Protective Effects of Vitamin D_3 on DGLA-Induced Ferroptosis in *C. elegans* and Implications for Redox Homeostasis

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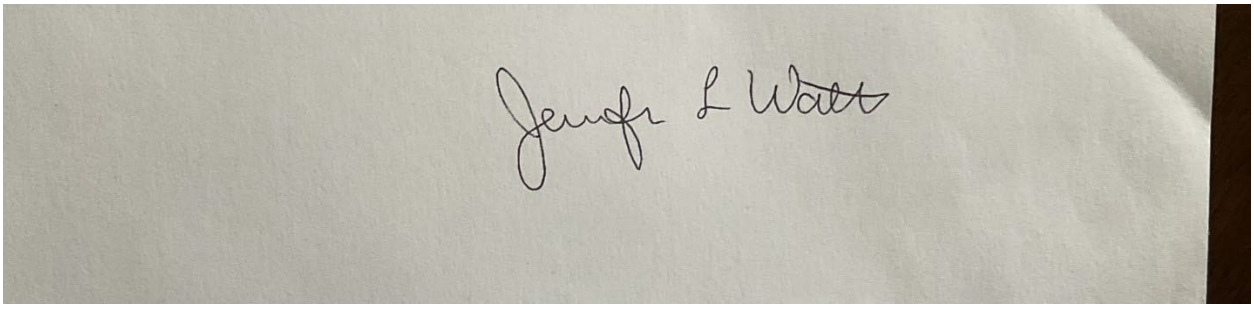
As thesis advisor for Christopher Infranco, I have read this paper and find it satisfactory



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PRÉCIS

Ferroptosis is an iron-dependent form of cell death that has been demonstrated in various species, most notably human cancer cells and the nematode worm *Caenorhabditis elegans.* Ferroptosis is thought to involve the chemical process of peroxidation on lipid constituents of the plasma membrane of a cell. Peroxidation involves the linking of molecular oxygen (O_2) to lipids containing an unpaired electron, with lipid peroxide formation in the membranes that line the periphery of cells resulting in disruption of membrane integrity and cell death (Mortensen et al., 2023). Iron participates in a characteristic reaction, called the Fenton reaction, that can lead to the formation of lipid peroxides and thus ferroptosis (Mortensen et al., 2023). It has been reported previously that treating *C. elegans* with the dietary lipid dihomo-gamma-linolenic acid (DGLA) can promote sterility through ferroptosis of germ cells (Perez et al., 2020). It has also been reported that vitamin D_3 can promote resistance to oxidative stress and improve the lifespan of *C. elegans* (Huggins & Farris, 2023). Given that lipid peroxidation operates through the formation of chemical species called radicals, which are characteristic of oxidative stress pathways, it is natural to wonder whether vitamin D_3 could improve the sterility outcomes of worms treated with DGLA. This study investigates the effects of co-treating *C. elegans* strains with vitamin D_3 and DGLA and comparing the differences in sterility levels of worm populations treated with both vitamin D_3 and DGLA with those treated with DGLA alone. When working with the wild-type N2 strain of *C. elegans,* it was found that co-treatment of vitamin D_3 with DGLA reduced sterility levels compared to those individuals treated with DGLA alone. The reduction in sterility was higher when using when using a higher concentration of vitamin D_3 to treat the worms, thus demonstrating a dose-dependent protective effect of vitamin D_3. Additionally, the effects of vitamin D_3 on sterility protection were investigated in the ferroptosis-

sensitive *gpx-1* mutant strain of *C. elegans.* Unlike the wild-type strain, these mutants had little response to vitamin D_3 when compared to DGLA alone. Given that ferroptosis has been demonstrated to occur in human cancer cells (Perez et al., 2020), hopefully gaining a greater understanding of the mechanisms controlling this process will enable more effective treatment of this disease in the future.

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INTRODUCTION

Vitamin D is a compound known to have diverse effects on human health and homeostasis. Currently recognized effects of vitamin D and its associated receptor on the human body include a role in bone mineralization, enhancements in the secretion of insulin, apoptosis of cells that have been infected by pathogens, and reductions in oxidative stress (Rebelos et al., 2023). There are two naturally occurring forms of vitamin D. Vitamin D_2 (ergocalciferol) can be obtained from the diet through consumption of fungi, while vitamin D_3 (cholecalciferol) can be obtained from animal products. Additionally, vitamin D_3 can be produced in human skin from exposure to UV radiation (Nair & Maseeh, 2012). The formation of biologically active vitamin D requires the product obtained from the diet or skin to be hydroxylated twice. One of these hydroxylations occurs in the liver under the influence of the enzyme vitamin D-25-hydroxylase, and the other occurs in the kidneys (Nair & Maseeh, 2012). It has been found that a deficiency of vitamin D in humans (defined to be < 50 nmol/L serum 25-hydroxyvitamin D) is associated with musculoskeletal defects and higher risk of infection (Amrein et al., 2020).

