A Novel Determination of the Relationship Between Exposure to Industrial Toxicant HFPO-DA and *pqm-1*-related Aging in *C. elegans*

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Research

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Abstract

Hexafluoropropylene oxide dimer acid (HFPO-DA, ammonium salt with trade name “GenX”) is an industrial toxicant that has recently been detected in the environment [1]. However, HFPO-DA’s potential aging-related effects on organisms of higher trophic levels, including worms and humans, have not been extensively explored. The purpose of this study is to quantify influences on *C. elegans* (free-living nematode) lifespan by HFPO-DA exposure, specifically via ingestion of food to simulate the mechanisms of toxicant exposure, through lower trophic-level organisms, commonly found in nature. *C. elegans* N2 (wild-type) samples were prepared with a uracil-based medium and *E. coli* OP50 (food source) at room temperature; *C. elegans* in the experimental, treated sample was fed *E. coli* OP50 incubated with 280 ng/L HFPO-DA. The target gene *pqm-1* was selected due to its role in an evolutionarily conserved insulin signaling pathway and in promoting development. Molecular biology laboratory techniques (RNA extraction, qRT-PCR, fluorescence tagging, etc.) were used to quantify *pqm-1* expression to yield four technical replicates for each sample. The data was analyzed through null hypothesis t-tests, heatmaps, protein interactions, and gene homology tools. HFPO-DA exposure through *E. coli* caused a statistically insignificant (0.811-fold) change in *pqm-1*-related aging in *C. elegans*. Future work includes investigating the effects of different levels of HFPO-DA exposure on *C. elegans* aging.

Introduction

Through this study, we sought to quantify the potential effects of HFPO-DA exposure on *pqm-1* gene expression in *C. elegans* (a free-living, transparent nematode) in order to determine whether such chemical stress could influence aging processes in *C. elegans*. HFPO-DA is an ammonium salt with the trade name “GenX” and is a potent toxicant used in manufacturing industries that has recently been detected in global river water ecosystems [1].

C. elegans was used as a model organism for various reasons. Its full genome has been sequenced, and *C. elegans* shares many of the same genes and biological pathways with humans [2]. *C. elegans* is relatively easy to grow in a short amount of time, producing more than 1,000 eggs daily and having a life cycle of only two weeks [2]. *C. elegans* is also inexpensive to grow and only requires cultures of bacteria and a nutrient source.

The target gene *pqm-1* was selected for several reasons. In *C. elegans*, the PQM-1 protein (encoded by *pqm-1*) is an important nuclear transcription activator that binds to the DAF-16 Associated Element (DAE) upstream of development (Class II) genes [3, 4]. In nature, reduced insulin/IGF-1-like signaling (IIS) leads to PQM-1 remaining in the cytoplasm and thus downregulation of Class II genes, increasing lifespan [3]. IIS is also conserved across many species, including mammals; therefore, the implications of IIS in *C. elegans* may reflect aging outcomes in humans.

Regarding the selection of the reference gene, previous studies have shown that *tba-1* (alpha tubulin subunit) expression is predominantly localized to developing germ cells and nervous system to maintain embryonic viability [5]. Therefore, experimental factors are unlikely to significantly affect *tba-1* expression in mature adult *C. elegans*, in which *tba-1* is only expressed at the minimum level for survival. A previous toxicology study also showed that *tba-1* expression in C. elegans was stable with exposure to nano-copper oxide (industrial toxicant similar to HFPO-DA), suggesting its reliability as a reference gene when measuring gene expression in C. elegans in response to HFPO-DA exposure [6].

It was hypothesized that the difference in relative *pqm-1* gene expression between the experimental and control groups will not be statistically significant and that HFPO-DA will induce a statistically significant difference in relative *pqm-1* gene expression.

Materials And Methods

Animal Care and Maintenance

C. elegans N2 (wild type) was fed *E. coli* OP50, maintained at a temperature of 23°C, and kept in a uracil-based growth medium. The experimental group of *C. elegans* was fed *E. coli* incubated with 280 ng/L HFPO-DA, simulating how *C. elegans* and humans may be exposed to HFPO-DA in nature via lower trophic-level organisms.

RNA Extraction and Quantification

RNA extraction was commenced by using the M9 buffer to wash *C. elegans* off of the NGM plates, transferring the worms into conical tubes with a pipette, centrifuging *C. elegans* to the bottom of the tubes, and removing the liquid supernatant. *C. elegans* cells were then disrupted with Buffer RLT Plus. Four beads were added to each sample tube, and all the tubes were loaded to the Bead Mill for homogenization. Lysis and purification were conducted through the RNeasy Plus Mini Kit. The RNeasy Plus Mini Kit handbook by Qiagen was followed to ensure adherence to recommended procedural guidelines. NanoDrop 2000 was used to assess the quality and quantity of the extracted RNA; if needed, the RNA samples were exposed to DNase I and protease for further RNA purification.

qRT-PCR was used to produce quantifiable gene expression results. For *pqm-1*, reverse transcriptase and the DNA forward and reverse primers CACCGCCGACTACTATGCC and TCGGCTGCATTAGGTTTACTGTG, respectively, were added to the RNA solutions. Taq polymerase, a heat-stable DNA polymerase found in *T. aquaticus*, was also added Solutions the solutions. The solutions were then subjected to multiple heat cycles to separate DNA strands, allowing Taq polymerase to bind to primers and synthesize complementary DNA strands. The qRT-PCR was designed such that the positive control would be the samples with the reference *tba-1* gene, the negative control would be samples missing a key cellular component, and the repeated measurements for each sample would serve as technical replicates.

