Effect of Vitamin D on Aging in *Caenorhabditis elegans*

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Abstract

Nutrigenomics, also referred to as nutritional genomics, is a branch of science aimed at discovering how various nutrients interact with the genome and potentially alter genetic expression. One nutrient of particular interest is vitamin D, deficiency of which has been linked to diseases and conditions that develop in various organ and muscle systems throughout the entire body. Based on its extensive actions within the body, vitamin D supplementation is sometimes warranted. *Caenorhabditis elegans* is a good model organism for examining optimal levels of vitamin D supplementation in relation to vitamin D's overall affect on aging since the DAF-12 nuclear hormone receptor in *C. elegans* is homologous to the Vitamin D Receptor (VDR) in humans. Lifespan assays were conducted to determine if vitamin D affects the lifespan of the worm. Results indicate that vitamin D significantly increases lifespan at a concentration of 1000 μg/ml. While it was hypothesized that vitamin D^3's interaction with a functional DAF-12 receptor may be required to mediate the effect of vitamin D on lifespan, results were not able to confirm this. Further studies should be done to test additional concentrations of vitamin D^3 and to further test the role of DAF-12.

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In the 10 years since the human genome was sequenced, a relatively new scientific field has emerged. Studies in the field of nutrigenomics, also referred to as nutritional genomics, aim to discover how various nutrients interact with the genome and potentially alter genetic expression. It has been theorized that the impact of these studies on society may have a greater impact than the human genome project itself (1).

Interest has recently been growing in one nutrient in particular – vitamin D (2, 3, 4, 5). In the past, vitamin D insufficiency was most commonly associated with the development of nutritional rickets in children or osteomalacia in adults, both of which occur as a result of insufficient mineralization of bone (3). In addition to bone health, vitamin D has been linked to diseases and conditions that develop in various organ and muscle systems throughout the entire body (2, 3, 4). Although labeled as a vitamin, vitamin D actually acts as a hormone in that it can be produced by the body, is transferred by means of the bloodstream, and acts on target tissues. Upon exposure to solar UVB radiation, 7-dehydrocholesterol in the skin is converted to previtamin D_3, which is transformed to vitamin D_3 upon heat exposure and then enters the blood circulation. In the liver, vitamin D_3 is converted into 25-hydroxyvitamin D_3, which is eventually converted into 1,25-dihydroxyvitamin D_3 (vitamin D hormone) in the kidneys. The hormone is then capable of acting on its target tissues. Increased intake of vitamin D hormone has been positively linked to the prevention of various cancers, such as colon, prostate, and breast cancer, as well as to the prevention of multiple chronic diseases, including cardiovascular disease, multiple sclerosis, type 1 diabetes mellitus, and rheumatoid arthritis (2). An inverse relationship between vitamin D levels and the occurrence of heart failure, mortality, high blood pressure, and suppression of the immune system has also been observed (4). This widespread functioning of vitamin D as a hormone is not surprising, as the Vitamin D Receptor (VDR) is found in nearly

all human tissues, including the skin, brain, breasts, heart, small intestine, colon, and gonads (2, 5). At the cell surface, Vitamin D hormone [$1,25(OH)_2D_3$] binds to the VDR and is transported to the nucleus, where it binds to the Retinoic Acid X receptor (RXR). The resulting 3-part complex then attaches to a specific area on the DNA called the vitamin-D response element (VDRE), and, with the aid of transcriptional factors, causes changes in the transcription of vitamin-D responsive genes, which ultimately control bone metabolism, calcium modulation, and the regulation of cell proliferation and differentiation (2).

Based on its extensive actions within the body, vitamin D supplementation is sometimes warranted, especially since the amount of some nutrients needed to prevent short-term deficiency symptoms may not be adequate enough to promote long-term health (6). There is still some debate as to how significant a role vitamin D plays in preventing or treating certain conditions and diseases. Inconsistent results were found among studies examining the use of vitamin D supplements to reduce the risk of allergies and asthma, mental illness, musculoskeletal pain, and renal disease (3). In addition, a study examining vitamin D supplementation and cardiometabolic outcomes found no statistically significant relationships (4).