Ferroptosis is a form of controlled, iron-mediated non-apoptotic cell death. The currently accepted mechanism of ferroptosis involves the peroxidation and subsequent loss of structural integrity of membrane lipids (Jiang et al., 2021). A common target for peroxidation is polyunsaturated fatty acids (PUFAs) incorporated into the lipids of cell membranes. Reaction of a membrane PUFA with a hydroxyl radical can cause the placement of a free electron on the carbon chain of the fatty acid. This lipid radical can further react with nearby membrane PUFAs, causing propagation of peroxides and disruptions in membrane integrity as a result (Mortensen et al., 2023). The iron-dependent Fenton reaction leads to the generation of the reactive hydroxyl

radicals as well as other species that can cause the peroxidation of PUFAs, thus implicating iron in the mechanism for cell death by ferroptosis (Mortensen et al., 2023). It has previously been shown that treatment of the nematode model organism *Caenorhabditis elegans* with exogenous PUFAs can induce sterility (Watts & Browse, 2006). In particular, the omega-6 PUFA dihomo-gamma-linolenic acid (DGLA) has been shown to cause the loss of germ cells, oocytes, and sperm for *C. elegans* grown in its presence. Later, this process was elaborated on to suggest that the mechanism of DGLA-induced sterility in *C. elegans* came through ferroptosis (Perez et al., 2020). This was demonstrated when *C. elegans* populations co-treated with both DGLA and the ferroptosis inhibitor ferrostatin-1 showed reduced levels of sterility compared to treatment with DGLA alone (Perez et al., 2020). Defense against lipid peroxidation in various organisms can come from the glutathione defense system, which utilizes the redox-active molecule glutathione to reduce peroxide species under the catalysis of the enzyme glutathione peroxidase (Sakomoto et al., 2014). As could be expected based on the presence of toxic redox species during ferroptosis, the *gpx-1* strain of *C. elegans,* which lacks a homologue to one of the human glutathione peroxidase enzymes, has been shown to be particularly sensitive to DGLA-induced sterility (Perez et al., 2020).

Separate to the sterility induced by DGLA, previous studies have demonstrated that vitamin D_3 (cholecalciferol) is capable of extending the lifespan of *C. elegans* (Huggins & Farris, 2023). It was shown that in *nhr-8* mutants containing a mutation in a protein orthologous to the human vitamin D receptor, treatment with exogenous vitamin D_3 was able to promote lifespan extensions as well as improve motility of these mutants. Curiously, vitamin D_3 was also shown to promote oxidative stress resistance in the *nhr-8* mutants (Huggins & Farris, 2023).

This study sought to merge the findings on the effects of vitamin D_3 on *C. elegans* health and DGLA on sterility by examining whether vitamin D_3 can alter the levels of sterility in individuals treated with DGLA. This process, in which populations co-treated with DGLA and vitamin D_3 were compared in their sterility to those treated with DGLA alone, was performed on the wild-type strain of *C. elegans*, known as N2, as well as the ferroptosis sensitive strain *gpx-1*.

MATERIALS AND METHODS

The *C. elegans* nematodes used in this study were grown on standard nematode growth media (NGM) seeded with *E. coli* OP50 at 20°C. In the preparation of the NGM used to treat the plates, each treatment dose was separately given a combination of DGLA, added from a stock such that its concentration in the final plates would be between 0.10 mM and 0.15 mM, and vitamin D_3. The DGLA stock was made at room temperature by dissolving the lipid in water, and was contained in a chamber protected from light exposure at a concentration of 100 mM. The stock was evacuated with nitrogen after use to prevent oxidation of the DGLA. The vitamin D_3 stock solution used ethanol as a solvent. The ethanol stock containing dissolved vitamin D_3 at a concentration of approximately 236 mM was added to each dose of agar solution such that the concentration in the agar and NGM plates was between 50 and 200 μM, depending on the experiment being conducted. The use of this range of vitamin D_3 concentrations was motivated by previous studies utilizing the 50 μM base concentration to test the effects of vitamin D_3 on *C. elegans* (Huggins & Farris, 2023). Once the NGM agar was added to the plates, stocks of each strain of *C. elegans* to be used were synchronized using the alkaline hypochlorite method, thus ensuring the individuals to be added to the NGM plates were young and all at approximately the