The iTaq™ Universal SYBR® Green One-Step Kit was used for quantification of RNA products, as it contains SYBR Green I (a chemical that binds to the minor grooves in double stranded DNA and emits green light under blue light). ROX (emits red light) was used to provide a baseline reflectance level and normalize the results.

Data Analysis

The Google Sheets software was used to calculate data based on the threshold number of cycles (Ct) (i.e., the number of cycles needed to achieve a statistically significant signal increase relative to the background level). The ΔCt (difference between Ct values of target and reference genes within a sample), average ΔCt's for each condition, ΔΔCt (difference between average ΔCt's of experimental and control conditions), and $2^{-\Delta\Delta Ct}$ (fold relationship between target RNA transcripts of experimental and control conditions) values were calculated. A two-tailed t-test was conducted for the ΔCt values to assess the significance of the results (with a p-value threshold of 0.05).

The JMP Pro software was used to create heatmaps, a technique used to visualize gene expression data. The STRING Database was used to construct gene-to-gene and protein networks based on the *pqm-1* gene and was also used to identify interactions and other pathways of interest. HomoloGene was used for gene homology analysis.

Results

The heatmap (refer to Fig. 1) shows a slight decrease in relative *pqm-1* expression in the experimental condition compared to in the control condition. After using Google Sheets for data analysis (refer to **Appendix A**), it was also revealed that the average ΔCt values for the experimental (HFPO-DA exposure) and control conditions were 5.694 and 5.392, respectively. The ΔΔCt value was 0.302, and the fold relationship ($2^{-\Delta\Delta Ct}$) value from the averaged ΔCt values was 0.811. The p-value from the aforementioned two-tailed null-hypothesis t-test was 0.770.

The STRING protein interactions analysis (refer to Fig. 2) depicted PQM-1's association with DAF-16 (transcription activator, binds to the DAF-16 Binding Element (DBE)) and DAF-2 (insulin-like receptor tyrosine kinase). PQM-1 was also associated with BLMP-1 (transcription factor regulating vulvar development), ELT-2 (promotes normal gut-specific differentiation), PHA-4 (transcription factor promoting pharyngeal cell determination), GEI-11 (needed for germ-line maintenance), and other transcription factors (e.g., NHR-77) related to development and growth [6].

Pqm-1 was found to be only exclusive to *C. elegans*; thus, the gene homology graph compares *pqm-1* with *F10B5.3*, another gene in *C. elegans*. Their query coverage and percent identity were 59% and 74.26%, respectively (refer to Fig. 3).

Discussion And Conclusion

As shown in the heatmap (Fig. 1), relative *pqm-1* expression was slightly lower in *C. elegans* exposed to HFPO-DA than in the control group by a fold change of 0.811. However, we fail to reject the null hypothesis that the difference in relative *pqm-1* gene expression between the experimental and control groups was not statistically significant ($p = 0.770 > 0.05$, two-tailed). It can thus be concluded that the results do not indicate a significant influence of HFPO-DA exposure, via *E. coli* OP50, on aging in *C. elegans* specifically in relation to *pqm-1*.

The STRING analysis suggests that PQM-1 is associated with not only members of the IIS pathway, but also other proteins that are involved in development, growth, and maturation of *C. elegans*. This underscores the integral role of *pqm-1* expression in contributing to Class II (development) gene expression and thus aging in *C. elegans*. Although humans and mammals share IIS pathways as *C. elegans*, no direct homologues for *pqm-1* were found outside of the *C. elegans* species. The *pqm-1* expression patterns analyzed in this study may not directly apply to humans; however, the STRING analysis strongly suggests that relationships between HFPO-DA exposure and aging in *C. elegans* and in humans share commonalities on an IIS pathway level. Thus, future research may aim to confirm the existence of such a similar relationship between HFPO-DA exposure and human aging on a developmental pathway level.

This study's limitations include the number of technical replicates that were used for each condition. No biological replicates were analyzed; thus aging variability across the *C. elegans* species may not have been accurately reflected in the results. Due to time constraints, RNeasy was

used for RNA extraction from the samples; however, the TRIzol protocol is more accurate and often results in higher yields.

Other future directions include exposing *E. coli* to different amounts of HFPO-DA and determining a potential threshold for HFPO-DA concentration and abnormal *C. elegans* aging. Longitudinal studies could also be conducted to explore the effects of direct HFPO-DA exposure on overall *C. elegans* development across the lifespan. Another possibility may be to repeat the experiment with different means of HFPO-DA exposure or in a higher order organism. This study's results may prove useful in future research on aging with other similar toxicants.