When investigating the potential benefits of dietary supplementation, it is most beneficial to run clinical trials with the desired supplement, but this is not always feasible. Humans are inherently hard to study when it comes to nutrient-gene interactions because of the great variation in genotype, a long life span, and the researchers' inability to completely control subjects' diets (1). In addition, when following human subjects throughout their lives, diet is not the only factor that must be taken into consideration. Lifestyle choices and environment can have a significant influence on research outcomes. Such lifestyle variations include the use of tobacco, alcohol consumption, level of physical activity, the presence of stress, and the level of air

pollution, among others (7). One approach to studying the effects of a nutrient such as vitamin D is to use model organisms which can be observed in a controlled environment. The model organism *Caenorhabditis elegans* is of particular interest in such a study for numerous reasons; it is a small nematode with a short lifespan, simple morphology, fully-sequenced genome, and can be manipulated to create age-synchronous populations (8). Also significant is the homology between certain nuclear hormone receptors in *Caenorhabditis elegans* and their counterparts in humans (9), suggesting this study's results may be applicable to understanding vitamin D's role in the human body as well.

Several studies using *Caenorhabditis elegans* have examined both genetic and environmental impacts on longevity (10). For example, activation of the Epidermal Growth Factor (EGF) receptor LET-23 by regulator proteins *hpa*-1 and *hpa*-2 is positively associated with increased life span and increased health span, respectively, in the worm (11). A second study demonstrated that genetically or chemically altering the worm's nutrient-sensitive pathways results in a 10-fold increase in lifespan. One important environmental factor is dietary restriction, which results in a 2- to 3-fold increase in lifespan in the worm and elevates its resistance to misexpressed toxic proteins (12). Vitamin E supplementation also extends the lifespan of the nematode, but a negative relationship between longevity and fecundity was observed (8).

Although vitamin D's many roles in the human body and longevity in *C. elegans* have been studied at length, they have until now remained as separate issues in separate fields of study. Thus, it was yet to be determined whether increased vitamin D intake was related to longevity in *Caenorhabditis elegans*. While *Caenorhabditis elegans* is an optimal model organism for many reasons, it is especially suited to a study examining vitamin D intake and

longevity because the vitamin D nuclear hormone receptor in humans is homologous to the longevity-related DAF-12 nuclear hormone receptor in *Caenorhabditis elegans* (9, 13). NCBI BLAST indicates that 45% of amino acids are identical and 64% of amino acids have similar functions. Since these receptors are quite homologous, it is possible that Vitamin D_3, a secosteroid hormone, will function as a ligand for the *C. elegans* DAF-12 receptor. This hypothesis is supported by Antebi (2006), who suggests that a steroid-derived hormonal mechanism of control for DAF-12 is likely (9). Although DAF-12 has been consistently described as a vitamin D receptor homolog (9, 13), a comparison in functionality had yet to be explored.

The goals of this study were to determine if vitamin D is indeed a ligand for DAF-12 and how various levels of supplementation of vitamin D influence aging in the worm. Our hypothesis was that vitamin D supplementation will increase the lifespan of *Caenorhabditis elegans* as a result of its interaction with the DAF-12 receptor. Thus, we expected the following: we would discover an optimal dose of vitamin D that affects *C. elegans* longevity, the worms exposed to vitamin D in the preliminary dose-response curve would live longer than those exposed only to OP50 bacteria, and the wild-type worms exposed to vitamin D in the more narrowed dose-response curve would live longer than the *daf-12* mutants exposed to the same concentrations of vitamin D.

Materials and Methods

Stock solution of vitamin D_3 (cholecalciferol) was bought from Sigma Chemical Co. and stored at 4° C in the dark. *Caenorhabditis elegans* (wild type and *daf-12* mutants) and OP50 *Escherichia coli* were obtained. The worms were kept at 20° C in 15ml of OP50/S-basal medium (10^9/ml *E. coli* in 2.5 mM K_2HPO_4, 2.5 mM KH_2PO_4, 10 mM NaCl, 1.0 mg/l cholesterol) on

petri dishes of 25ml solidified nematode growth media (50 mM NaCl, 17 g/l Bactoagar, 2.5 g/l peptone, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 2.5 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2 mg/l uracil, 5 mg/l cholesterol) (8). Wild-type age-synchronous worms were obtained by completing a standard synchronizing technique using bleach and M9 Buffer (14).