same age. In this protocol, adult worms were added to a bleach solution in which eggs were able to be isolated from adult individuals, and then these eggs were cultured in a buffer such that they hatched but remained in the first larval stage until addition to NGM plates (Porta-de-la-Riva et al., 2012). Before nematodes were added to NGM plates, *E. Coli* OP50 was seeded to the plates and allowed to grow. Once the young nematodes of each strain were ready to be plated, worms were added to the appropriate NGM plates such that each plate contained approximately 200 individuals. Upon the addition of worms to each plate, the plates were incubated at 20°C for approximately 2-3 days to allow the worms to reach adulthood before being scored for sterility. Scoring the worms for sterility involved visual analysis under a light microscope for the presence of deformities in reproductive structures. The transparent nature of the nematodes enables simple detection of an individual as fertile or sterile based on whether eggs can be seen within an individual's uterus, a state that implies fertility. On each plate, 50 worms were analyzed and labelled as fertile or sterile, and the percent sterility was determined for each plate. Averaging the percent sterility for each plate of a given treatment then yielded a value which was then compared between treatment groups, labelled in the graphs in this report simply as "%Sterility."

In some experiments, worms from each treatment were collected for analysis by DAPI imaging. In a given treatment group of 5 plates of nematodes, 5 µL of *C. elegans* suspended in water, condensed from typically two plates of worms, were added to a microscope slide and treated with a DAPI solution, such that they could be observed under high-resolution microscopy and analyzed more carefully for the presence of reproductive abnormalities. *C. elegans* has both an anterior and posterior gonad that is responsive to DGLA. Samples of DAPI images for sterile and fertile worms can be seen below, in Figure 1 (Perez et al., 2020). For the worms labelled as sterile, little gonad morphology can be seen. For those marked as fertile, worms have gonads

illuminated within the body under DAPI microscope (Perez et al., 2020).

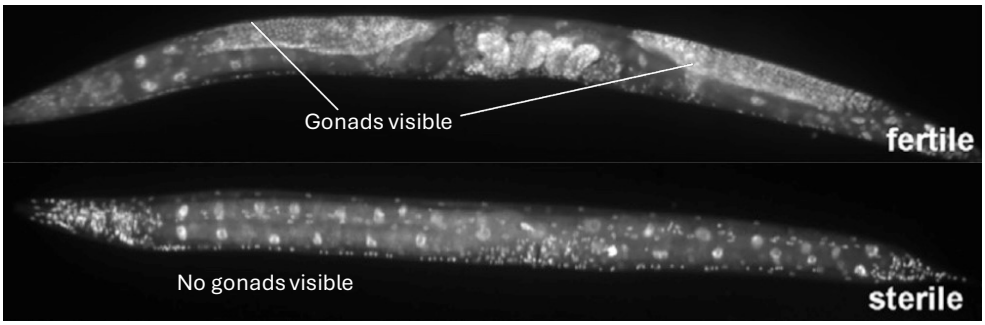

Figure 1: Examples of DAPI-imaged *C. elegans* individuals. The top image showcases a fertile worm, with two clearly visible gonads on the left and right sides of the individual's body. Embryos can also be seen as bean-shaped clusters towards the center of the body. The bottom image showcases a worm that would be denoted sterile, as no gonad morphology can be viewed and the individual has a characteristic "hollow" appearance under microscopy. Images taken from Perez et al., 2020.

RESULTS

Vitamin D_3 Reduces DGLA-Induced Sterility in Wild-Type *C. elegans*

The initial experiment conducted was intended to investigate the role of vitamin D_3 in altering the sensitivity of the N2 wild-type (WT) strain of *C. elegans* to DGLA-induced sterility. This procedure involved 4 treatment groups of nematodes grown on *E. coli* OP50. One treatment contained plates of nematodes given no exogenous DGLA or vitamin D_3, another was treated with a 0.10 mM concentration of DGLA with no vitamin D_3, one had no DGLA yet 50 µM exogenous vitamin D_3, and a final treatment contained 0.10 mM DGLA and 0.50 µM vitamin D_3. Each treatment group contained 5 plates of approximately 200 nematode individuals. The treatment containing no D_3 or DGLA was intended to serve as a negative control for this experiment, with little sterility levels expected, and the treatment containing 0.10 mM DGLA with no vitamin D_3 was intended to serve as a positive control, as relatively high sterility levels were to be expected based on treatment with DGLA alone. As in all experiments described in this report, the agar solution for the treatment groups not treated with exogenous vitamin D_3 were given an equivalent volume of pure ethanol, meant to serve as a vector that would account for any unexpected effects of the ethanol the vitamin D_3 was dissolved in. As expected, the worms treated with no DGLA or vitamin D_3 saw little levels of sterility relative to those treated with only DGLA, and the worms treated with 50 µM vitamin D_3 showed little change in sterility relative to those treated without DGLA or vitamin D_3 (Figure 2). Interestingly, as can be seen in Figure 2, worms treated with both vitamin D_3 and DGLA showed significantly ($P < 0.05$) lower levels of sterility relative to those treated with DGLA alone (Figure 2). These results suggest a protective effect of vitamin D_3 on DGLA-induced sterility in *C. elegans*.

