Dietary cholesterol shortens lifespan but enhances egg laying in *Caenorhabditis elegans* via DAF-2 dependent mechanism

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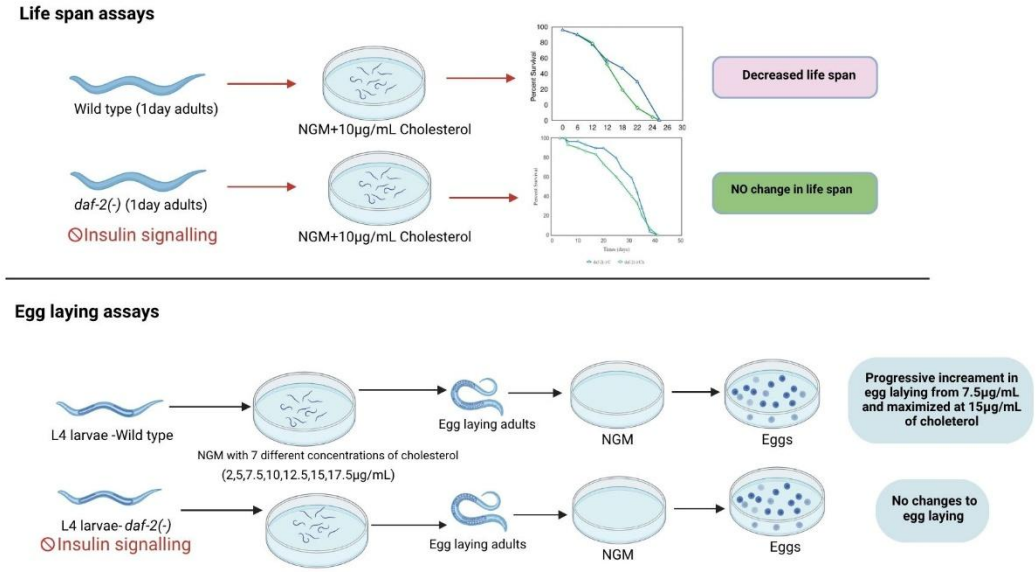

Abstract

Determining the variables and mechanisms that govern aging is important for facilitating healthy aging. Dietary fats, specifically cholesterol, possibly play a critical role in the aging of organisms. Additionally, dietary cholesterol may influence the reproduction of organisms. The insulin signalling/IGF pathway plays a vital role in longevity in *C. elegans*. Nevertheless, the relationship between cholesterol metabolism and the insulin/IGF signalling pathway is well known. Hence, cholesterol metabolism possibly has an indirect impact on the aging process of organisms. In this study, wild-type *C. elegans* and an insulin receptor mutant, *daf-2(-)*, were employed to investigate the effect of cholesterol on aging and egg laying. For the lifespan study, synchronised L4 larvae of wild type and daf-2(-) were exposed to 10 µg/mL cholesterol, a concentration twice the standard level routinely used for the maintenance of *C. elegans*. Life span assays were conducted using three biological replicates, each consisting of ten worms per plate. The egg laying of the above *C. elegans* strains was assayed at 7 different concentrations ranging from 2.5 µg/mL to 17.5 µg/mL of cholesterol, using 30 L4 larvae per treatment (n=30). Survival plots of the lifespan assay were created using the Kaplan-Meier estimator. Despite its negative impact on the life span of wild-type *C. elegans,* cholesterol has no significant effect on the life span of *daf-2* mutants, suggesting that cholesterol-mediated lifespan shortening is DAF-2 mediated. Egg laying data was statistically analysed using one-way ANOVA followed by the Tukey post HSD pairwise comparison, showing that there was a progressive increase in the egg laying starting from 7.5 µg/mL and effects maximised at the 15µg/mL concentration. The lower dose (2.5 µg/mL) and the highest concentration (17.5 µg/mL) did not exert a significant effect on egg laying in wild type *C. elegans*. No discernible changes in *daf-2(-)* egg laying were seen at any cholesterol concentration. Hence, both life span reduction at 10 µg/mL concentration of cholesterol and the enhancement of the egg laying are both DAF-2 dependent, while the effects on egg laying showed a dose dependency.