The first experiment consisted of exposing 7 sets of 50 wild-type, synchronized worms to differing doses of vitamin D_3 in OP50 bacteria for the duration of their lifespan, beginning at the L1 larval stage. Before completion of this component of the experiment, the next experiment was begun. Three sets of age-synchronous wild-type worms and 3 sets of age-synchronous *daf-12* mutant worms were prepared. Ninety L1-stage nematodes were taken from each group to create 3 sets of 30 wild-type nematodes and 3 sets of 30 *daf-12* mutants. Two sets of each type of nematode were exposed to vitamin D_3 at 1 ug/ml and 100 ug/ml, respectively, for the duration of their lifespan, while the remaining set was exposed only to the OP50 bacteria. All worms in the second experiment were transferred to NGM plates as L1s, but were moved to plates containing 0.1mg/ml FUdR as L4s, which prevented the worms from reproducing and thus eliminated the need to separate the adult worms from their progeny. The doses were determined based on a preliminary analysis of the data from the first experiment. All nematodes were moved to fresh media as needed and were observed every 1-3 days for signs of death. Death was indicated by a straight body and a lack of movement by the nematode upon prodding. Any worms that exhibited internally hatched progeny or that had been lost to burrowing or crawling off the agar were removed from the study.

Data were entered into PASW (Predictive Analytics Software) (2009) version 18.0 from IBM (Armonk, NY). Analysis of data included descriptive statistics, Tukey's t-test with a Bonferroni post hoc adjustment, ANOVA, and correlation. Graphical analyses and tables were

prepared using Excel (2007) from Microsoft (Redmond, Washington). Statistical significance was determined at $p<0.05$ or 95% confidence, but a Bonferroni correction was used on the experiment #1 data due to the multiple comparisons being made between the vitamin D concentrations, thereby altering the statistical significance to $p<0.008$ or 99.2% confidence.

Results and Discussion

The goals of the experiments were to determine whether vitamin D^3 supplementation influenced longevity in the worm and, if so, if the DAF-12 receptor was required to mediate this process. Two lifespan experiments were completed – the first focusing on N2 *C. elegans* and resulting in a dose-response curve, and the second comparing the effect of vitamin D between *daf-12* and N2 *C. elegans*. The first experiment indicated that exposure to vitamin D^3 resulted in a positive effect on lifespan in N2 *C. elegans* (Figure 1, Figure 2). All but one of the experimental groups of N2s displayed a greater mean number of days lived as compared to the control (Table 1). This result differs from a previous study on mice in which restricting dietary intake of vitamin D resulted in mitigation of certain premature aging phenotypes (15). The study examined the relationship between vitamin D, FGF-23, and *klotho* (a gene involved in premature aging) though, so is not necessarily comparable to our study. A different study with mice found that an analogue of vitamin D (22-Oxa-1α, 25 –dihydroxyvitamin D_3) was effective in increasing longevity, on average, by 150% (16). It was hypothesized that this was due to prevention of disease in the organism, which is a theoretical possibility in this study, though *C. elegans* nematodes live such short lives, it's not clear that significant disease contributes to their morbidity. The worms in our study that were exposed to 0.1 μg/ml of vitamin D^3 did not live as long as even the control; however, it should be noted that the number of worms studied in that group was less than half of the number of worms in the other groups.

Overall, only those worms exposed to 1000 µg/ml of vitamin D^3 showed a statistically significant greater mean lifespan, living about 28% longer than the control group (Figure 2). To put this percentage into perspective, a similar increase in life span would equate to almost 22 more years of life for humans (based on the current average life span being about 78 years according to the CDC). Some statistically significant differences between experimental groups were also seen (Table 2). Both 100 and 1000 µg/ml concentrations of vitamin D^3 resulted in significantly greater mean lifespans compared to 0.1 µg/ml of vitamin D^3, and 1000 µg/ml vitamin D^3 resulted in significantly greater mean lifespan as compared to 1.0 µg/ml of vitamin D^3. These results support the hypothesis that exposure to vitamin D^3 has a positive effect on life span in wild-type *C. elegans* hermaphrodites. What these results do not explain is whether the extended lifespan is a result of vitamin D^3 binding the DAF-12 receptor or acting via a different effector. The second experiment of the study was completed in an attempt to answer this question.