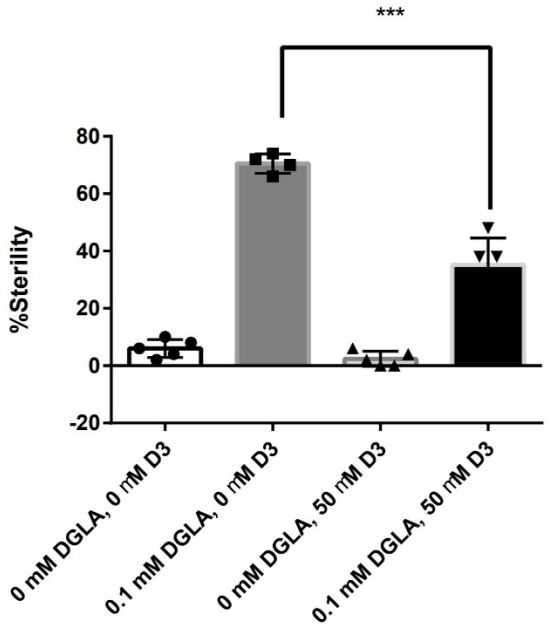

Figure 2: Sterility results for WT *C. elegans* nematodes treated with varying combinations of vitamin D_3 and DGLA. The vitamin D_3 concentration used for the treatment groups that contained it was 50 μM, obtained by dilution of a 236.3436 mM stock to 50 mL of NGM agar solution, which was then distributed to the appropriate plates. The DGLA concentration used was 0.10 mM, obtained by dilution of a 100 mM stock to 50 mL of NGM agar solution. Average sterility was measured as the mean percent sterility for all the plates of each treatment group, which were scored by visual observation of nematode individuals under a light microscope. The data showed a significantly lower sterility level for the nematodes treated with DGLA and vitamin D_3 compared to DGLA alone. Results highlighted with one or more asterisks show statistically significant differences in average percent sterility ($P < 0.05$). Higher numbers of asterisks indicate greater statistical significance.

The same experiment was replicated and a similar result was found in which the plates treated with 0.10 mM DGLA and 50 μM vitamin D_3 had lower average sterility levels than those treated with DGLA alone, although these results were not tested for statistical significance. However, worms from each treatment were analyzed using DAPI microscopy to more closely

compare the effects of DGLA and vitamin D_3 on the reproductive morphology of the nematodes. The gonad morphology of nematodes treated with no DGLA or vitamin D_3**,** or vitamin D_3 alone, was clearly visible under DAPI microscope, as both the anterior and posterior gonads of these individuals could be seen. However, for individuals that had been grown with 0.10 mM DGLA, the germ cells were absent, showcasing the previously demonstrated effect of DGLA on disrupting reproductive structure and function in *C. elegans* (Watts & Browse, 2006). For individuals treated with vitamin D_3 along with DGLA, a restoration of gonad structure was seen with the DAPI imagery (Figure 3). Therefore, direct visualization of *C. elegans* individuals under DAPI microscope re-affirms the observations noted in direct scoring analysis – that vitamin D_3, when present in a 50 µM concentration, can have a protective effect against DGLA-induced sterility in *C. elegans.*

