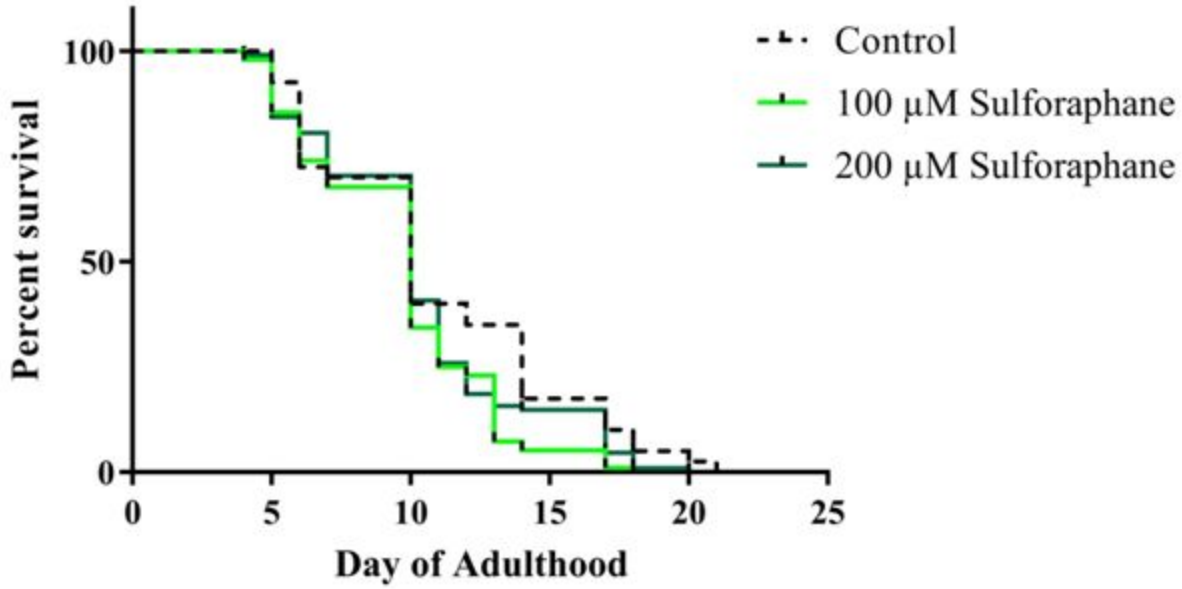
Sulforaphane	Control	100 µM	200 µM
Median survival	7	12	12
Maximum Survival	14	19	19

Fig. 3: Survival curve of wild-type (N2) *C. elegans* treated with SFN and 5 mM paraquat. Nematodes were treated with SFN (100 µM and 200 µM) and paraquat starting from the L4 stage (day 0) and the survivals were recorded every other day until all the worms died. Log-rank analysis showed that SFN-treated worms did live longer than the control group ($P < 0.0001$). n = 106-201/group.

Sulforaphane has no effect on lifespan and fat accumulation

To determine if SFN affected lifespan, concentrations of 100 µM and 200 µM were tested (Fig. 4A). Neither concentration had a significant effect on the median lifespan compared to the control.

To test if SFN affected fat accumulation, the worms were treated with 100 µM and 200 µM SFN along with a glucose and no glucose treatment. Triglyceride analysis indicated that glucose increased fat accumulation but SFN had no impact on triglyceride levels (Fig. 4B).



Sulforaphane	Control	100 µM	200 µM
Median survival	10	10	10
Maximum Survival	21	18	20

Fig. 4A: Survival curve of wild-type (N2) *C. elegans* treated with SFN. Nematodes were treated with SFN (100 µM and 200 µM) starting from the L4 stage (day 0) and the survivals were

recorded every other day until all the worms died. Log-rank analysis showed that SFN-treated worms did not live more than the control group. n = 40-108/group.

