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Sulforaphane Prevents Cadmium Chloride-Induced Reproductive Toxicity in *Caenorhabditis elegans*

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Abstract

Cadmium (Cd) is a highly toxic heavy metal that disrupts development and reproduction, primarily through oxidative stress. In this context, sulforaphane (SFN), an antioxidant compound, may serve as a promising agent to counteract Cd-induced oxidative damage and prevent developmental and reproductive abnormalities. This study aimed to evaluate the effect of SFN on reproductive toxicity induced by cadmium chloride ($CdCl_2$) in the nematode *Caenorhabditis elegans* (*C. elegans*). Five experimental groups were established: (I) Control: no treatment, (II) dimethyl sulfoxide (DMSO): 48 h with 0.01% DMSO, (III) $CdCl_2$: 24 h with 4600 µM $CdCl_2$, (IV) SFN + $CdCl_2$: 24 h with 100 µM SFN followed by 24 h with both SFN and $CdCl_2$, and (V) SFN: 48 h with 100 µM SFN. Co-exposure to SFN and $CdCl_2$ prevented the reduction in the percentage of adult nematodes and increased egg-laying. It also significantly improved hatching rates, allowing more embryos to reach the larval stage, and prevented reductions in body size. However, no effects were observed on glutathione S-transferase-4 (GST-4) levels in the transgenic CL2166 strain. In conclusion, SFN substantially prevents Cd-induced reproductive toxicity in *C. elegans*. Future studies should investigate the molecular mechanisms by which SFN enhances egg-laying and offspring viability in this model.

Keywords: reproductive toxicity; cadmium; sulforaphane; *Caenorhabditis elegans*; heavy metals

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1. Introduction

Heavy metal pollution, including cadmium (Cd), poses a significant threat to food security and public health [1]. Cd is a highly toxic metal with no known biological function in living organisms. Its toxicity impacts multiple body systems, leading to diseases such as chronic kidney disease, end-stage renal failure, diabetes, cancer, and even death [1–5]. Notably, Cd exposure is strongly associated with adverse reproductive outcomes in both males and females, disrupting fundamental processes that lead to infertility, hormonal imbalances, and developmental disorders [6–8]. The primary mechanism by which Cd

interferes with reproduction is through the excessive production of reactive oxygen species (ROS), resulting in oxidative stress and cellular damage [6,9]. Additionally, Cd acts as an endocrine disruptor, mimicking or inhibiting the actions of endogenous hormones and interfering with steroidogenesis [6,10,11]. It has also been shown to induce epigenetic changes that alter the expression of genes involved in reproductive function, thereby perpetuating its toxic effects [6].

On the other hand, sulforaphane (SFN) is a bioactive compound widely recognized for its cytoprotective, antioxidant, and anti-inflammatory properties [12,13]. Several studies have reported that SFN attenuates the toxic effects of heavy metals [14,15]. It has been shown that SFN reduces apoptosis and necrosis in human lymphocytes, as well as inflammation and oxidative stress in HepG2 cells, thereby decreasing Cd-induced hepatotoxicity [14–16]. Furthermore, our research group previously demonstrated that SFN protects *Caenorhabditis elegans* (*C. elegans*) from disruptions in redox homeostasis and mitochondrial function caused by cadmium chloride ($CdCl_2$) [17]. However, there is still limited evidence regarding the specific effects of SFN on Cd-induced reproductive toxicity.

In this context, the nematode *C. elegans* is an ideal model for reproductive toxicity studies due to its simple and highly conserved reproductive system, short life cycle, and high fecundity, which enable rapid experiments and statistically significant data collection [18–20]. *C. elegans* is also highly sensitive to toxic compounds such as heavy metals and endocrine disruptors, and its response to these toxins is relevant for understanding human toxicity [18,21]. Moreover, exposure to Cd in *C. elegans* has already been reported to negatively affect reproduction. Cd exposure impairs oogenesis and fertility, induces apoptosis in germ cells, and reduces the number of offspring produced [22,23]. Additionally, Cd disrupts nutrient absorption and alters egg-laying behavior, further decreasing reproductive success [24–26]. Therefore, the objective of this study is to investigate whether SFN supplementation can mitigate $CdCl_2$-induced reproductive toxicity in the nematode *C. elegans*.

2. Materials and Methods

2.1. Reagents

Sulforaphane (SFN-S8044) was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Cadmium chloride ($CdCl_2$, C2544), yeast extract (70161), cholesterol (C3045), dimethyl sulfoxide (DMSO), and streptomycin sulfate (S6501) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium hypochlorite (NaClO) was purchased from Cloralex (Oakland, CA, USA). Levamisole hydrochloride 12% (Levamisole HCl 12 g, Q-0021-006) was obtained from PARFAM S.A. (Mexico City, Mexico). Bacto™ agar (214010), Bacto™ peptone (211677), and Bacto™ tryptone (211705) were purchased from GIBCO (Thermo Fisher Scientific; Waltham, MA, USA). Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, 2500-01), monobasic potassium phosphate (KH_2PO_4, 3246-01), disodium phosphate (Na_2HPO_4, 3828), sodium hydroxide (NaOH, 46697), and sodium chloride (NaCl, 7647) were purchased from JT Baker (Xalostoc, State of Mexico, Mexico). Potassium chloride (KCl, 6858) and dibasic potassium phosphate (K_2HPO_4, 7088) were purchased from Mallinckrodt AR (St. Louis, MO, USA).

2.2. C. Elegans Strains and Maintenance

The *C. elegans* strains used in this study were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). The strains employed in this experiment include: N2 (wild-type, Bristol [27] and CL2166 dvIs19 [(pAF15)gst-4p::GFP::NLS]III [28].

C. elegans was cultured on Nematode Growth Medium (NGM) plates [composition: 0.3% NaCl, 1.7% agar, 2.5% peptone, 0.1% potassium phosphate buffer (1 M, pH 6.0), 5 µg/mL cholesterol, 1 mM $CaCl_2$, and 1 mM $MgSO_4$] seeded with a lawn of *Escherichia coli* strain OP50-1 (CGC, University of Minnesota, Minneapolis, MN, USA). *E. coli* cultures were grown overnight at 37 °C in lysogeny broth (LB: 10 g Bacto™ tryptone, 5 g yeast extract, 5 g NaCl, and 1000 mL ddH_2O supplemented with streptomycin), and then diluted to an optical density at 600 nm (OD600) of approximately 0.3, as previously described [29]. Subsequently, 1000 µL of the *E. coli* suspension was seeded onto 60 mm NGM plates and dried overnight at room temperature. Worms were maintained following standard protocols [30] at an incubation temperature of 20 °C.