Keywords: *Life span, Egg laying, Cholesterol, C. elegans, Insulin signaling*

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Introduction

Caenorhabditis elegans is a small (1 mm in length at the adult stage), transparent, free-living soil nematode that feeds on bacteria such as *Escherichia coli*. It possesses various traits that make it an ideal model system in research (Strange, 2006). *C. elegans* plays a prominent and promising role in aging research. Studies on *C. elegans* have provided a wealth of information on aging (Zhang et al., 2020).

Cholesterol is a structural component that influences the fluidity and permeability of membranes and is a precursor to steroid hormones (Estahani and Swany, 1939). While cholesterol is needed in minute amounts for proper physiological function, excessive amounts can cause problems such as atherosclerosis. In humans, cholesterol affects the life span indirectly via some diseases such as heart diseases, atherosclerosis, *etc*. (Akoh, 2005). However, the direct effect of dietary cholesterol on human lifespan remains unclear. This research gap underscores the need for mechanistic studies in a model organism. *C. elegans* lacks *de novo* cholesterol synthesis. Hence, it requires exogenous cholesterol for survival and development. This makes it an exceptionally suitable model for studying the impacts of dietary cholesterol on lifespan and reproduction (Kurzchalia and Ward, 2003).

Recent scientific investigations have explored the relationship between cholesterol metabolism and the insulin signalling pathway. The transcription of the cholesterol 7α-hydroxylase enzyme, crucial in cholesterol metabolism, is upregulated when the insulin signalling pathway is downregulated (Pak and Park, 2011). This observation suggests a possible link between cholesterol metabolism and the aging process, implying that cholesterol may directly influence longevity through modulation of conserved aging pathways like the insulin signalling/IGF pathway.

Several studies have examined the effects of cholesterol on the aging and reproduction of *C. elegans*. According to Ruaud et al., 2011 low cholesterol levels cause life span reduction, decreased reproduction, and impaired movement ability. Furthermore, total cholesterol starvation reduced the average life expectancy of *C. elegans* by 40% (Merris et al., 2004). Conversely, Yuan et al., 2019 revealed that cholesterol supplementation increases the brood size of *C. elegans*, while, according to Korta et al. (2012), a cholesterol-lowering genetic mutation in *C. elegans* reduces worm brood size by reducing the number of germ cells developing in the gonad. Collectively, these studies demonstrate that cholesterol deficiency is detrimental to both longevity and reproduction in *C. elegans*. However, a critical gap exists in current knowledge of the impact of elevated cholesterol levels on *C. elegans* lifespan and reproduction. Most existing studies are focused on comparing cholesterol starvation or low levels without systematically investigating whether excessive cholesterol concentration might also be detrimental. Additionally, the molecular mechanism underlying the direct effects of cholesterol on aging, particularly through the insulin signalling pathway, also remains incompletely understood.

Thus, the purpose of this study is to investigate how dietary cholesterol affects the lifespan of *C. elegans* at a dose of 10 µg/mL, which is twice the standard 5 µg/mL supplement dose used for typical maintenance of *C. elegans*. Additionally, to do a comprehensive analysis of the effects of cholesterol on the egg laying of *C. elegans* under a series of cholesterol levels, and to elucidate the contribution of the insulin signalling pathway to those effects.

Materials and Methodology

Worm strains and bacterial strains

Caenorhabditis elegans wild-type Bristol isolate (N2), along with insulin signalling pathway mutant CB1370 *daf-2(-)* and *Escherichia coli* OP50, which is a uracil auxotrophic mutant as a food source for *C. elegans,* were obtained from the Caenorhabditis Genetics Center (CGC), which is supported by the National Institutes of Health - Office of Research Infrastructure Programs (P40 od010440).

C. elegans were grown and maintained on Nematode Growth Medium (NGM), which was prepared using the method described in Brenner (1974).