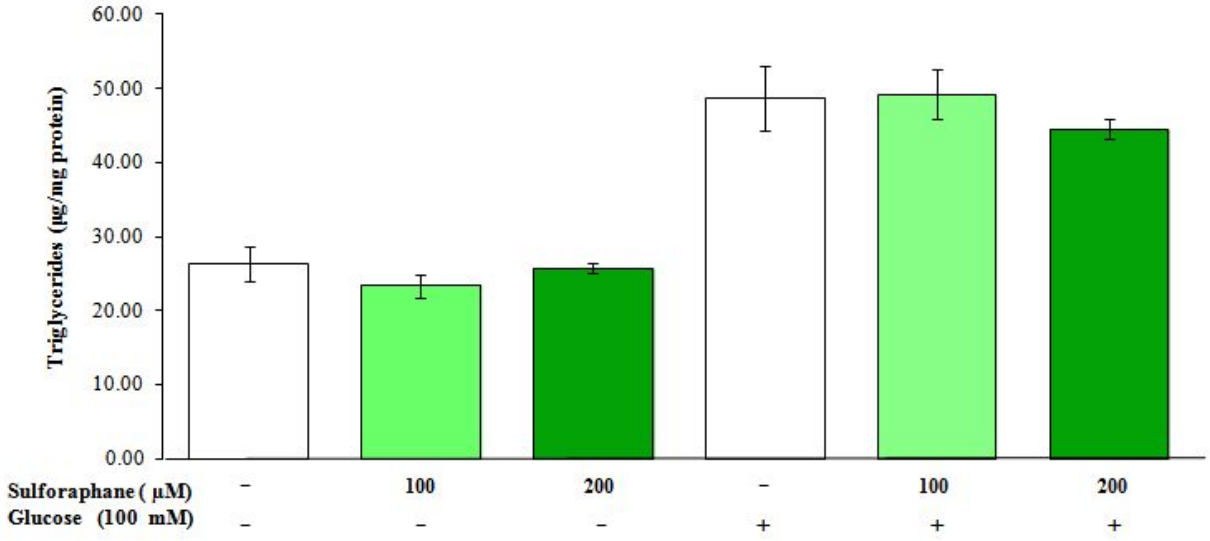


Fig. 4B: SFN had no effect on fat accumulation compared to the control. When wild-type (N2) *C. elegans* were treated with glucose, the overall triglyceride levels were significantly higher compared to the no glucose treatment. n = 4/group

Sulforaphane delays development and growth

The *C. elegans* physiological functions were analyzed to investigate whether SFN had any effects on the growth rate, worm size or locomotive behavior of the nematodes. After 48 hours of incubation of L1 stage worms, 85.7% of the nematodes treated with 200 µM SFN were L4, 7.6% were L3, 1.3% were L2 and 5.5% were L1. In the control treatment, 92.6% were L4, 6.8% were L3 and 0.6% were L2 ($P < 0.05$; Fig. 5A). This indicates that 200 µM SFN delayed the growth rate of *C. elegans*.

Worm size information was obtained by the WormLab tracking system, and it was found that SFN delayed the growth of L1 stage nematodes after 48 hours of treatment with SFN ($P < 0.001$; Fig. 5B). SFN did not, however, effect locomotive behavior. Altogether, the data suggest that 200 µM SFN does alter some physiological states in *C. elegans*.

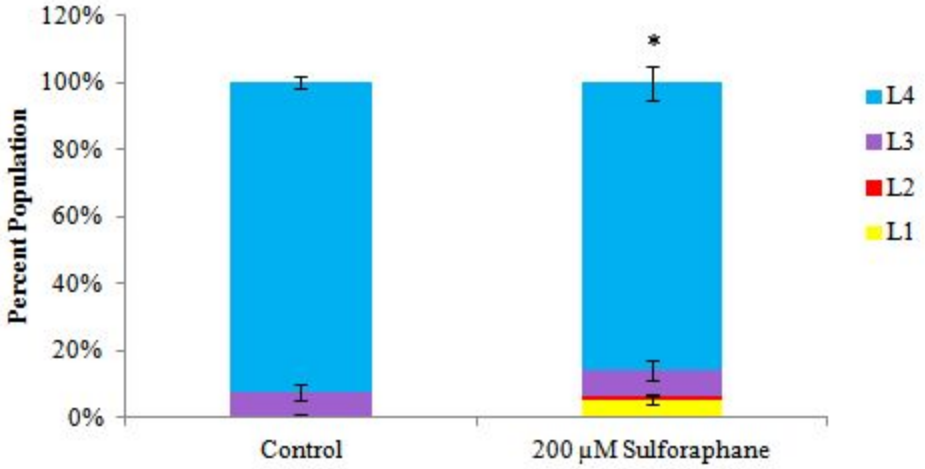


Fig. 5A: 200 µM SFN delayed the growth rate of synchronized L1 stage *C. elegans*, resulting in more L1 and L2 stage worms when treated with SFN than in the control (* $P \leq 0.05$). n = 2 collected from 57-128 worms