2.3. Experimental Design

C. elegans were collected from NGM plates using M9 buffer (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4 \cdot 7\ H_2O$, and 1000 mL ddH_2O), and cultures were synchronized using a bleaching solution (0.5% NaClO and 0.5 M NaOH, mixed at a 2:1 ratio) [31]. Subsequently, eggs were allowed to hatch on NGM plates seeded with *E. coli* to ensure larval development. Twenty-four hours after synchronization, L1-stage larvae were transferred to 6-well microplates containing K medium (52 mM NaCl and 32 mM KCl), *E. coli* (1:10 dilution), and the respective treatments. Plates were incubated at 20 °C under constant agitation (Table 1). Five experimental groups were established: (I) Control, untreated; (II) DMSO, vehicle for SFN; (III) $CdCl_2$; (IV) SFN + $CdCl_2$; and (V) SFN. The concentrations used in this study were previously established by our research group [17]. To prepare the SFN working concentrations, a 100 mM stock solution was prepared in DMSO and diluted 1:1000 in K medium. For $CdCl_2$, a 1 M stock solution was prepared in Milli-Q water and subsequently diluted to 50 mM in K medium [17,32,33].

Table 1. Experimental groups.

Well	Treatment 1: for 24 h	Treatment 2: Another 24 h
Control	Vehicle only	Vehicle only
DMSO	DMSO (0.01%)	DMSO (0.01%)
$CdCl_2$	Vehicle only	$CdCl_2$ (4600 µM)
* SFN + $CdCl_2$	SFN (100 µM)	SFN (100 µM) + $CdCl_2$ (4600 µM)
SFN	SFN (100 µM)	SFN (100 µM)

Abbreviations: DMSO = dimethyl sulfoxide; SFN = sulforaphane; $CdCl_2$ = cadmium chloride; Vehicle only: K medium with bacteria. * The co-exposure group (SFN + $CdCl_2$) received SFN alone during the first 24 h, followed by a renewal of SFN and the addition of $CdCl_2$ during the final 24 h of treatment. For all experimental groups, a medium change with fresh bacteria was performed at the 24 h mark.

Upon completion of the treatment exposures, evaluations were conducted following the protocol outlined in Figure 1. Evaluation Day 0 was designated as the time point marking the conclusion of the treatment phase. Throughout the assessment period, nematodes were maintained on Nematode Growth Medium (NGM) agar plates seeded with *E. coli* OP50-1 and incubated at 20 °C to ensure consistent environmental conditions.

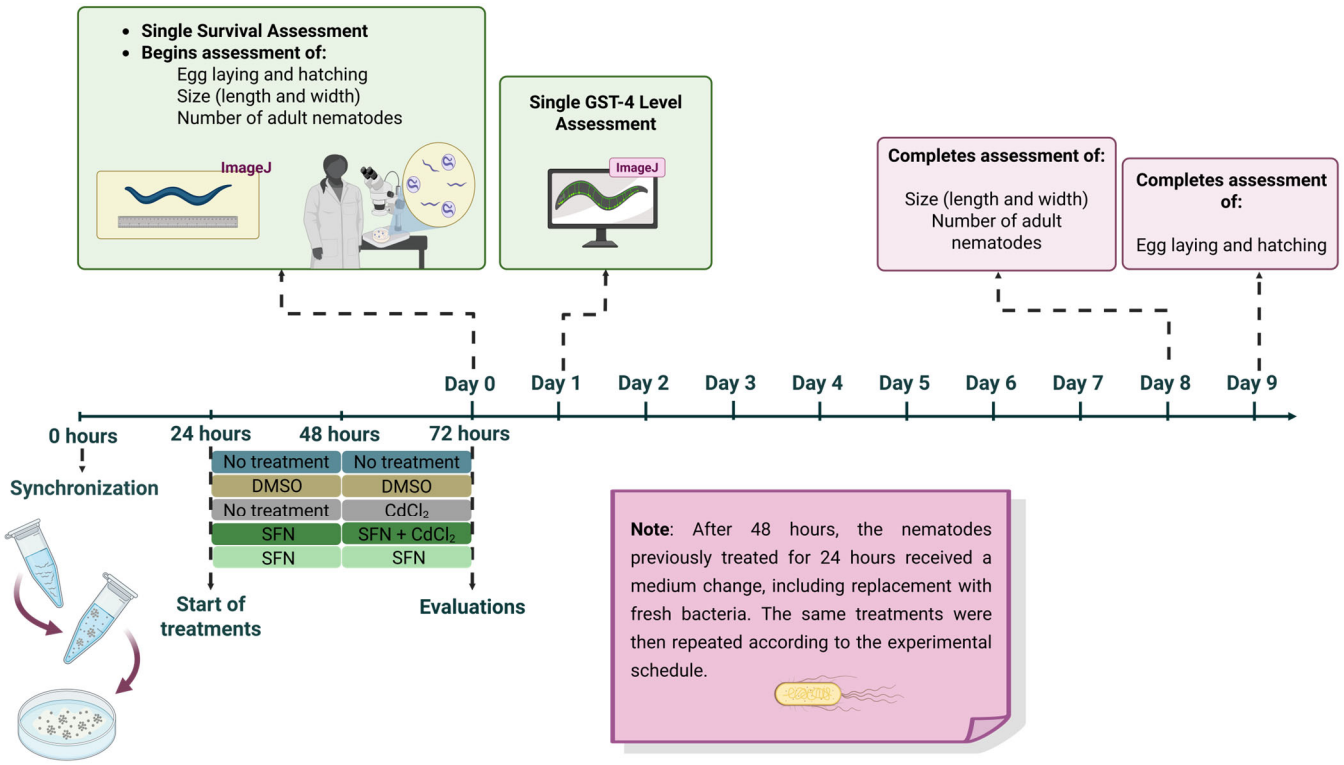

Figure 1. Experimental design to evaluate the protective effects of sulforaphane (SFN) against cadmium chloride ($CdCl_2$)-induced reproductive toxicity in *Caenorhabditis elegans* (*C. elegans*). Nematodes were synchronized at 0 h, and treatments were initiated 24 h post-synchronization. At 48 h, nematodes previously treated for 24 h underwent a media change, including the addition of fresh bacteria, followed by reapplication of the treatments. At 72 h post-synchronization, a single survival assessment was conducted, and evaluations of egg-laying, hatching, body size (length and width), and the number of adult nematodes were initiated. Oxidative stress marker GST-4 expression was assessed on day 1. Measurements of nematode size and adult count were completed on day 8, while egg-laying and hatching evaluations concluded on day 9. DMSO: dimethyl sulfoxide. Created in BioRender. Chaverri, J. (2025) BioRender.com/610n0h0 (accessed on 20 May 2025).

2.4. Survival

After treatment exposure, N2 nematodes were classified as alive or dead by observation under a dissecting microscope (Nikon SMZ-645, Nikon Instruments Inc., Melville, NY, USA). Nematodes were considered alive if they exhibited mobility in a liquid medium, and dead if they showed no movement, even after gentle agitation [34]. Five independent experiments were conducted, each including three technical replicates per condition.