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Life span assay

Life span assay was carried out according to a modified method described by Kenyon and Hsin (1999), and all life span experiments were performed at 15 °C. For life span assays, hermaphrodite *C. elegans* populations were synchronized and maintained until they reached the one-day adult stage. This synchronised one-day adult population was used for the lifespan assay. Individual *C. elegans* were transferred to NGM agar plates containing 10 µg/mL cholesterol using a sterile worm picker. The control group was provided with 5µg/mL cholesterol. The plates have been seeded with *E. coli* OP50. To prevent progeny interference and to maintain consistent conditions, *C. elegans* were transferred to new plates every other day until death. Three replicates per treatment were performed, with 10 worms per plate. The experiment was performed separately for wild-type and daf-2 mutants. *C. elegans* were considered dead when they ceased moving and responding to prodding. *C. elegans* that crawled off are considered censored. "Bag of worms" were considered dead. This step incorporated those *C. elegans* into the data set until their censor date.

Egg laying assay

The effects of cholesterol on egg-laying behaviour in *C. elegans* were determined by the modified method of Thomas *et al*. (1995). Larvae at the L4 stage were exposed to 7 different cholesterol concentrations (2.5, 5, 7.5, 10, 12.5, 15, 17.5 µg/mL). For that, larvae at the L1 stage were produced with hermaphrodite animals of N_2 and *daf-2(-)* by using the method to produce a synchronized worm population. L1 larvae were allowed to grow on *E. coli-seeded* plates with standard NGM. It takes 48 hours to develop from L1 to L4. L4 *C. elegans* on standard NGM media were washed with 1-2 mL of M9 buffer and transferred into plates of NGM with different cholesterol concentrations. After 24 hours of exposure to the different cholesterol concentrations at the young adult stage (one day adult), *C. elegans* were transferred into standard NGM plates under a dissecting microscope with a sterile worm picker, with 10 worms per plate, with three replicates for each treatment. After two hours of incubation at 15°C, eggs in each plate were counted.

Statistical analysis

Survival plots were generated using the online platform OASIS. (https://sbi.postech.ac.kr/oasis/), where the Kaplan-Meier estimator was applied to generate survival curves along with the censored data. Within OASIS, statistical significance between two groups was evaluated using the log-rank test and the weighted log-rank test. For egg-laying assays, group differences were first assessed using a one-way ANOVA followed by a Tukey HSD post hoc test to do the pairwise comparison while controlling the family-wise error rate.

Results

Effects of dietary cholesterol on the life span of C. elegans

Results of the experiment demonstrated that there is a significant reduction (P= 0.0155) in the life span of wild-type *C. elegans* provided with 10µg/ml dietary cholesterol, while there is no significant (P=0.3512) effect on the life span of *daf-2(-)* fed with cholesterol (Table 1 and Figure 1).

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Table 1: Data of life span assays for the determination of the effects of cholesterol on the life span and the involvement of the insulin signalling pathway. (WT = wild type) In Table 1, the % change in lifespan and *P* values were calculated relative to the same control data.
Increase (+) or decrease (-) in lifespan was indicated

Strain and treatment	Mean life span ± S.E.M (days)	Change of lifespan	P value
WT/ Control	18.3 ± 1.26		
WT/Cholesterol 10µg/mL	15.49 ± 0.92	-15	P = 0.0155
***daf-2(-)*/Control**	31.03 ± 1.46		
***daf-2(-)*/Cholesterol 10µg/mL**	27.67 ± 1.79	-11	P = 0. 3512