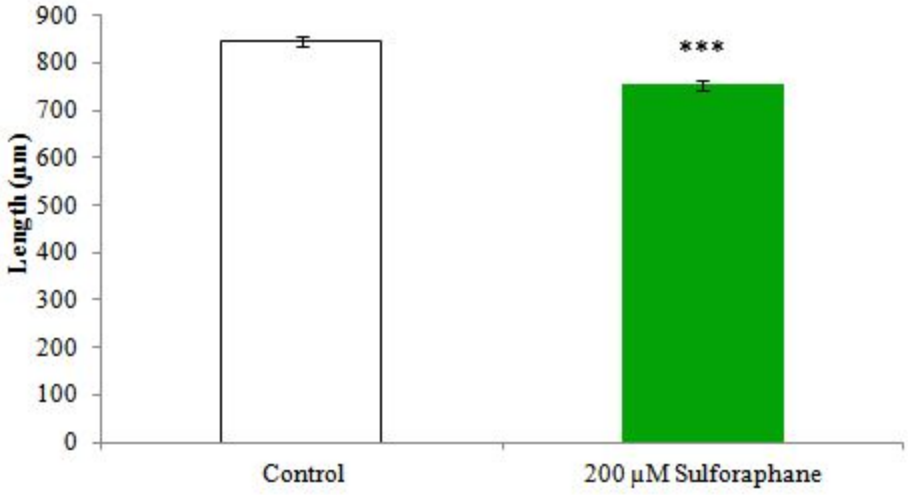


Fig. 5B: 200 µM SFN delayed growth in L1 stage worms compared to the control (*** $P \leq 0.001$). n = 62-66 nematodes.

Sulforaphane does not affect age-related phenotypes

The effect of SFN on pumping rate and locomotive behavior, two age-related phenotypes[13], were examined. 200 µM SFN (both with and without glucose) had no effect on the pumping rate in nematodes compared to the control (Fig. 6A). Furthermore, SFN did not affect the energy expenditure in the worms, with and without glucose, compared to the controls. This was represented through locomotive behavior (speed and maximum amplitude) and determined using the WormLab tracking system (Fig. 6B).