2.5. Number of Eggs Laid

Following treatment exposure, individual N2 hermaphrodites were transferred to NGM plates seeded with *E. coli* [35]. Every 24 h, the parent worms were removed and placed on a new plate under the same conditions until the end of the experiment. The total number of eggs per day was recorded by counting both the eggs and the larvae present on each plate (to include eggs that had already hatched). Counting was performed manually using a hand tally counter and a dissection microscope (Nikon SMZ-645, Nikon Instruments Inc., Melville, NY, USA). Seven independent experiments were conducted, each including three technical replicates per condition.

2.6. Progeny Quality

To assess progeny quality, the number of hatched eggs was determined. After treatment exposure, individual N2 hermaphrodites were transferred to NGM plates seeded with *E. coli*. Each worm was transferred daily to a new plate under the same conditions for nine consecutive days. This allowed us to isolate the eggs laid by each worm per 24 h interval. The number of hatched larvae and unhatched eggs on each plate was recorded daily under a dissecting microscope. An egg was considered hatched when a larva had visibly exited the eggshell. At the end of the experiment, the total number of eggs and hatched larvae per worm (summed across all plates from day 1 to day 9) was used to calculate the hatching percentage. Three independent experiments were conducted, each including three technical replicates per condition.

2.7. Size and Development of C. Elegans

Nematodes were collected at the designated evaluation time points and transferred to black 96-well plates with clear flat bottoms (cat. no. sc-204468; Santa Cruz Biotechnology Inc., Dallas, TX, USA). Subsequently, worms were immobilized using 5 mM levamisole, and images were captured using the Cytation™ 5 imaging system and multimode plate reader (BioTek Instruments, Inc., Winooski, VT, USA), with a 4× objective and brightfield filter. The acquired images were analyzed using Fiji ImageJ software version 1.54f [36]. With these nematodes and using the 20× objective of the Cytation™ 5, the worms were classified as non-adults (no visible eggs in the vulval region) or adults (visible eggs in the vulval region). Three independent experiments were conducted.

2.8. Expression of Glutathione S-Transferase-4 (GST-4)

GST-4 expression was assessed on day 1 of the experiment using the *C. elegans* strain CL2166 dvIs19 [(pAF15)*gst-4p*::GFP::NLS]III. Nematodes were collected after exposure to the different treatments and transferred to black 96-well plates with clear flat bottoms. Worms were then immobilized with 5 mM levamisole for 15 min, and fluorescence images were captured using the Cytation™ 5 imaging system and multimode plate reader (BioTek Instruments, Inc., Winooski, VT, USA), with a 4× objective and a green fluorescence filter (465 nm).

The images obtained were analyzed using Fiji ImageJ software version 1.54f [36]. A macro was developed to automate the analysis, which included the following steps: conversion of images to 8-bit grayscale, segmentation of worms using automatic thresholding, and manual selection of the corresponding outlines. Each outline was stored in the Region of Interest (ROI) manager, and fluorescence intensity was measured in the appropriate channel for each worm. To account for the background signal, random rectangles were placed in non-fluorescent regions and designated as ROIs. Background fluorescence was subtracted from each fluorescence measurement, and the resulting values were normalized to those of the control group. Furthermore, fluorescence values were adjusted according to the size of each nematode to ensure accurate and reliable comparisons across experimental conditions.

2.9. Statistical Analysis

Results are presented as the mean ± standard error of the mean (SEM). The Kolmogorov–Smirnov test was used to assess normality. Differences between experimental groups were analyzed using one-way or two-way analysis of variance (ANOVA), as appropriate, followed by Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism 9™ software (GraphPad Software, Inc., San Diego, CA, USA). Additional details regarding the number of biological and technical replicates, the number of worms

or eggs analyzed, and the associated statistics for each experiment are provided in the Supplementary Tables.

3. Results

3.1. SFN Prevented $CdCl_2$-Induced Reduction in Survival

C. elegans (Bristol-N2) were exposed to the different treatments described in Table 1, and survival was assessed for 48 h post-treatment. As illustrated in Figure 2, the control group demonstrated a survival rate of 96.4 ± 1.2%. Treatment with $CdCl_2$ led to a marked decrease in survival, reducing it to 53.3 ± 3.1% ($p < 0.001$ vs. control), indicating a 46.7% reduction relative to the control group. Conversely, co-treatment with SFN and $CdCl_2$ resulted in a partial restoration compared to the group exposed only to $CdCl_2$. Importantly, SFN administration alone had no significant effect on survival when compared to the control group (99.8 ± 0.1%; $p = 0.88$).