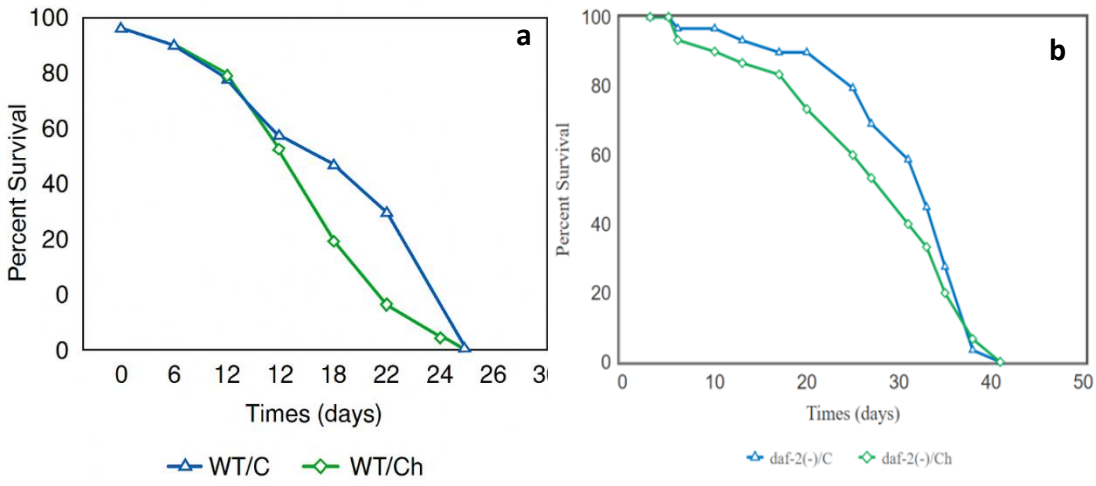

Figure 1: Survival patterns of wild-type and daf-2 mutants on Cholesterol (These figures were created using the online survival assay platform, OASIS: https://sbi.postech.ac.kr/oasis/). **Figure 1(a)** Survival pattern of wild type C. *elegans* treated with 10µg/mL cholesterol and control. WT/c stands for wild-type control, and WT/ch stands for 10 µg/mL cholesterol fed wild-type *C. elegans*. Excess dietary cholesterol shows a significant change in the life span of wild-type *C. elegans.* **Figure 1(b)** Survival pattern of *daf-2(-)* treated with 10µg/mL cholesterol and control. A diet containing 10µg/mL cholesterol did not show any significant effect on the life span of *daf-2(-)* animals. *daf-2*(-)/c stands for control of *daf-2(-)* and *daf-2*(-)/ch stands for 10µg/mL cholesterol fed *daf-2(-).*

Effects of dietary cholesterol on egg laying of C. elegans

The results of the egg-laying assay showed that there is an increasing trend in the mean number of eggs per worm of wild-type *C. elegans* starting from 7.5 µg/mL up to 15 µg/mL (Figure 2b), and those changes are statistically significant according to the Tukey post HSD test, compared to the control (Table 2). Interestingly, the mean number of eggs per worm at the highest cholesterol concentration (17.5µg/mL) was not significantly different from the control. Although higher cholesterol levels show a significant effect on egg laying of wild-type *C. elegans,* there is no significant

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effect on egg laying of *daf-2* mutants at any concentration of cholesterol used in this experiment (Figure 2a).

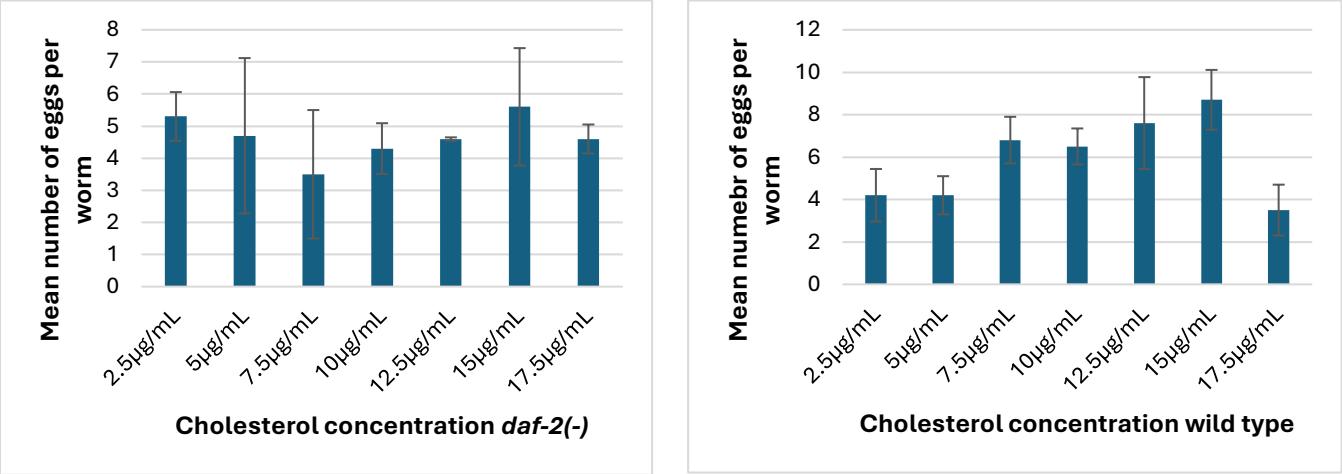

Figure 2: (a) Graph of the mean no of eggs per worm at different concentrations of cholesterol in *daf-2(-) C. elegans*. The mean number of eggs per worm is not statistically different from the control at any of the cholesterol concentrations. (b) Graph of the mean number of eggs per worm at varying concentrations of cholesterol in wild-type *C. elegans*. Post hoc Tukey HSD comparisons showed that concentrations of 7.5, 10, 12.5, and 15 µg/mL significantly increased egg laying compared to the control.