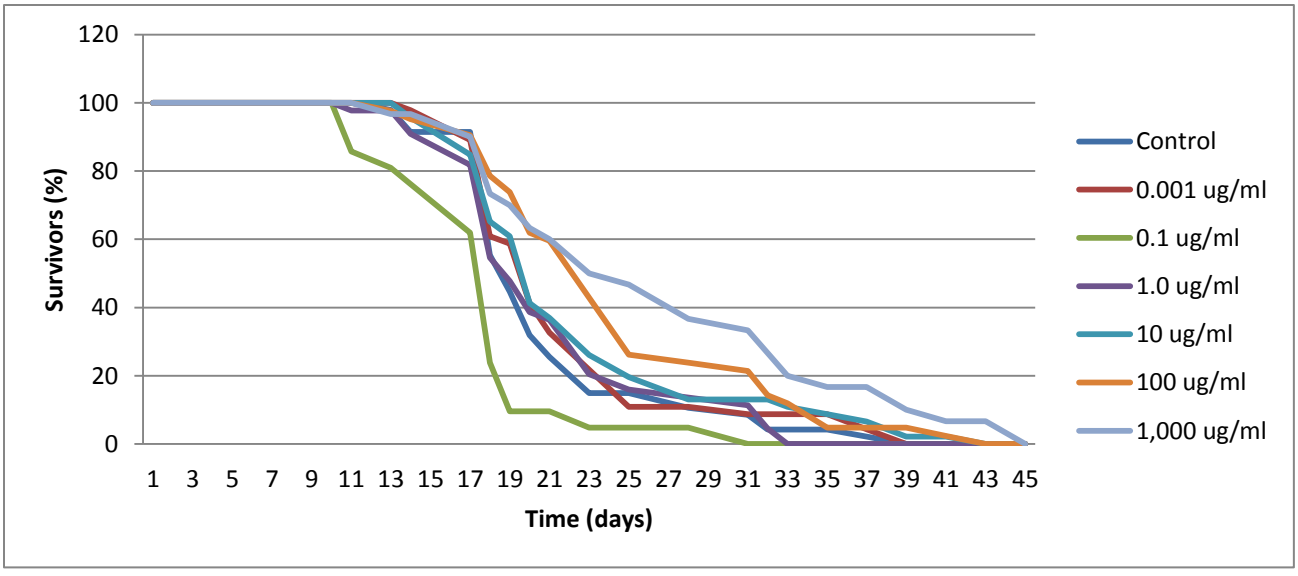
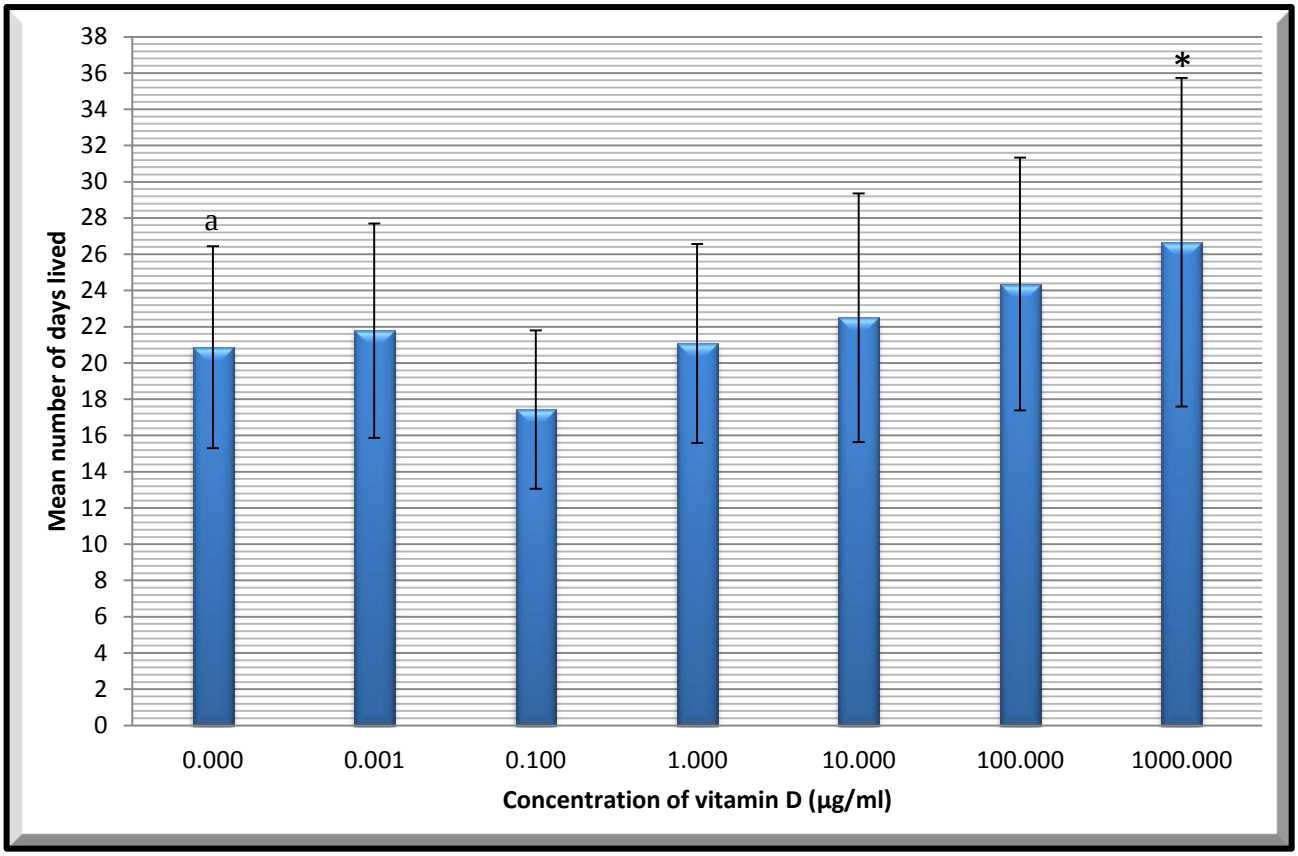