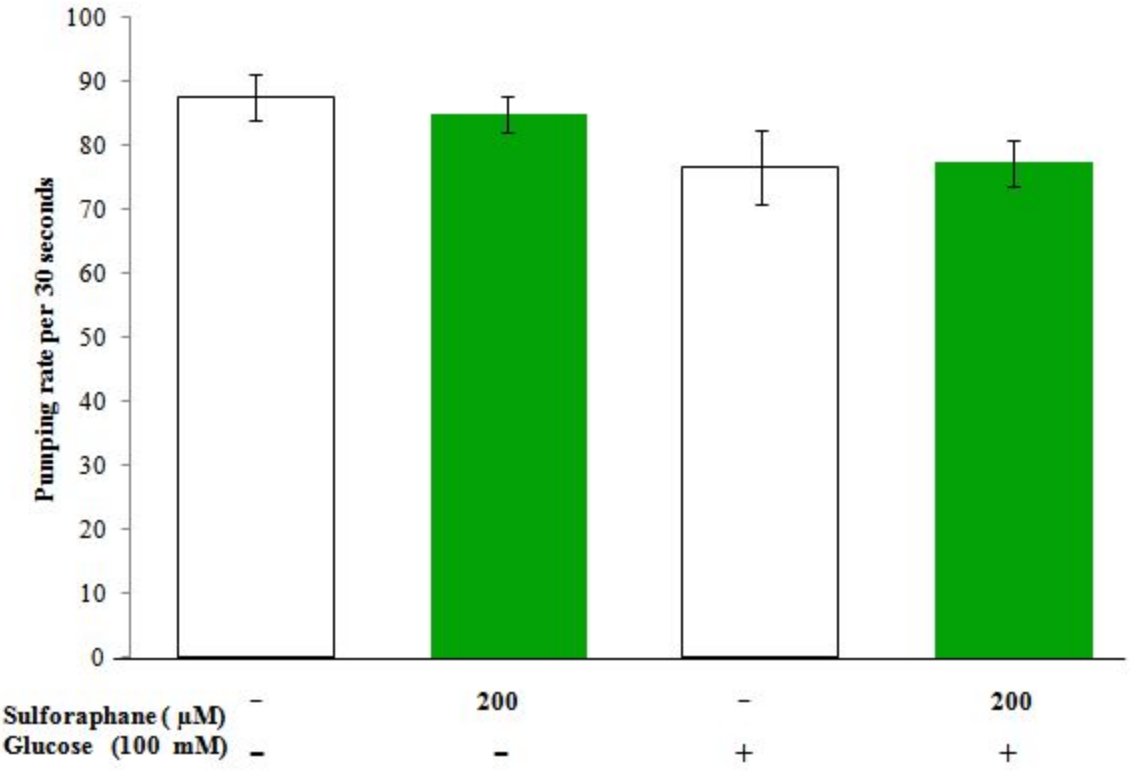


Fig. 6A: Synchronized L1 *C. elegans* treated with 200 µM SFN exhibited no change pharyngeal pumping compared to the control. n = 10

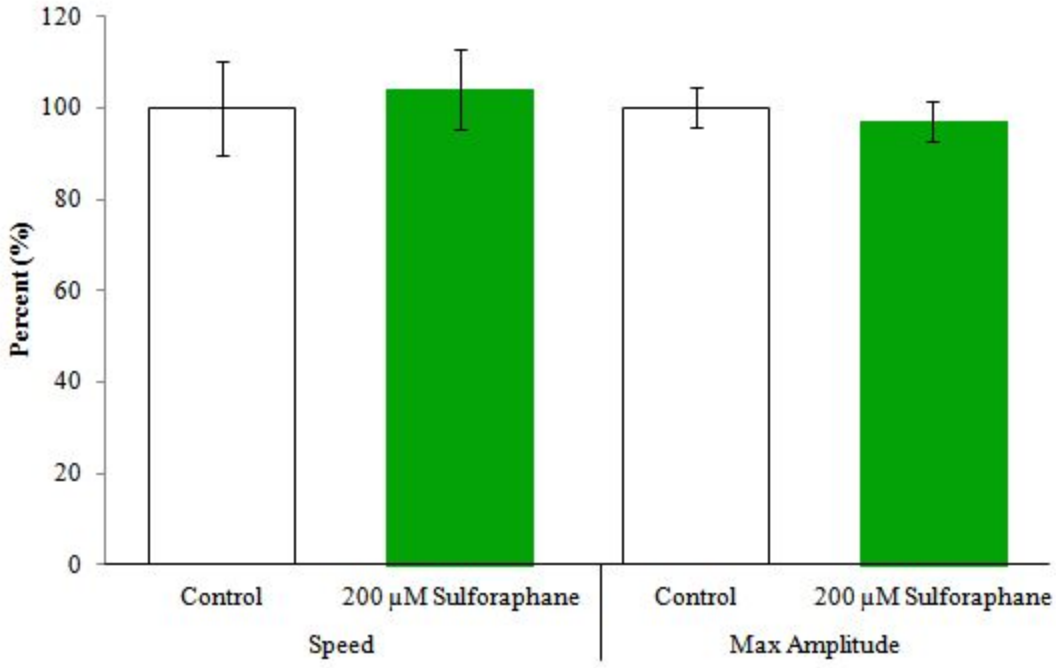


Fig. 6B: 200 µM SFN had no effect on the locomotive behavior (speed and maximum amplitude) of *C. elegans* compared to the control. n = 62-66 nematodes.