Table 2: Mean number of eggs per worm of *C. elegans* at different concentrations of cholesterol and their mean difference compared to the control and adjusted P value obtained from Tukey post hoc analysis after the one-way ANOVA. (WT= Wild type, Cho = Cholesterol)

Treatment and Strain	**Mean no of eggs per worm ± SEM**	**Mean difference**	**Adjusted P value with Tukey post hoc analysis**
WT/Cho 2.5 µg/mL	4.16 ± 0.22	- 0.10	0.99
WT/Cho 5 µg/mL (Control)	4.26 ± 0.16	0	
WT/Cho 7.5 µg/mL	6.8 ± 0.18	+ 2.54	0.015
WT/Cho 10 µg/mL	6.46 ± 0.15	+ 2.20	0.035
WT/Cho 12.5 µg/mL	7.6 ± 0.39	+ 3.34	0.002
WT/Cho 15 µg/mL	**8.7 ± 0.25**	**+4.44**	**<0.001**
WT/Cho 17.5 µg/mL	3.53 ± 0.21	- 0.73	0.85
***daf-2(-)*/Cho 2.5 µg/mL**	5.33 ± 0.13	+ 0.63	0.45
***daf-2(-)*/Cho 5 µg/mL (Control)**	4.7 ± 0.44	0	
***daf-2(-)*/Cho 7.5 µg/mL**	3.5 ± 0.37	- 1.8	0.86

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***daf-2(-)*/Cho 10 µg/mL**	4.3 ± 0.14	- 0.4	0.89
***daf-2(-)*/Cho 12.5 µg/mL**	4.96 ± 0.10	+ 0.26	0.81
***daf-2(-)*/Cho 15 µg/mL**	5.67 ± 0.33	+ 0.97	0.86
***daf-2(-)*/Cho 17.5 µg/mL**	4.6 ± 0.08	- 0.1	0.89

Discussion

Effect of cholesterol on the lifespan of C. elegans

The cholesterol concentration of 10 µg/mL was selected for the lifespan assay because it represents a supra-physiological level relative to standard maintenance conditions. The typical cholesterol concentration used for routine maintenance of *C. elegans* is 5 µg/mL. Therefore, 10 µg/mL constitutes a two-fold increase over the maintenance dose. Importantly, previous studies have demonstrated that this concentration is excessive for *C. elegans* and is associated with adverse effects, including neurodegenerative effects (Ramnathan and Deshmuk, 2009). Hence, this concentration was chosen to examine the impact of elevated cholesterol on lifespan.

Aging in *C. elegans* is a complex process contributed by many genes. The most described gene is *daf-2,* which encodes the DAF-2, insulin-like growth factor receptor. Mutations in *daf-2* lengthen the lifespan of *C. elegans*. Another prominent gene known to regulate the lifespan of *C. elegans* is *age-1,* which is a downstream target of the insulin signalling pathway and encodes the AGE-1 homolog of phosphatidylinositol 3-kinase (William, 1998).

daf-2 and *age-1* mediated lifespan changes through the downregulation of the IIS pathway mainly depend on DAF-16, which is the homolog of the FoxO transcription factor. Nuclear localisation of DAF-16 due to downregulation of the IIS pathway extends lifespan in *C. elegans* through transcriptional changes in downstream targets of DAF-16 (Masharu and Eisuke, 2016). However, the oxidative stress-responsive gene SKN-1 has been shown to regulate various lipid metabolic genes, is a downstream target of DAF-2, and has its activity regulated by DAF-2. To validate the claim that cholesterol-mediated life span reduction is mediated by the nuclear-localised DAF-16, transgenic worms of daf-16 fused with GFP can be used.