Figure 1 – Survival curves for N2 *C. elegans* in first experiment. Seven sets of 50 wild-type, synchronized worms were exposed to differing doses of vitamin D_3 in OP50 bacteria for the duration of their lifespan, beginning at the L1 larval stage. Worms were scored for death every 1 to 3 days.



[a]Standard deviation
*Statistically significant at $p<0.008$

Figure 2 – Dose-response curve for N2 *C. elegans* in first experiment. All groups of worms lived a comparable length of time, save those exposed to 1000 µg/ml. This group lived significantly longer than the control group. A slight positive relationship between vitamin D exposure and longevity should be noted.

Group (ug/ml)	n	μ +/- S.D. (d)	Min (d)	Max (d)
0.000	47	20.87 +/- 5.57	13	39
0.001	46	21.78 +/- 5.91	14	39
0.100	21	17.43 +/- 4.37	11	31
1.000	44	21.07 +/- 5.49	11	33
10.000	46	22.50 +/- 6.86	14	43
100.000	42	24.36 +/- 6.98	13	43
1000.000	30	26.67 +/- 9.07	13	45

Table 1 – Effect of vitamin D on lifespan of N2 C. elegans –descriptive statistics for data gathered in experiment #1. All but one of the experimental groups of N2s displayed a greater mean number of days lived as compared to the control.

Group 1	Group 2	df	T	p value	Bonferonni	95% CI
0	0.001	91	-0.764	0.447	1.000	(-3.28, 1.46)
0	0.1	66	2.505	0.015	0.899	(0.70, 6.19)
0	1	89	-0.169	0.866	1.000	(-2.50, 2.11)
0	10	91	-1.257	0.212	1.000	(-4.20, 0.94)
0	100	87	-2.615	0.011	0.240	(-6.13, -0.84)
0	1000	75	-3.477	0.001	0.003*	(-9.11, -2.47)
0.001	0.1	65	3.015	0.004	0.228	(1.47, 7.24)
0.001	1	88	0.593	0.554	1.000	(-1.68, 3.11)
0.001	10	90	-0.537	0.592	1.000	(-3.37, 1.93)
0.001	100	86	-1.872	0.065	1.000	(-5.31, 0.16)
0.001	1000	74	-2.846	0.006	0.029**	(-8.30, -1.46)
0.1	1	63	-2.661	0.01	0.718	(-6.37, -0.91)
0.1	10	65	-3.107	0.003	0.065**	(-8.33, -1.81)
0.1	100	61	-4.151	0.0	0.002*	(-10.27, -3.59)
0.1	1000	49	-4.322	0.0	0.000*	(-13.53, -4.94)
1	10	88	-1.091	0.278	1.000	(-4.04, 1.18)
1	100	84	-2.435	0.017	0.393	(-5.97, -0.60)
1	1000	72	-3.308	0.001	0.006*	(-8.97, -2.22)
10	100	86	-1.258	0.212	1.000	(-4.79, 1.08)
10	1000	74	-2.277	0.026	0.132	(-7.81, -0.52)
100	1000	70	-1.221	0.226	1.000	(-4.11, 2.47)