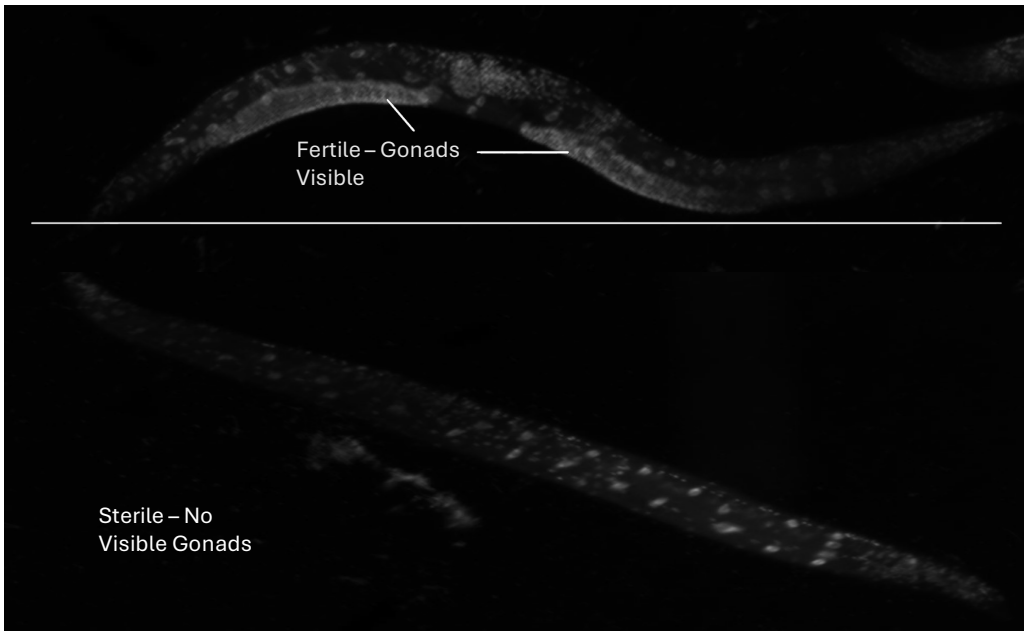

Figure 3: DAPI imaging results from WT nematodes treated with varying levels of DGLA and vitamin D_3. The panel on the bottom shows an individual grown in 0.10 mM exogenous DGLA. As can be seen, the gonads of this

individual are not clearly visible, indicating sterility. However, the top panel shows an individual grown in the presence of 0.10 mM DGLA and 50 µM exogenous vitamin D_3. The gonads of this individual are more clearly visible than for the individual grown in only DGLA, thus supporting the protective effect of vitamin D_3 on DGLA-induced sterility in *C. elegans.*

The Protective Effect of Vitamin D_3 on DGLA-Induced Sterility Exhibits Dose-Dependency

The next experiment aimed to determine whether the protective effect of vitamin D_3 on DGLA-induced sterility in *C. elegans* exhibits a dose-dependent effect. To determine this, WT *C. elegans* populations were grown on *E. coli* OP50 in conditions of either 0.15 mM DGLA or no exogenous DGLA. Additionally, within each dose of DGLA, worms were grown in either 0 µM, 50 µM, 150 µM, or 200 µM exogenous vitamin D_3. The use of a higher concentration of DGLA compared to previous experimentation was warranted by a desire to generate higher levels of base sterility, such that any possible dose-dependency of DGLA could easily be observed. 5 plates of nematodes were prepared for each treatment, and after the worms were allowed to grow to adulthood (roughly 3 days), their sterility was measured through direct observation, similar to previous experiments. No addition of exogenous DGLA resulted in low levels of sterility, as could have been expected based on earlier results (Figure 4). However, the 0.15 mM dose of DGLA rendered nearly all of the *C. elegans* populations sterile in the absence of vitamin D_3 (Figure 4). The presence of exogenous vitamin D_3 significantly ($P < 0.05$) lowered the sterility of the worms treated with DGLA, and higher doses of vitamin D_3 correlated with lower levels of sterility (Figure 4). These results suggest that vitamin D_3 exhibits dose-dependent protection against DGLA-induced sterility in *C. elegans,* such that higher concentrations of vitamin D_3 exhibit a greater degree of protection against sterility compared to lower concentrations.