As *daf-2* is a major governing gene of the lifespan of *C. elegans,* to determine the contribution of cholesterol to lifespan changes through the insulin signalling pathway, the same experiment was carried out for *daf-2* mutants. *daf-2* mutants have downregulated insulin signalling pathway due to a lack of DAF-2 receptors.

The observation of a reduction in lifespan due to cholesterol in the wild type was not observed in *daf-2* mutants. This implies that the lifespan reduction by cholesterol requires a functional DAF-2 insulin receptor. This is due to upregulated DAF-16 in *daf-2(-)* in the absence of DAF-2.

C. elegans metabolizes cholesterol that they take from outside sources to produce the steroid hormone dafachronic acid, which is essential in the development of *C. elegans* into a reproductive adult (Goncalves *et al*., 2020). DAF-9, encoded by the *daf-9* gene, is a component of the pathway in which *C. elegans* synthesises sterols from cholesterol obtained from external sources (Riddle *et al*., 2002). DAF-9 is the *C. elegans* homolog of the cytochrome P450 and is transcriptionally regulated by nuclear-localised DAF-16. Hence, in *daf-2(-),* sterol biosynthesis occurs at a higher rate due to upregulated DAF-16, which, in turn, increases transcription of *daf-9* (Jeong *et al*., 2010). Sterol synthesis from cholesterol takes place at a lower rate in wild-type worms than in *daf-2(-)* due to upregulated DAF-16 and DAF-9. Consequently, in *daf-2(-),* excess cholesterol is rapidly removed, whereas in the wild type, it is not. Therefore, this can be a possible reason for the shortening of life span in wild-type *C. elegans*. Excess cholesterol in wild-type *C. elegans* that is not utilised may accelerate aging. *In vitro* studies by Ramanthan and Deshmuk (2009) show that exposure to high

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cholesterol levels (10 µg/mL) has a neurodegenerative effect, which reduces the cell count with time. This can be taken as a reason for the shortened lifespan of the wild-type animals. Cholesterol 7α-hydroxylase (CYP7A1) is the human ortholog for DAF-9, which plays an important role in maintaining cholesterol homeostasis and bile acid biosynthesis. DAF-9 (CYP7A1) is regulated mainly at the transcription level by various transcription factors. Activated IIS pathway inhibits the transcription of CYP7A1 *via* the inactivation of FOXO1 (Park and Pak, 2011). This suggests that the IGF pathway regulates cholesterol levels through the transcriptional regulation of DAF-9 (CYP7A1).

Most of the lifespan assays of *C. elegans* were done under the influence of the chemical 5-fluoro-2-deoxyuridine (FUdR) to inhibit the reproduction of worms. This avoids the need to transfer worms into new plates. In this study, we did not use FUdR to prevent any effects of reproductive inhibition on lifespan. Some previous studies have shown a direct connection between *C. elegans* reproduction and longevity. According to Kenyon et al. (1999), removing germline cells increased the lifespan of *C elegans* by 60%. Moreover, the downregulated IIS pathway, specifically through the DAF-2 mutation, has been shown to delay reproductive development, indicating a connection between the IIS pathway and reproductive development (Kenyon et al., 1993). Furthermore, DAF-12 nuclear receptor conjugates with DAF-16 in the absence of reproductive signals to extend lifespan (Larsen et al., 1995). Hence, in this study, we aimed to observe the lifespan of *C. elegans* without inhibition of reproduction.

Effect of cholesterol on egg laying of C. elegans

Cholesterol is crucial for the synthesis of steroid hormones, such as dafachronic acid, which is required for activating the nuclear hormone receptor DAF-12, which regulates processes related to aging, development, and reproduction (Merris et al., 2022).