*significant at the $p<0.008$ level
**$p<0.10$, trending toward statistical significance

Table 2 – T-test results for data gathered in experiment #1 in which the effect of vitamin D on lifespan of N2 *C. elegans* was tested. One thousand μg/ml was a statistically significant concentration of vitamin D^3 as compared to both the control and 1 μg/ml vitamin D^3. One

thousand µg/ml and 10 µg/ml vitamin D^3 trended toward significant when compared to the effects of 0.001 µg/ml and 0.1 µg/ml, respectively.

To determine if Vitamin D^3 acts to extend lifespan via the DAF-12 receptor, the *daf-12* mutant was used in a lifespan assay. This experiment was started prior to the completion of the first experiment. The effect of 1.0 µg/ml vitamin D^3 was compared in control and *daf-12* mutants. We initially also set out to test the effect of 100 µg/ml of vitamin D^3 on *daf-12* worms, but the worms being exposed to this concentration of vitamin D^3 were accidentally transferred to the wrong plates early in the experiment and had to be removed from the study. Thus, no data were collected for *daf-12* worms exposed to 100 µg/ml (Table 3).

No statistically significant differences were found in the second experiment, as evidenced by the ANOVA (Table 5). Our hypothesis stated that the DAF-12 receptor was necessary for any increase in lifespan; however, our results with 1.0 µg/ml vitamin D^3 did not support the hypothesis. Indeed, all groups of worms lived a comparable number of days. Upon reflection, this is not surprising, as exposure to 1.0 or 100 ug/ml vitamin D^3 did not result in any significant increase in lifespan in the first experiment.