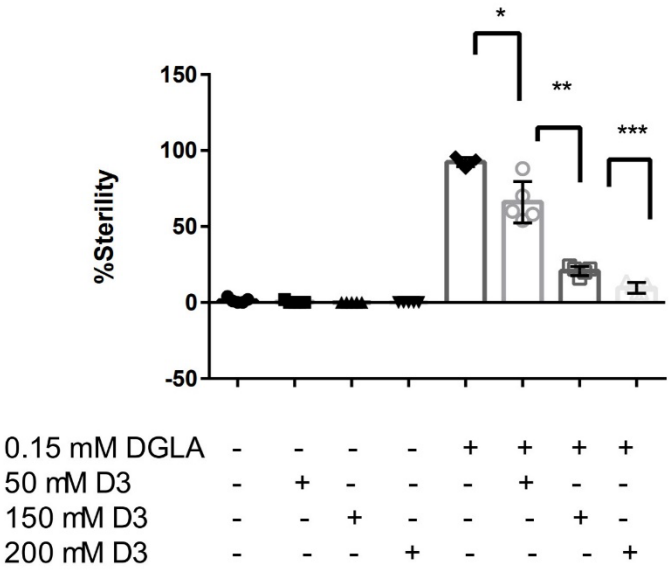

Figure 4: Dose-dependency of vitamin D_3-induced protection against sterility in WT *C. elegans.* Plates treated with exogenous DGLA were given a 100 mM stock such that dilution would produce a 0.15 mM concentration, and plates treated with vitamin D_3 were given either varying volume of a 236.3436 mM stock of vitamin D_3, to obtain a 50, 150, or 200 µM concentration. The treatments not given the full dose of vitamin D_3 were given an amount of pure ethanol that would equal a 84.62 µL addition of both vitamin D_3 and ethanol, to account for any effects of the ethanol on nematode growth**.** Percent sterility was measured in the same way as it was for the data in Figure 3. Administration of exogenous 0.15 mM DGLA to nematodes resulted in near-complete sterility. However, addition of vitamin D_3 to the treatment plates alongside DGLA resulted in reduced sterility in a dose-dependent manner, with 50 µM, 150 µM, and 200 µM vitamin D_3 consistently showing statistically significant ($P < 0.05$) reduced sterility with higher doses. These results suggest the protective effect of vitamin D_3 against DGLA-induced sterility exhibits dose-dependency.

Vitamin D_3 Fails to Protect Against DGLA-Induced Sterility for *gpx-1* Mutant Strains of *C. elegans*

The next experiment aimed to test if vitamin D_3 could exhibit a protective effect against DGLA-induced sterility in a ferroptosis-sensitive strain of *C. elegans.* The strain used for this test was *gpx-1,* which contains a deletion in the gene for glutathione peroxidase, an enzyme involved in maintaining cellular redox homeostasis through the utilization of glutathione to reduce peroxides (Ferguson & Bridge, 2019). The *gpx-1* worms were grown on *E. coli* OP50 plates treated with either no exogenous DGLA or 0.15 mM DGLA. Additionally, each plate contained a dose of exogenous vitamin D_3 that was either 0 µM, 50 µM, 150 µM, or 200 µM. 5 plates of nematodes were prepared for each treatment, except for the group given no DGLA or D_3, which had 3 plates due to difficulties in plate preparation**.** The *gpx-1* worms that received no exogenous DGLA exhibited low levels of base sterility higher than those seen in WT worms (Figure 5). This result can be expected given that *gpx-1* individuals have impaired homeostatic mechanisms for redox balance and therefore are likely more sensitive to ferroptosis even under conditions of no exogenously added DGLA. With no exogenous DGLA, vitamin D_3 was largely ineffective in lowering the base sterility of these individuals (Figure 5). When the worms were grown in 0.15 mM exogenous DGLA, sterility was substantially higher than when no DGLA was added. Additionally, vitamin D_3 was ineffective in driving a substantial reduction in the sterility levels of *gpx-1* individuals treated with exogenous DGLA (Figure 5). Although sterility was slightly lower when DGLA-treated worms were given 50 µM vitamin D_3 compared to no vitamin D_3, and likewise were lower for a 200 µM D3 dose compared to 150 µM, the sterility of the 150 µM group were higher than the 50 µM group. The difference in sterility between the

nematodes treated with 0.15 mM DGLA and 0 µM vitamin D_3 and those treated with 0.15 mM DGLA and 200 µM vitamin D_3 did show to be significant ($P < 0.05$), but due to the variability in sterility levels among the other concentrations vitamin D_3 used, it appears that any dose-dependent effects of vitamin D_3 on sterility in *gpx-1* nematodes are weak. There were also significant differences in sterility between DGLA-treated *gpx-1* nematodes given a certain dose of vitamin D_3 and the corresponding WT treatment group given the same dose. These results as a whole suggest that vitamin D_3 is not able to induce a substantial protective effect against DGLA-induced sterility in the *gpx-1* strain of *C. elegans.*