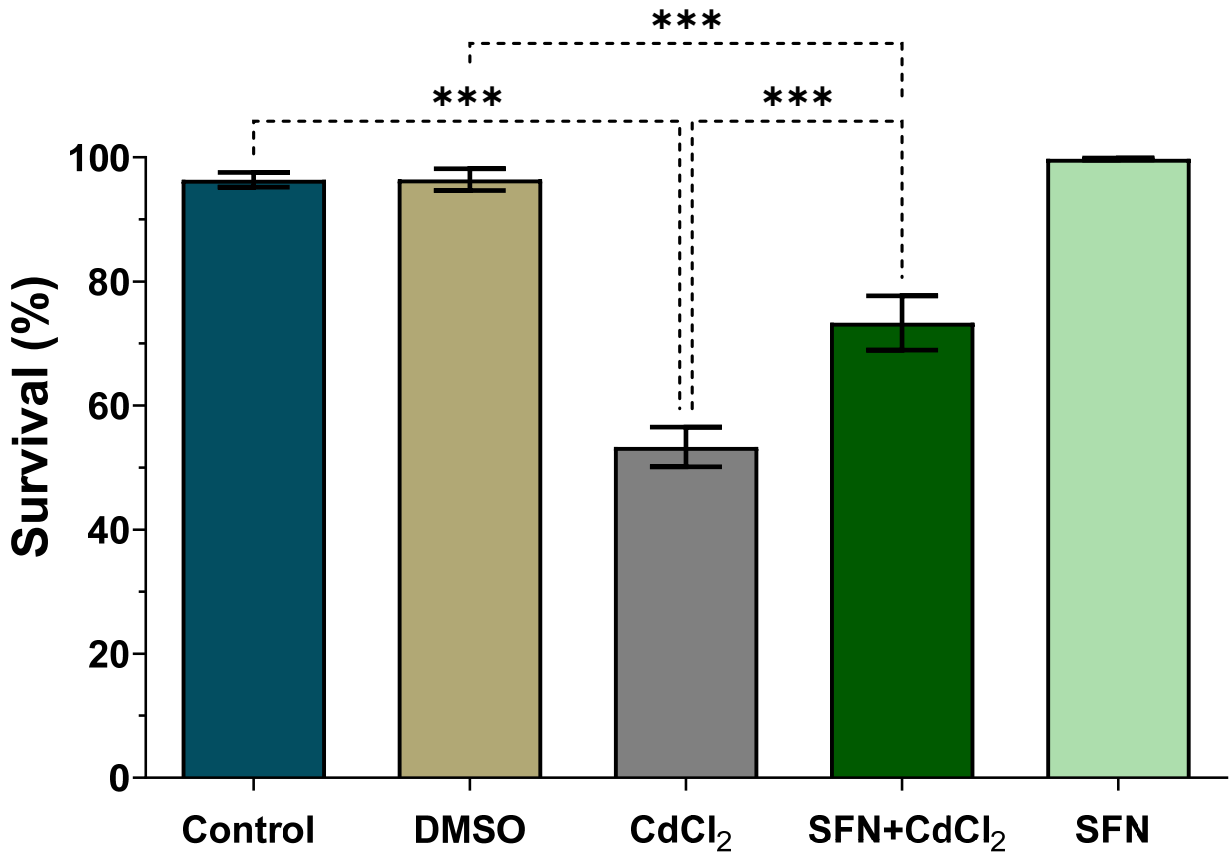

Figure 2. Effect of sulforaphane (SFN) on the survival of *Caenorhabditis elegans* (*C. elegans*) exposed to cadmium chloride ($CdCl_2$). L1-stage N2 nematodes were exposed to: (1) Dimethylsulfoxide (DMSO, 0.01%), (2) $CdCl_2$ (4600 µM), (3) SFN (100 µM) + $CdCl_2$ (4600 µM), and (4) SFN (100 µM). Following treatment, nematodes were scored under a dissecting microscope as alive if they exhibited movement or as dead if they failed to respond to gentle prodding. Percent survival was calculated based on collected data. Results are presented as mean ± standard error of the mean (SEM), $n = 5$ independent replicates. Statistical analysis: one-way ANOVA followed by Tukey's post hoc test. *** $p < 0.001$.

3.2. SFN Prevented $CdCl_2$-Induced Alterations in Egg Deposition

Egg counts were performed daily from day 0 (the day on which exposure to the treatments ended) through day 5 [37,38]. However, due to the developmental delay induced by $CdCl_2$ (see subsequent results), egg counting was extended to day 9. Nematodes in the control group laid a cumulative total of 157 ± 15 eggs by day 9, with a hatching rate of 98.8 ± 0.4%. In contrast, exposure to $CdCl_2$ reduced cumulative egg laying to 56.4 ± 4.5, representing a 64.1% decrease compared to the control. Likewise, the hatching rate dropped to 41.1 ± 1.2% ($p < 0.001$ vs. control), equivalent to a 58.4% reduction relative to the control group. Conversely, nematodes exposed to SFN + $CdCl_2$ exhibited egg-laying rates similar to the $CdCl_2$-only group during the first five days. However, from day 8 onward, a significant recovery was observed, reaching 118.6 ± 10.01 eggs by day 9 ($p < 0.05$ vs. $CdCl_2$). Similarly, the hatching rate was 75.07 ± 2.6% ($p < 0.001$ vs. $CdCl_2$), reflecting a 28.6% increase in progeny viability. These findings suggest that SFN not only partially restores egg laying but also enhances embryonic survival in the presence of $CdCl_2$. Treatment with DMSO showed a downward trend in oviposition, though not statistically significant, with a cumulative total of 128.2 ± 12.2 eggs by day 9 ($p = 0.49$ vs. control). However, it moderately reduced the hatching rate to 86.7 ± 1.4% ($p < 0.01$ vs. control). In contrast, exposure to SFN alone significantly increased the hatching rate to 98.01 ± 0.5% ($p < 0.01$ vs. DMSO) (Figure 3).