Sulforaphane increases SKN-1 nuclear translocation

SFN cytoprotective effects are known to be associated with the upregulation of Nuclear factor E2-related factor 2 (Nrf2).[1] Since *C. elegans* have SKN-1, the ortholog of Nrf2, experiments were conducted to determine if SKN-1 was responsible for SFN's positive improvement of oxidative stress response, since previous studies have reported that SKN-1 and Nrf2 promotes oxidative stress resistance.[1] First, the effect of SFN on the mRNA levels of the *skn-1* gene in *C. elegans* was determined using qrt-PCR (Fig. 7A). However, the results were insignificant, which suggests that SFN does not impact mRNA amount. Then, using a transgenic strain of SKN-1, which expressed a Green Fluorescent Protein (GFP), nuclear translocation of SKN-1 was used to determine if SFN regulated SKN-1 functions (Figs. 7B-7C); 18% of nematodes in the control group exhibited nuclear translocation compared to 44% in the 200 µM SFN -treated group ($P < 0.01$; Fig. 7D), indicating that SFN's positive effect (specifically with oxidative stress resistance) comes from the post-translational regulation of SKN-1.

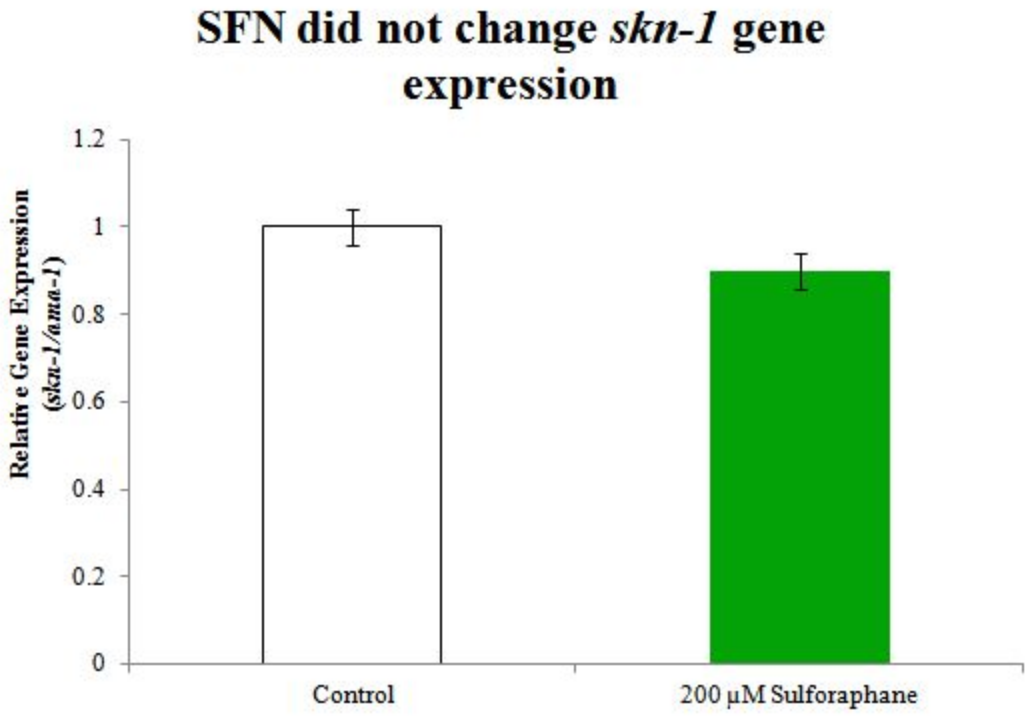


Fig. 7A: Through qrt-PCR, it was determined that 200 µM SFN had no effect on the mRNA levels of the *skn-1* gene in *C. elegans* compared to the internal control (*ama-1*). n = 3