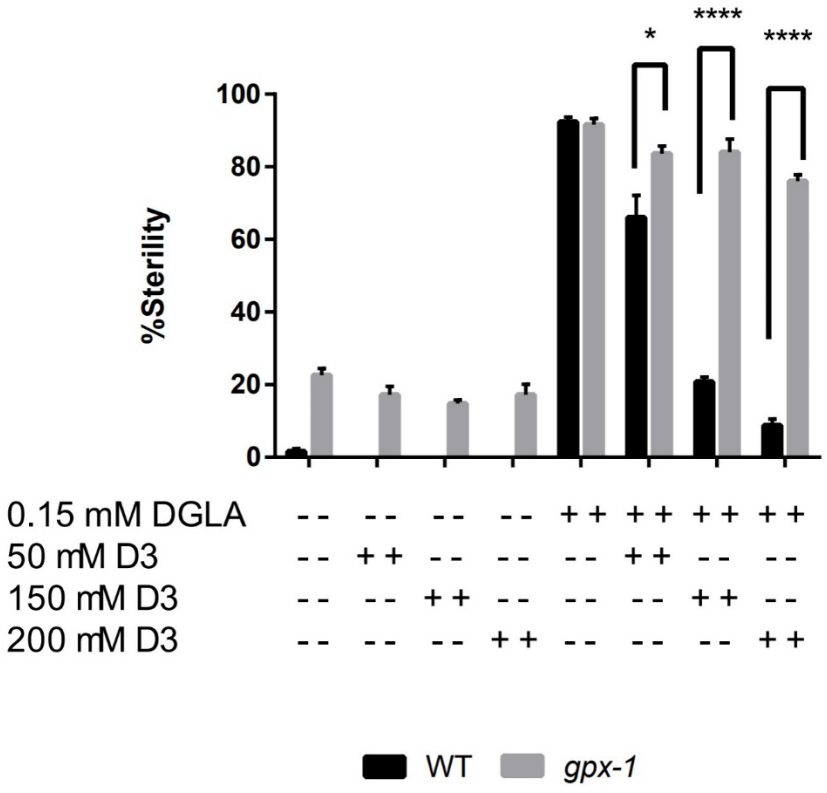

Figure 5: Sterility results for experimentation using individuals of the *gpx-1* strain of *C. elegans,* compared to those using the WT strain. Volumes and stocks added to treatments in order to obtain the desired concentrations of vitamin D_3 and DGLA were the same as for the experiment described in Figure 4**.** Sterility results are higher for individuals in the presence of 0.15 mM DGLA compared to when no exogenous DGLA was added. However, both with and without DGLA, sterility levels remained steady regardless of the concentration of exogenously added vitamin D_3. Although there did show to be a significant difference in sterility between the lowest and highest vitamin D_3 concentrations used alongside DGLA, the changes in sterility throughout the dose range were inconsistent. The sterility was significantly ($P < 0.05$) higher for *gpx-1* samples given a particular dose of vitamin D_3 alongside DGLA and the WT worms that were given the same doses. This suggests WT and *gpx-1* nematodes differ in their response to vitamin D_3 with regards to the protective effects of this compound against oxidative stress.

DISCUSSION

This study examined the influence of vitamin D_3 on DGLA-induced sterility in *C. elegans.* It has been previously demonstrated that treatment of *C. elegans* with exogenous DGLA leads to sterility and disrupted gonad morphology through the controlled form of cell death known as ferroptosis (Perez et al., 2020). It was also previously found that vitamin D_3 can improve both longevity and resistance to oxidative stress in *C. elegans* (Huggins & Farris, 2023).

This experiment demonstrated that wild-type *C. elegans* had lower levels of sterility when co-treated with vitamin D_3 and DGLA compared to treatment with DGLA alone. This relationship was also later found to be dose-dependent, with higher concentrations of vitamin D_3 promoting greater protection against sterility when treatment with DGLA was held at a constant concentration. However, vitamin D_3 did not have a protective effect against DGLA-induced sterility when tested with the *gpx-1* strain of *C. elegans.*

Curiously, the finding by Huggins & Farris that vitamin D_3 can promote oxidative stress resistance and improved longevity in *C. elegans* was only found to apply to the *nhr-8* mutant strain, which lacks an orthologous protein to the human vitamin D receptor. The results were not found to be significant for the WT strain (Huggins & Farris, 2023). Thus, it could be further investigated whether the results found in this study, in which WT worms were responsive to vitamin D_3 in improvements in sterility rates, could be replicated in the *nhr-8* mutant.

Previous research in humans has found that vitamin D supplementation can raise glutathione peroxidase-1 protein levels (Ansari et al., 2020). Other work has shown that vitamin D and its associated receptor are involved in the up-regulation of glutathione peroxidase-3 expression in human kidney cells (Wu et al., 2022). Wu et al. found that in human kidney cells, the *GPX3* gene was under transcriptional control of the vitamin D receptor, with VDR binding sites located throughout the *GPX3* promoter. Vitamin D was thus able to regulate oxidative stress in these kidney cells through regulation of glutathione peroxidase-3 at the transcriptional level. Wu also found that the vitamin D receptor agonist paricalcitol was unable to restore redox balance in *GPX3* knockdown cells (Wu et al., 2022). This result implies that vitamin D is implicated in the genetic regulation of glutathione peroxidase-3, yet knockdowns in the gene encoding the glutathione peroxidase-3 enzyme are unable to respond to vitamin D or its receptor with improved resistance to oxidative stress in human kidney cells. These results from Wu imply that a potential explanation for the inefficacy of vitamin D_3 in protecting against DGLA-induced ferroptosis in the *gpx-1* strain of *C. elegans* could be through transcriptional upregulation of a gene encoding a redox-balancing enzyme related to human glutathione peroxidase by an ortholog to the human vitamin D receptor. By this mechanism, exogenous vitamin D in the culture of WT *C. elegans* could increase transcription of the *gpx-1* gene and resultingly improve the ability of