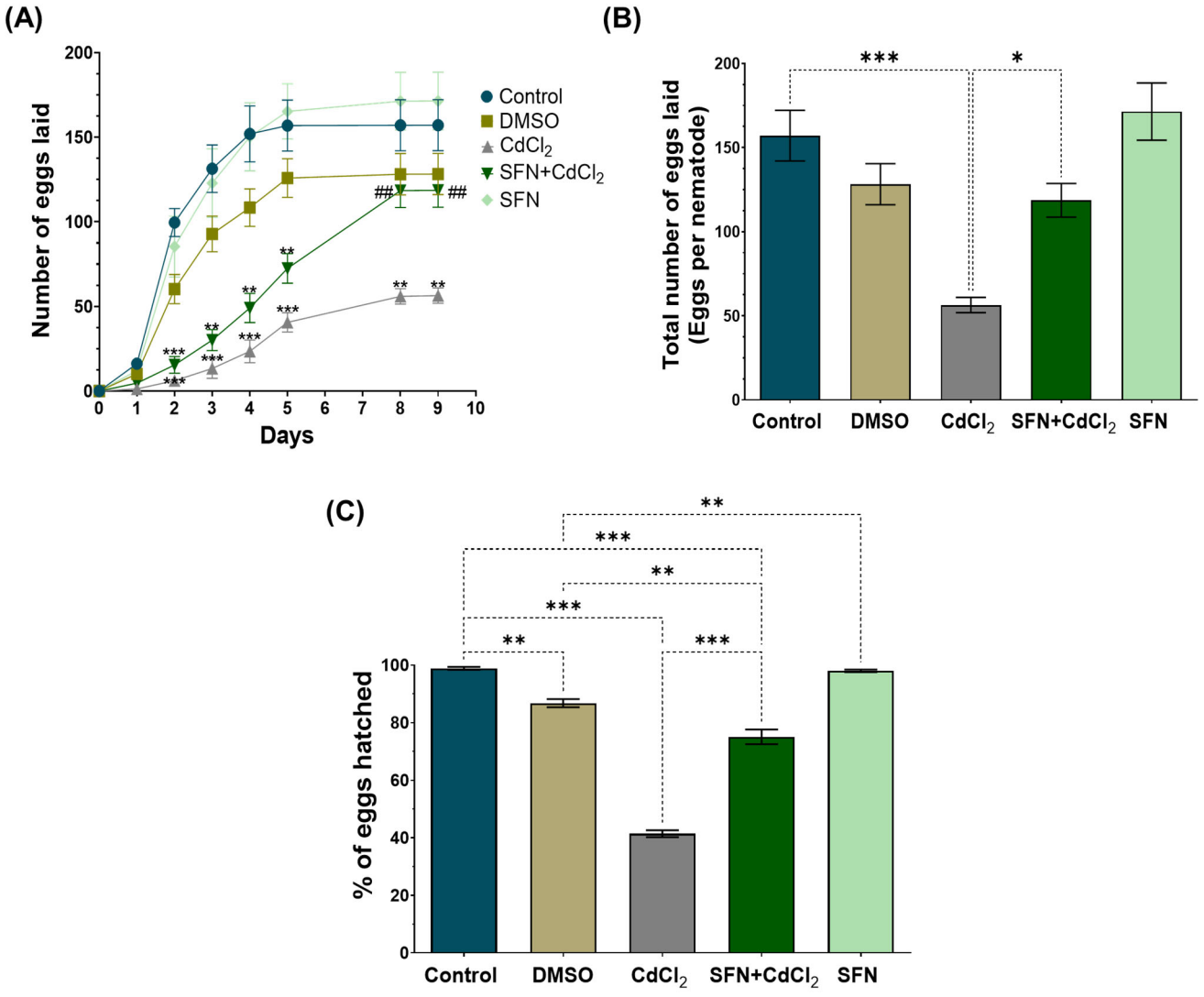

Figure 3. Effect of sulforaphane (SFN) on egg-laying and egg quality in *Caenorhabditis elegans* (*C. elegans*) exposed to cadmium chloride ($CdCl_2$). N2 nematodes were exposed to (1) Dimethyl sulfoxide

(DMSO, 0.01%), (2) $CdCl_2$ (4600 µM), (3) SFN (100 µM) + $CdCl_2$ (4600 µM), and (4) SFN (100 µM). Following exposure, evaluations were performed, considering day 0 as the time point at which treatments concluded. (**A**) Cumulative number of eggs recorded daily from day 0 to day 9 post-treatment. Data are presented as mean ± standard error of the mean (SEM). Statistical analysis: two-way ANOVA followed by Tukey's post hoc test. ** $p < 0.01$, *** $p < 0.001$ vs. control group; ## $p < 0.01$ vs. $CdCl_2$ group. (**B**) Total number of accumulated eggs on day 9. Data are presented as mean ± SEM. Statistical analysis: one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, *** $p < 0.001$. $n = 7$ independent biological assays with 3 replicates each. (**C**) Cumulative percentage of hatched eggs. Data are presented as mean ± SEM, $n = 7$ independent biological assays with 3 replicates each. Statistical analysis: one-way ANOVA followed by Tukey's post hoc test. ** $p < 0.01$, *** $p < 0.001$.

3.3. SFN Prevents the Reduction in Size and Developmental Delay in C. elegans Exposed to $CdCl_2$

The body length and width of *C. elegans* were measured to assess their potential relationship with reduced egg-laying. On day 8 of evaluation, nematodes in the control group exhibited an average length of 0.950 ± 0.015 mm and a width of 0.066 ± 0.001 mm. In contrast, nematodes exposed solely to $CdCl_2$ showed a marked reduction, with a length of 0.600 ± 0.012 mm and a width of 0.038 ± 0.001 mm ($p < 0.001$ vs. control). Nematodes co-exposed to SFN + $CdCl_2$ exhibited partial recovery of their body dimensions, with a length of 0.790 ± 0.014 mm ($p < 0.001$ vs. $CdCl_2$) and a width of 0.051 ± 0.001 mm ($p < 0.001$ vs. $CdCl_2$). Interestingly, signs of recovery became apparent by day 4 for body length solely with $CdCl_2$. Additionally, worms exposed only to SFN exhibited a modest increase in size relative to the control group, with a length of 1.030 ± 0.026 mm ($p < 0.05$ vs. control) and a width of 0.060 ± 0.002 mm ($p = 0.012$ vs. control) (Figure 4). These findings suggest that $CdCl_2$ exposure leads to significant reductions in both body length and width, whereas co-treatment with SFN partially mitigates these morphological alterations.