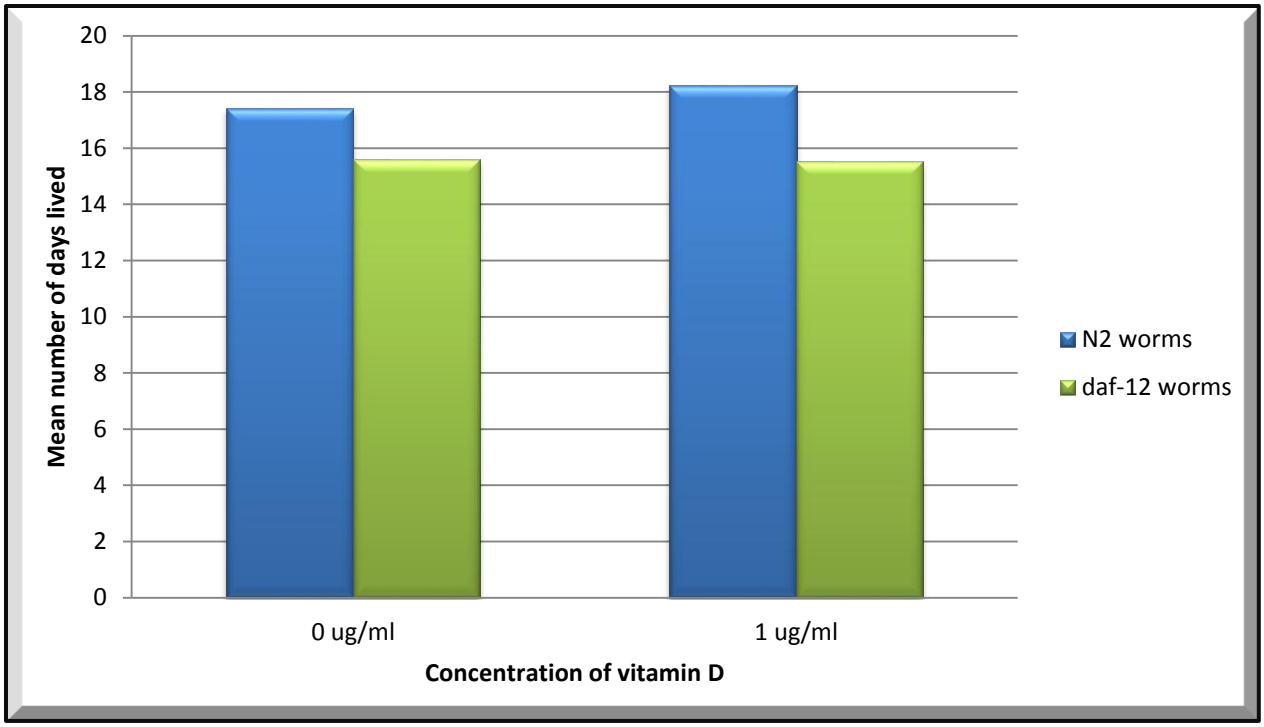


Figure 3 – Comparison of effect of vitamin D on lifespan between N2 and *daf-12 C. elegans.* The effect of 1.0 µg/ml vitamin D^3 was compared in control and *daf-12* mutants in order to determine if a functional DAF-12 receptor may be required to mediate the effect of vitamin D on lifespan. Worms in experimental group were exposed to the vitamin D^3 throughout their entire lifespan, beginning at the L1 larval stage. Results were inconclusive. All groups of worms lived a comparable number of days.

Group (ug/ml)	n	µ +/- S.D. (d)	Min (d)	Max (d)
0.000 N2	29	17.41 +/- 5.89	13	35
1.000 N2	26	18.23 +/- 6.28	11	35
100.000 N2	29	16.10 +/- 3.28	13	27
0.000 daf-12	29	15.59 +/- 4.14	8	27
1.000 daf-12	29	15.52 +/- 4.71	8	29

Table 3 – Comparative effect of vitamin D on lifespan of N2 and *daf-12 C. elegans* – descriptive statistics for data gathered in experiment #2. N2 worms appear to have lived slightly longer than their *daf-12* counterparts, but not significantly so.

Group 1	Group 2	df	T	p value	95% CI
0 (N2)	1 (N2)	53	-0.498	0.621	(-4.11, 2.47)
0 (N2)	100 (N2)	56	1.047	0.3	(-1.20, 3.82)
1 (N2)	100 (N2)	53	1.599	0.116	(-0.54, 4.80)
0 (N2)	0 (daf-12)	56	1.368	0.177	(-0.85, 4.50)
1 (N2)	1 (daf-12)	53	1.825	0.074**	(-0.27, 5.70)
0 (daf-12)	0 (daf-12)	56	0.059	0.953	(-2.26, 2.40)

**p<0.10, trending toward statistical significance

Table 4 – T-test results for data gathered in experiment #2 in which the effect of vitamin D on lifespan was compared between N2 and *daf-12 C. elegans*. There was no significant difference between any of the groups, but the difference in lifespan between the N2 and *daf-12* worms exposed to 1.0 µg/ml was trended toward significance, with the worms in the N2 group living slightly longer on average (see Table 3).

Group	df	F	p value
Experiment #1	6	5.709	0*
Experiment #2 N2s	3	1.145	0.323

*statistically significant at p<0.008

Table 5 – Analysis of Variance (ANOVA) on number of days lived by groups in each experiment within the study. The statistically significant p-value for experiment #1 confirms that

there were statistically significant results found within that study. Refer to tables 2 and 4 for specific results within each experiment and between each experimental group.

Before a definitive rejection or acceptance of the hypothesis can be made, further studies are warranted. It is necessary to expand the dose-response curve by testing even higher concentrations of vitamin D^3 in order to discover if there is a concentration at which longevity peaks. In addition, further testing on higher concentrations of vitamin D^3 will have to be done with *daf-12* mutants in order to determine if the DAF-12 receptor plays a role in increasing longevity induced by vitamin D exposure. The worm model may be most useful in eventually contributing to an understanding of the effects of Vitamin D at a cellular level.

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