For egg laying assays, several cholesterol concentrations ranging from 2.5 µg/mL to 17.5 µg/mL were selected to establish a dose-response relationship. The lowest concentration represents the minimum amount of cholesterol required for the survival of *C. elegans*. Then progressively higher concentrations were selected from the standard 5µg/mL, extending up to 17.5 µg/mL, to examine the impact of higher cholesterol levels on egg laying of *C. elegans*. A progressive increase in the mean number of eggs laid per worm was observed with rising cholesterol concentrations from 7.5 µg/mL to 15 µg/mL, with the maximum effect observed at 15 µg/mL. These observations suggest that elevated cholesterol levels within this range enhance the egg production capacity in *C. elegans*. This observation can be explained by the fact that cholesterol is an essential precursor for sterol-derived hormones and signalling molecules in *C. elegans*, including dafachronic acids, which regulate reproductive development and germline functions. Dafachronic acids bind and regulate DAF-12, which acts as the switch between reproductive growth and dauer formation in *C. elegans*. (Motola et al., 2006). Thus, elevated cholesterol levels promote steroid signalling pathways and enhance egg-laying behaviour in *C. elegans*. In contrast, higher concentrations, such as 17.5 µg/mL, did not show any effects on egg laying, suggesting an inhibitory or toxic effect at higher concentrations of cholesterol. This progressive increment in egg laying was not observed in *daf-2(-)*, suggesting that these effects of cholesterol on egg laying are through the insulin signalling pathway and mediated by DAF-2. This effect on egg laying can be further explained by the behaviour of nematode sterol-binding protein-1 (NSBP-1), which links cholesterol metabolism of *C. elegans* into the insulin signalling pathway. According to Cheong et al., 2013 NSBP-1 is one of the downstream targets of DAF-2, which translocates into the nucleus in the absence of DAF-2 and binds with DAF-16 to control metabolic activities and fat accumulation. The binding of NSBP-1 to DAF-16 is mediated by the cholesterol concentration. Cholesterol-mediated egg-laying boost was only observed in wild type *C. elegans* and was not apparent in *daf-2(-)* due to the translocation of NSBP-1 into the nucleus in the absence of DAF-2. Furthermore, the concentration-related effect on egg laying is supported by the binding of NSBP-1, which is influenced by cholesterol concentration and subsequently downstream processes controlled by DAF-16 (Ihara et al., 2017). Koyiloth and Gummadi (2022) suggest that, unlike other

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organisms, *C. elegans* requires only a minute amount of cholesterol for the maintenance of physiological activities and reproduction. This was further supported by Merris et al. 2022. However, the cholesterol deprivation causes a significant negative impact on *C. elegans* egg laying, viability of eggs and the germline cells (Shim et al., 2002: Cheong et al, 2010: Merris et al., 2022).

In general, cholesterol plays a central role in endocrine signalling in *C elegans*, providing the precursor for the dafachronic acid synthesis. Dafachronic acid is the primary steroid hormone in *C. elegans*. Supplemented cholesterol is enzymatically converted into dafachronic acid through multiple steps, in which DAF-36 and DAF-9 play a crucial role as enzymes. These dafachronic acids bind and activate the downstream transcription factor DAF-12, which is the major transcriptional regulator that mediates environmental and metabolic signals to the growth and development, reproduction and lifespan in *C. elegans* (Gerisch et al., 2001; Dumas et al., 2015; Aguilaniu et al., 2016). Importantly, studies showed that the effects of cholesterol on *C. elegans* physiology depend on its conversion to dafachronic acid and subsequent activation of DAF-12 (Cheong et al., 2010; Mukherjee et al., 2018). It has been shown that *C. elegans* brood size was significantly reduced upon cholesterol starvation. Additionally, embryonic survival rates reduced over generations due to cholesterol starvation (Shim et al., 2002). Hence, studying the viability of embryos and the brood size of worms would be beneficial.

Conclusions

Cholesterol reduces the life span of wild type *C. elegans* but does not affect the life span of *daf-2(-)*. This is possibly due to DAF-16 mediated active metabolism of cholesterol. The results of the egg-laying assay indicated that cholesterol significantly increases reproduction in *C. elegans.* As these effects are mediated through the insulin signalling pathway, it greatly influences reproduction. This study reveals a possible effect of cholesterol on lifespan and reproduction, underscoring the importance of diet for quality of life. Limitations of this work are that it does not explicitly address the underlying molecular mechanism or the role of steroid hormones in these effects, although alterations in lifespan and egg laying were observed and the relevance of DAF-2 and the insulin signalling pathway was clarified. The main reproductive result in this study is egg laying. However, it is also crucial to consider other factors, including brood size and embryo viability.

Conflict of interest

There is no conflict of interest.

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