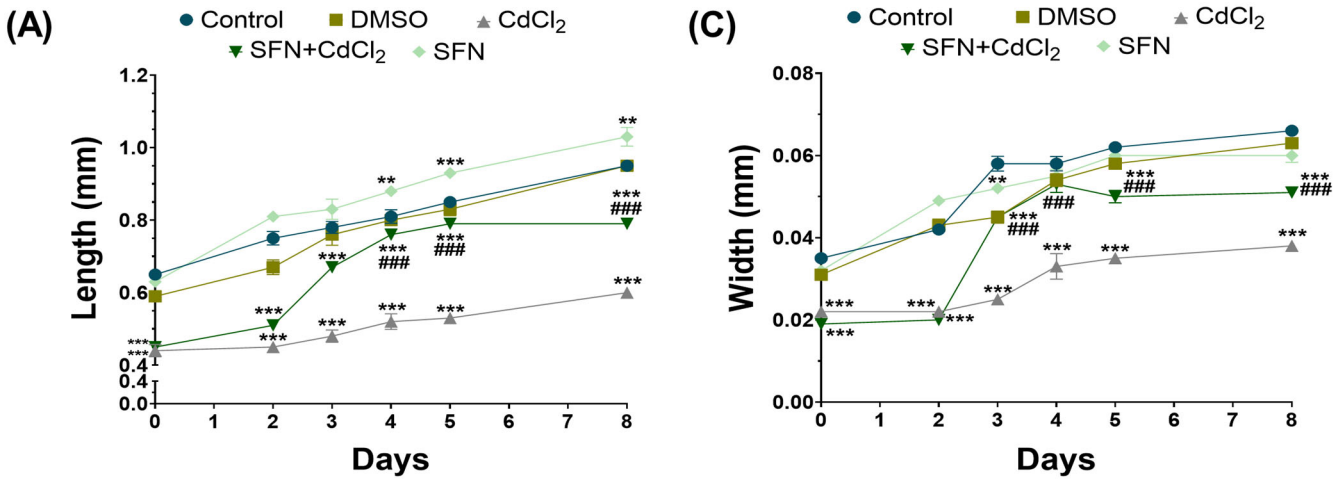

Figure 4. *Cont.*

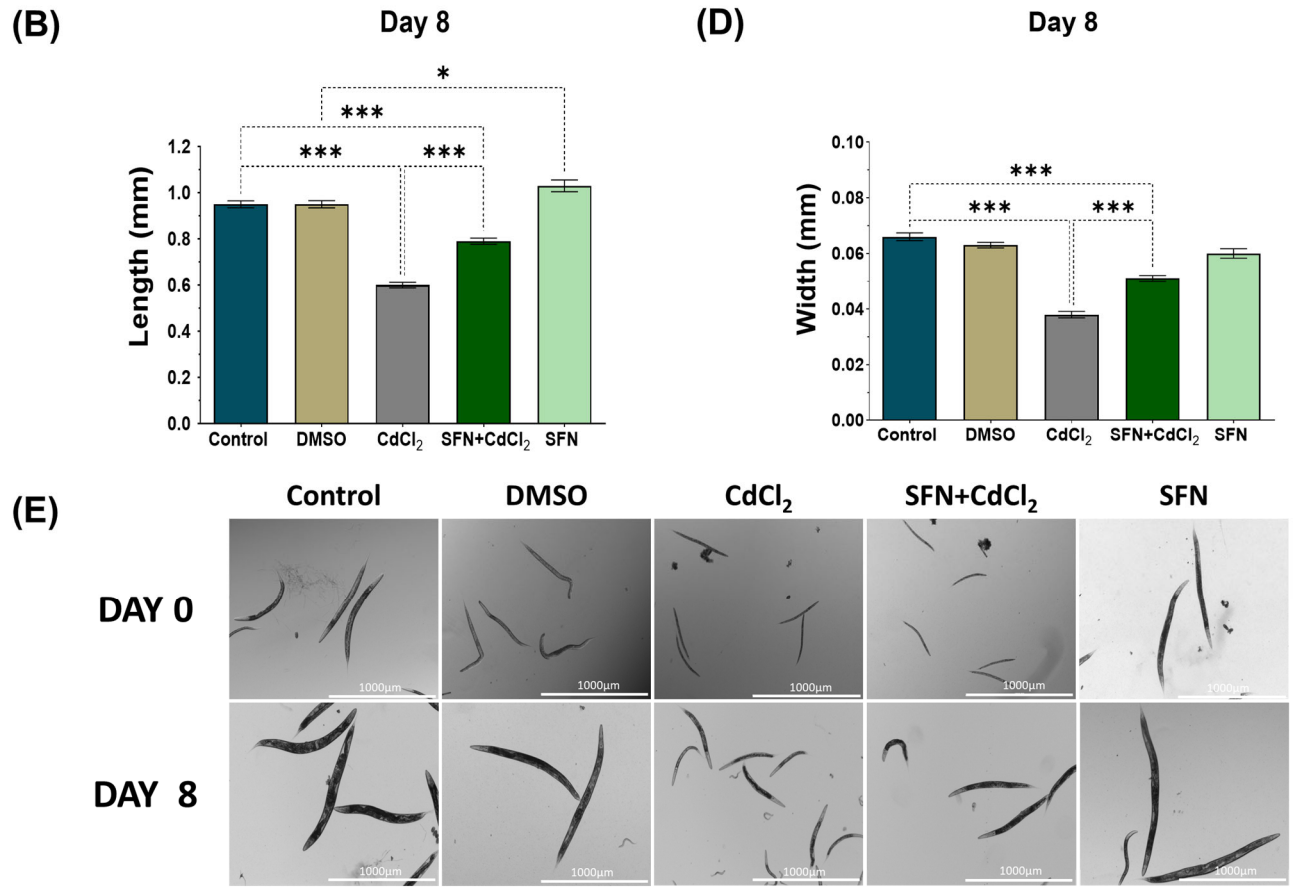

Figure 4. Effect of sulforaphane (SFN) on the body length and width of *Caenorhabditis elegans* (*C. elegans*) exposed to cadmium chloride ($CdCl_2$). N2 nematodes were exposed to four conditions: (1) Dimethyl sulfoxide (DMSO, 0.01%), (2) $CdCl_2$ (4600 µM), (3) SFN (100 µM) + $CdCl_2$ (4600 µM), and (4) SFN (100 µM). Day 0 was defined as the point at which treatments concluded. (**A**) Body length measured daily from day 0 to day 8. (**B**) Body length on day 8. (**C**) Body width measured daily from day 0 to day 8. (**D**) Body width on day 8. (**E**) Representative micrographs of nematodes on days 0 and 8 (4× objective = 1000 µm). Data are presented as mean ± standard error of the mean (SEM). Statistical analysis: two-way ANOVA followed by Tukey's post hoc test for longitudinal data (**A**,**C**), and one-way ANOVA followed by Tukey's post hoc test for comparisons on day 8 (**B**,**D**). (**A**,**C**): ** $p < 0.01$, *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. $CdCl_2$. (**B**,**D**): * $p < 0.05$, *** $p < 0.001$. $n = 3$ independent experiments.

Figure 5 shows the effects of the different treatments on the proportion of nematodes that reached the adult stage. Both the control group and the DMSO and SFN groups exhibited full maturation (100%) by day 4, with no statistically significant differences among them. In contrast, $CdCl_2$ treatment caused a sustained developmental delay, with only 45.8 ± 3.2% of nematodes reaching adulthood by day 8 ($p < 0.001$ vs. control). Co-exposure to SFN and $CdCl_2$ partially attenuated this toxic effect, as approximately 60% of nematodes reached adulthood from day 5 onward, and by day 879.8 ± 3.7% had reached maturity ($p < 0.001$ vs. $CdCl_2$), although this percentage remained below the 100% observed in the control group. These results indicate that $CdCl_2$ significantly delays larval development to the adult stage, while co-administration of SFN partially mitigates this toxicity by promoting developmental progression.