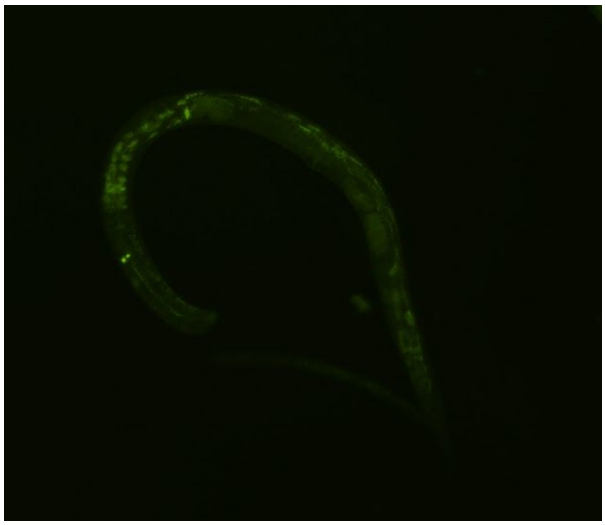


Fig. B: SKN-1::GFP nematode treated with 0 µM SFN

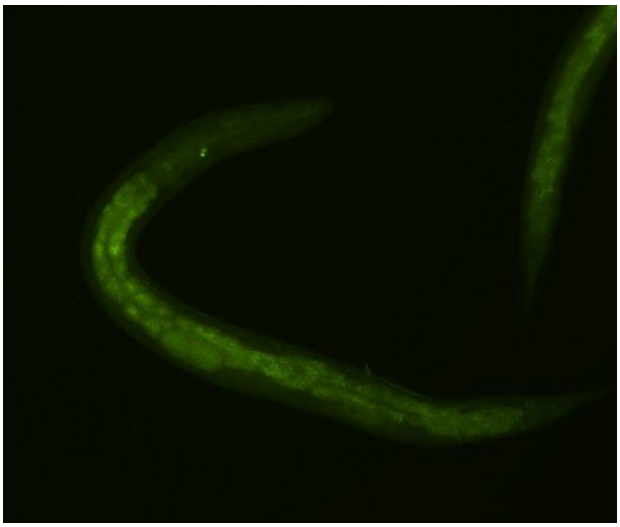


Fig. C: SKN-1::GFP nematode treated with 200 µM SFN

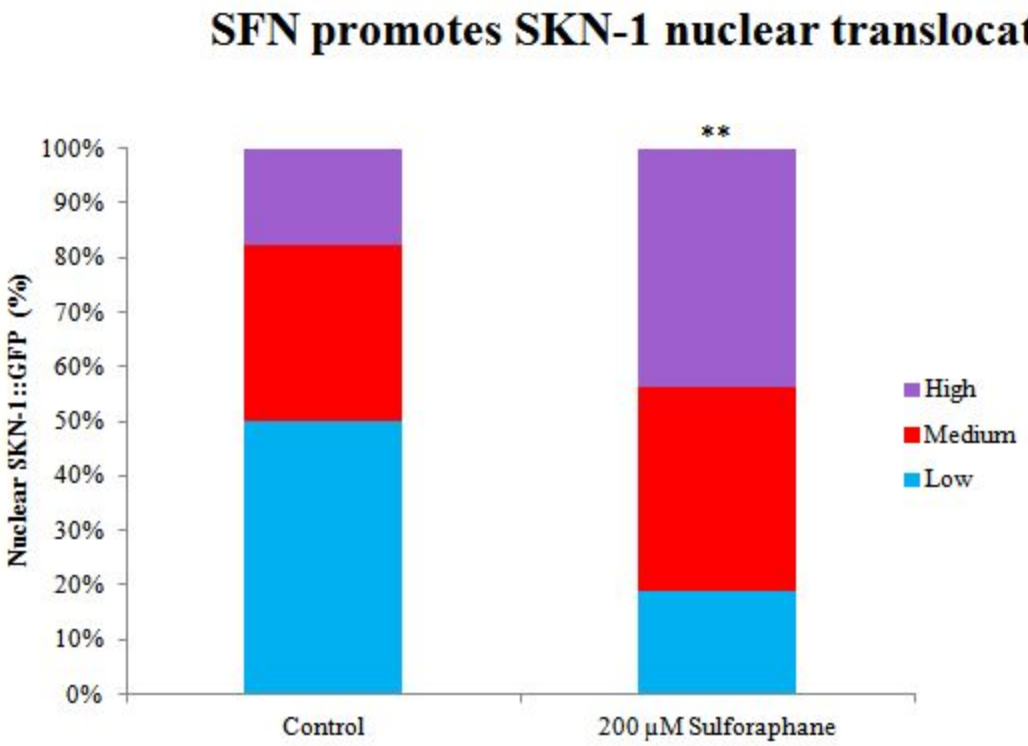


Fig. 7D: 200 µM SFN induced nuclear translocation of SKN-1: 18% of nematodes in the control group exhibited nuclear translocation compared to 44% in the 200 µM SFN-treated group (** $P \leq 0.01$). The stacked graph represents the percent of worms exhibiting each type of nuclear translocation (high, medium and low). n = 67-83