Declarations

Ethics Approval and Consent to Participate

All experimental subjects were used in adherence to approved ethical standards.

Consent for Publication

Not applicable.

Availability of data and material

Not applicable. All relevant data is included in the body of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

VN initiated the study, performed all data analysis, and authored and edited the manuscript.

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Author's information

VN is a student at Stony Brook University in the highly selective Scholars for Medicine Combined 4+4 Bachelor's/MD program. VN has extensive research experience in projects conducted with internationally-renowned research professionals and through high profile research institutions and entities; including the University of California, San Diego Extension; the BioScience Project; and the Boz Research and Teaching Institute. VN's work and research has been recognized through publications and presentations by the International Neuroethics Society, Nature Protocol Exchange, Open Journal of Genetics, International Youth Research Summit, California Science & Engineering Fair, and California Humanities (partner of National Endowment for the Humanities). VN has also been selected as a member of the prestigious New York Academy of Sciences. Through research and writing, VN hopes to advance medicine and science and contribute to the greater scientific community.

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Appendices

Appendix A

Sample Name	Target Name	Ct	ΔCt	Average ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$ from Averaged ΔCt
HFPO-DA1	*tba-1*	20.354	5.578	5.694	0.302	0.811
	pqm-1	25.931				
HFPO-DA2	*tba-1*	18.353	4.753			
	pqm-1	23.106				
HFPO-DA3	*tba-1*	18.672	6.238			
	pqm-1	24.91				
HFPO-DA4	*tba-1*	22.899	6.206			
	pqm-1	29.105				
Control1	*tba-1*	25.55	3.635	5.392		
	pqm-1	21.781				
Control2	*tba-1*	28.095	6.359			
	pqm-1	31.908				
Control3	*tba-1*	31.133	6.181			
	pqm-1	34.276				

Data analysis of qRT-PCR results generated through Google Sheets from four and three technical replicates of experimental and control groups, respectively. Blue boxes, yellow boxes, and white boxes correspond to data pertaining to solely the experimental group, solely the control group, and both the experimental and control groups, respectively.

Figures

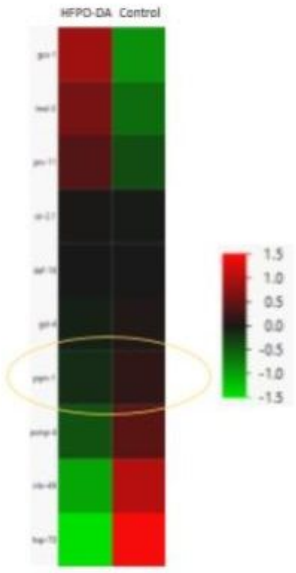

Figure 1

Heatmap generated through JMP Pro showing relative pqm-1 gene expression (circled in yellow), as normalized to tba-1 gene expression, in C. elegans. As shown in the legend on the right, box color is indicative of the magnitude of relative gene expression, ranging from red (higher expression) to green (lower expression).

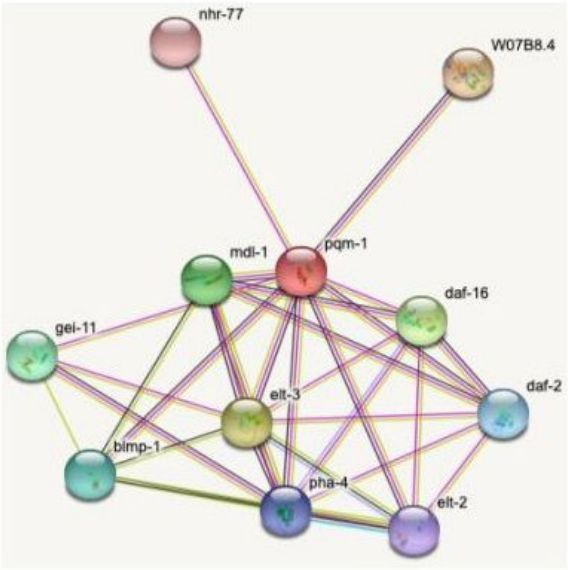

Figure 2

STRING protein interaction network centered on PQM-1 (10 network edges in total).

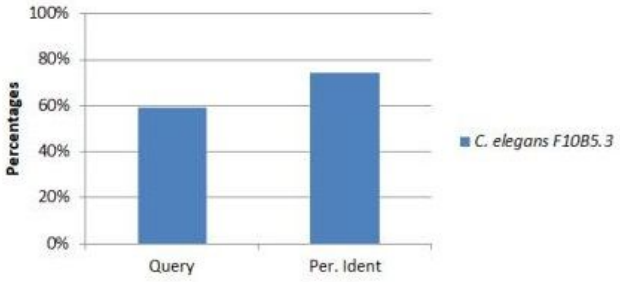

Figure 3

Gene Homology Between pqm-1 and F10B5.3 in C. elegans Legend Gene homology graph showing similarities between pqm-1 and F10B5.3 in C. elegans. Query coverage refers to the degree of overlap between pqm-1 and F10B5.3, and percent identity refers to the degree of sequence similarities between the two genes.