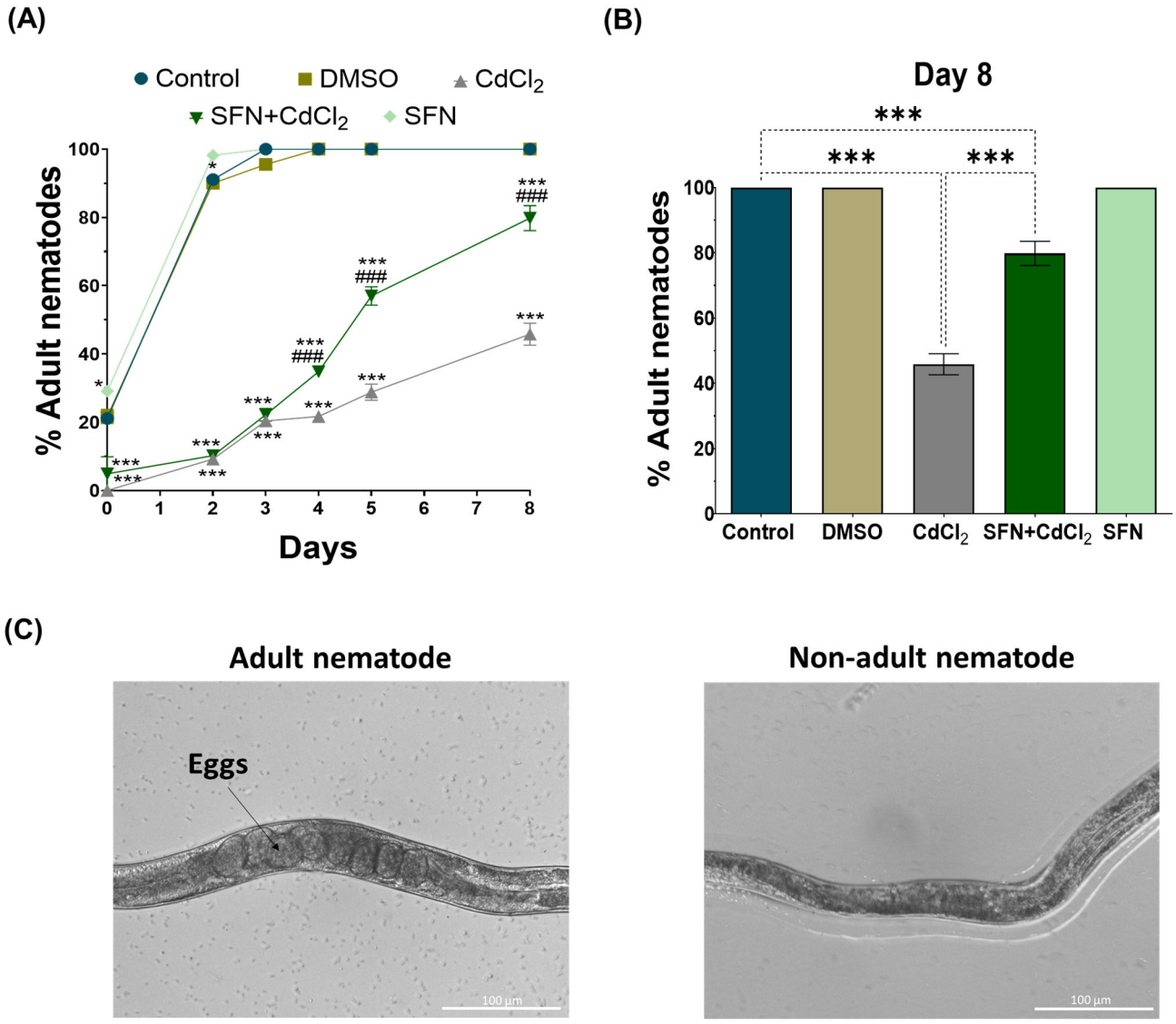

Figure 5. Effect of sulforaphane (SFN) on the development of *Caenorhabditis elegans* (*C. elegans*) exposed to cadmium chloride ($CdCl_2$). N2 nematodes were exposed to four conditions: (1) Dimethyl sulfoxide (DMSO, 0.01%), (2) $CdCl_2$ (4600 µM), (3) SFN (100 µM) + $CdCl_2$ (4600 µM), and (4) SFN (100 µM). Day 0 was defined as the point at which treatments concluded. (**A**) Percentage of adult nematodes assessed daily from day 0 to day 8. (**B**) Percentage of adult nematodes on day 8. Data are presented as mean ± standard error of the mean (SEM). (**C**) Representative micrographs of adult nematodes (with visible eggs inside) vs. non-adult nematodes (20× objective = 100 µm). Statistical analysis: two-way ANOVA followed by Tukey's post hoc test for longitudinal data (**A**), and one-way ANOVA with Tukey's post hoc test for comparisons on day 8 (**B**). (**A**): * $p < 0.05$ vs. control; *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. $CdCl_2$. (**B**): *** $p < 0.001$. $n = 3$ independent experiments.

3.4. GST-4 Expression Is Not Associated with the Protective Effect of SFN Against $CdCl_2$-Induced Damage

Figure 6 shows green fluorescent protein (GFP) levels, which indicate GST-4 expression in the CL2166 strain. Fluorescence was normalized to the control group, which was set at 1.0 ± 0.021. Exposure to DMSO did not significantly alter the signal (1.033 ± 0.022; $p = 0.852$ vs. control). In contrast, exposure to $CdCl_2$ induced a significant increase in fluorescence to 1.147 ± 0.024 ($p < 0.01$ vs. control), indicating activation of the antioxidant response mediated by GST-4. When nematodes were co-exposed to SFN and $CdCl_2$, fluorescence rose to 1.193 ± 0.012 ($p < 0.01$ vs. control); however, this increase only showed a trend toward being higher than $CdCl_2$ alone ($p = 0.60$) and did not reach statistical significance.