Discussion

Through these experiments, it was determined that SFN improved the oxidative stress response in *C. elegans*. SFN did not influence the lifespan or triglyceride amounts in the nematodes. Furthermore, SFN delayed development and growth in the worms, yet did not impact pumping rate or locomotive behavior, which are two age-related phenotypes. The nuclear translocation of SKN-1 due to SFN was observed, indicating that SFN's cytoprotective effects are enhanced by this protein.

Previous studies had reported that SFN improved the oxidative stress response in rodent models,[2] which means that this result can also be applied to the *C. elegans* model. Furthermore, because SFN does not impact the RNA levels of *skn-1* in the nematode but affects the protein amount SKN-1, a conclusion can be drawn that SFN impacts the post-translational regulation of SKN-1. Therefore, because previous studies have determined that SFN's cytoprotective effects come from the upregulation of Nrf2[1], which is in the ortholog of SKN-1, SFN's positive effects may come from that post-translational regulation of SKN-1.

The statistically significant result that SFN appears to delay growth and development implies that it may have a toxic or negative effect on the cells in *C. elegans*, impacting some phase of their growth that causes them to be smaller and take longer to reach their expected size. This may be explained by SFN's previously noted effect of inducing apoptosis in cancerous cells[1]; *C. elegans* is considered an animal model for human cancer research given that many pathways and human genes involved in cancer are conserved in the nematodes.[19] If certain cell mutations in the worms cause SFN to perceive the cells as cancerous, it may cause apoptosis to occur in those cells and thus delay growth and development. However, the cause of this delay could simply be that some pathway in the *C. elegans* was either being upregulated or downregulated because of the SFN. More testing is needed to draw a firm conclusion.

SFN had no statistically significant effect on lifespan or fat accumulation, which means that it does not reduce the nematodes lifespan or reduce triglyceride amounts. While the data is insignificant, it also suggests that SFN does not have any toxic effects in the *C. elegans*. Additionally, SFN did not cause a decrease in energy expenditure. Higher energy levels would result in a lower lifespan and triglyceride levels, whereas a decrease in energy levels would cause

a longer lifespan and higher triglyceride levels. Therefore, the insignificant energy expenditure data support the previously mentioned results that SFN had no impact on lifespan or fat accumulation.

There are limitations within this study to be addressed, such as with the SKN-1 nuclear translocation assay. The *skn-1* and *ama-1* primers used for qrt-PCR may have certain sensitivities unknown prior to experimentation that resulted in insignificant data. Furthermore, the nuclear translocation of SKN-1 may cause the GFP amounts to appear higher. Because few experiments have implemented this procedure, the subjective analysis may have limited conclusions due to possible errors. Also, due to sulforaphane's importance of oxidative stress resistance in the nematodes, it should also have elongated lifespan[1]; however, this was not the case, suggesting procedural errors. Lastly, the sulforaphane used in this treatment itself has many possible limitations given the experimentally recorded instability of the compound in certain temperatures, pHs and when in various solvents and formulations.[20]

Future experiments should further look into connections between skn-1 and growth and development in larvae and adult stage *C. elegans*, determining if development is halted by SFN. Since SFN promotes apoptosis in cancerous cells, it may also promote apoptosis in the nematodes. Additionally, looking at SFN 's effect on ROS production would confirm and further explain its positive oxidative stress response, especially by looking at SFN's effect on SKN-1::GFP mutant worms.

In conclusion, the findings from this study indicate that SFN improves oxidative stress response in *C. elegans* through post-translational regulation of SKN-1. Because SKN-1 is the ortholog of Nrf2, and human beings have the Nrf2 gene, the findings from these experiments could have beneficial implications for humans consuming SFN.

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