these worms to respond to DGLA-induced oxidative stresses that could otherwise lead to ferroptosis and cell death in the germ cell line. For *gpx-1* mutants, which have a knockdown in the glutathione peroxidase ortholog, vitamin D-induced transcriptional activation would be ineffective in increasing levels of this enzyme, thus rendering the nematodes equally susceptible to DGLA-induced ferroptosis whether treated with or without exogenous vitamin D_3.

One potential caveat to this explanation is that the original results implicating the role of the vitamin D receptor in activating the transcription of a glutathione peroxidase enzyme was that it was only demonstrated for the glutathione peroxidase-3 enzyme, rather than for the ortholog to the gene product of *gpx-1* in *C. elegans*, which is the human GPX 4 enzyme (Kishore et al., 2020). Additionally, given that the results were found in human kidney cells, there would have to exist similar mechanisms in *C. elegans.* Thus, more research investigating this phenomenon is necessary. A potential further direction for research is to explore *C. elegans* mutants lacking an ortholog to the human vitamin D receptor, such as *nhr-8.* Assuming that the *gpx-1* gene is under control from an orthologous protein to the human vitamin D receptor, knocking down such an ortholog should render *C. elegans* individuals with the mutation unresponsive to vitamin D with regards to its protective effect against DGLA-induced ferroptosis.

Mammalian cells have been shown to undergo ferroptosis in response to DGLA (Perez et al., 2020). Given that vitamin D has been demonstrated to have an effect on the redox control of humans (Ansari et al., 2020), it should be investigated whether or not the protective effects of vitamin D_3 in DGLA-induced ferroptosis could also be found in mammalian cells. Given that human cancer cells have been shown to undergo ferroptosis (Perez at al., 2020), learning more

about the regulation of this process and the factors controlling it are imperative to exploiting its properties in the treatment of human disease.

CONCLUSION

This study investigated the role of vitamin D_3 in the regulation of DGLA-induced ferroptosis in *C. elegans.* It has been demonstrated previously that treatment of *C. elegans* with exogenous DGLA results in sterility caused by ferroptosis in the germ cell line (Perez et al., 2020). Vitamin D_3 has also previously been implicated in redox homeostasis in *C. elegans,* with *nhr-8* mutant individuals of *C. elegans* having been shown to have higher levels of oxidative stress resistance upon treatment with vitamin D_3. Given that ferroptosis is driven by redox imbalances within a cell, this study sought to mend these two findings by exploring the role of exogenous vitamin D_3 on DGLA-induced sterility in *C. elegans.* It was found that co-treatment of wild-type *C. elegans* individuals with exogenous vitamin D_3 and DGLA resulted in lower levels of sterility compared to those treated with DGLA alone. This finding was further supported through DAPI imagery, which showed improved gonadal morphology in those individuals co-treated with DGLA and vitamin D_3 compared to those treated with only exogenous DGLA.

Furthermore, the effect of vitamin D_3 on DGLA-induced sterility exhibited a dose dependency, with higher concentrations of vitamin D_3 showing a stronger protective effect against DGLA-induced sterility. Although the results showing a protective effect of vitamin D_3 were consistent for WT worms, an experiment was also performed investigating the effect of vitamin D_3 on *gpx-1* mutant worms, which are ferroptosis-sensitive and have impaired redox

homeostasis. It was shown that vitamin D_3 was ineffective in protecting these individuals against DGLA-induced sterility. This suggests a potential glutathione peroxidase-dependent mechanism by which vitamin D_3 can protect against redox imbalances in WT nematodes. Based on research in human kidney cells (Wu et al., 2022), it is thought this mechanism could potentially be one in which a vitamin D receptor ortholog serves as a transcriptional activator for the *gpx-1* gene, thus leading to higher levels of a glutathione peroxidase homologue and higher levels of redox protection for individuals grown in conditions rich in vitamin D_3. However, this proposed mechanism requires further investigation and testing. Given that DGLA-induced ferroptosis has been shown to occur in human cancer cells (Perez et al., 2020), the effect of vitamin D_3 on ferroptosis in mammalian cells also ought to receive further attention and exploration.

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