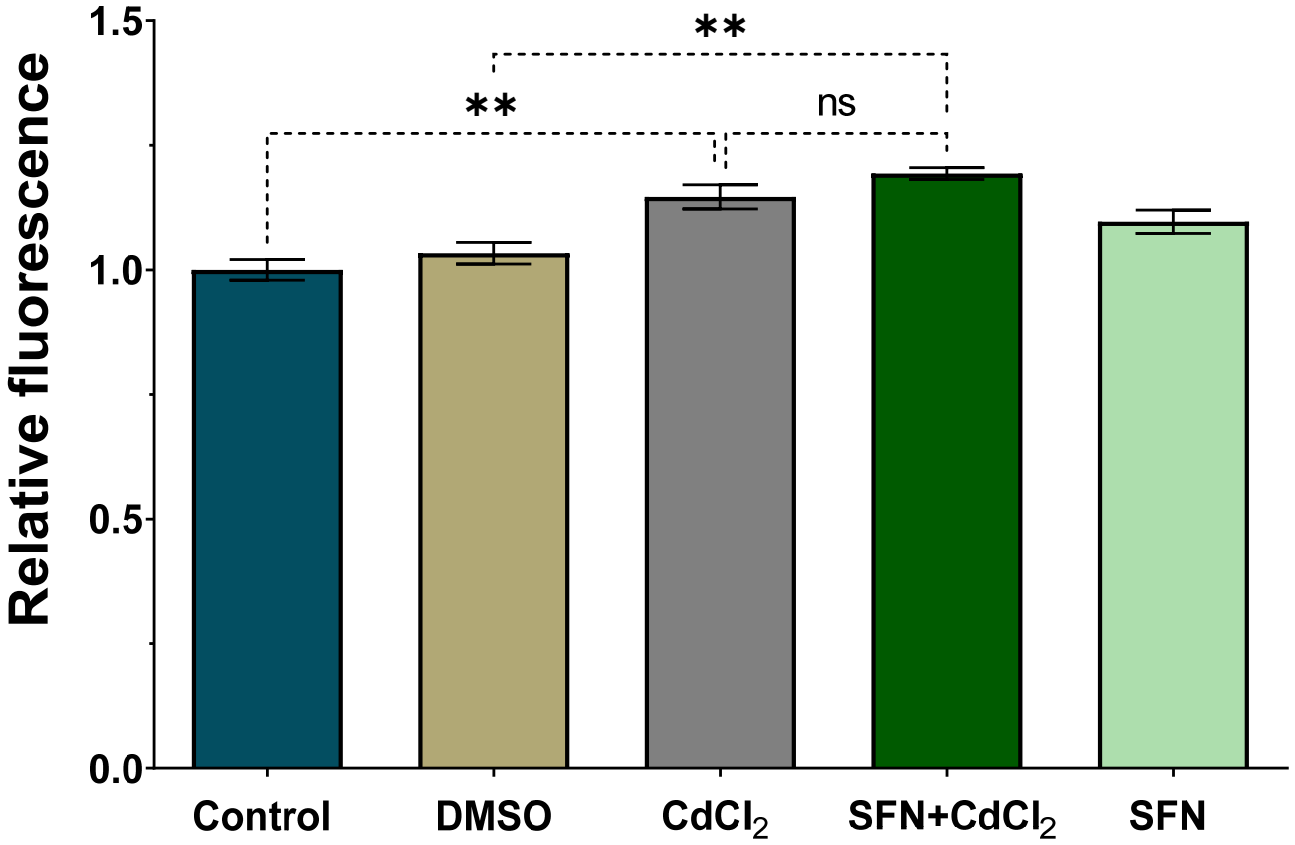

Figure 6. Effect of sulforaphane (SFN) on GST-4 expression in *Caenorhabditis elegans* (*C. elegans*) exposed to cadmium chloride ($CdCl_2$). CL2166 nematodes were exposed to four conditions: (1) Dimethyl sulfoxide (DMSO, 0.01%), (2) $CdCl_2$ (4600 µM), (3) SFN (100 µM) + $CdCl_2$ (4600 µM), and (4) SFN (100 µM). Data represent the relative of green fluorescent protein (GFP) in the CL2166 strain and are presented as mean ± standard error of the mean (SEM). Statistical analysis: one-way ANOVA followed by Tukey's post hoc test (n = 3 independent experiments). ** $p < 0.01$ and ns: not significant.

4. Discussion

This study aimed to evaluate the effect of SFN on $CdCl_2$-induced reproductive toxicity in the nematode C. elegans. To this end, we first validated the $CdCl_2$ toxicity model and the protective effect of SFN. In previous studies conducted by our research group, the median lethal concentration (LC_{50}) of $CdCl_2$ was determined to be approximately 4600 µM, and a concentration of 100 µM SFN was found to be effective in protecting against the $CdCl_2$-induced decrease in survival [17]. In the present study, the same concentrations were used, successfully replicating previously reported effects, with SFN conferring an 18.7% increase in survival (Figure 3).

Subsequently, the effects of Cd on reproduction and development in *C. elegans* were evaluated. Exposure to $CdCl_2$ caused a significant reduction in egg production, hatching rate, body size, and the percentage of nematodes reaching adulthood. As shown in Figures 3 and 5, $CdCl_2$ delayed nematode development, which may be directly related to a delay in egg laying, extending up to day 8. It is worth noting that although the reproductive period appeared prolonged, overall egg production was ultimately reduced by $CdCl_2$ exposure. These effects have been linked to oxidative stress and gonadal damage, where meiotic alterations and DNA fragmentation in germ cells and oocytes have been observed [10,22]. Furthermore, Cd accumulates in reproductive organs, resulting in sterility or adverse effects on offspring [39,40].

The reduction in egg-laying may also be attributed to the degeneration of motor neurons, leading to impaired serotonin synthesis and oviposition [10,41]. In turn, the decreased

hatching rate may be explained by Cd interference with oogenesis and embryogenesis: when administered 48 h post-synchronization, corresponding to the L3–L4 larval stages, Cd disrupts gamete formation and exerts mutagenic and teratogenic effects during embryonic development [39,42,43].

Additionally, Cd impairs nutrient absorption and assimilation by disrupting mitochondrial function in the pharynx and intestine. This leads to cellular damage, formation of inclusion bodies, and morphological changes in cytosomes, ultimately limiting energy availability required for growth [26,44]. Altogether, these findings indicate that Cd adversely affects reproduction, development, and growth in *C. elegans* through multiple mechanisms, including direct cellular damage, oxidative stress, nutrient uptake disruption, and energy metabolism alterations. These mechanisms converge to produce a phenotype characterized by reduced fecundity and arrested somatic development.

Considering the pivotal involvement of oxidative stress and mitochondria impairment in Cd-induced reproductive toxicity, identifying strategies to counter these effects is of paramount importance. In this regard, antioxidant agents such as SFN have shown considerable potential due to their capacity to alleviate oxidative damage and support mitochondrial function [45,46].

In our study, simultaneous exposure to SFN and $CdCl_2$ alleviated several of the reproductive and developmental impairments induced by cadmium. Notably, SFN treatment helped maintain a higher proportion of adult nematodes, which was associated with an increased number of eggs laid compared to the $CdCl_2$-only group. Moreover, SFN significantly enhanced egg viability, as evidenced by a higher hatching rate. Consequently, the treated population not only exhibited greater reproductive output but also achieved a higher rate of successful larval development. Furthermore, SFN prevented reductions in body size and adult numbers, parameters commonly associated with Cd-dependent toxicity, that, when restored, contribute to sustaining reproductive performance.

These findings are consistent with observations in mammals, where SFN has been shown to protect reproductive function from Cd-or obesity-induced damage by inhibiting oxidative stress via the Nrf2/ARE signaling pathway [47,48]. Similarly, protection against reproductive toxicity induced by phthalates such as DBP has been reported [48], along with enhanced proliferation of ovarian granulosa cells in mice [49].

Mechanistically, SFN exerts its effects by activating endogenous antioxidant systems. In mammals, this occurs through the activation of the Nrf2 transcription factor, whereas in *C. elegans*, it involves the activation of the orthologs *skn-1* and *daf-16* [17,47]. Hernández-Cruz et al. [17] reported that this activation reduces reactive oxygen species (ROS) formation, extends lifespan, and prevents Cd-induced mitochondrial dysfunction, positioning Insulin/IGF-like Signaling (IIS) pathway regulation as a key mechanism for metabolic protection.

Finally, we sought to explore the relationship between oxidative stress and reproduction in *C. elegans* by evaluating the activity of the GST-4 enzyme. Cd exposure significantly increased GST-4 levels, consistent with several studies showing this enzyme is upregulated in response to Cd-induced oxidative stress, acting as a detoxification mechanism [50,51]. However, in the SFN + $CdCl_2$-treated group, although a tendency for increased expression was observed, no significant differences were found compared to Cd exposure alone. Since SFN is known to induce the expression of various antioxidant enzymes through Nrf2 pathway activation, it would be relevant to assess the activity of other enzymes such as superoxide dismutase (SOD) and catalase (CAT) to determine whether SFN's protective effect is mediated through these. Additionally, performing time-course analyses would help better characterize the temporal dynamics of the antioxidant response.

5. Conclusions

Our results support that SFN not only prevents the loss of adult nematodes and deterioration in body size but also enhances both egg production and hatching rates, resulting in a comprehensive recovery of reproductive capacity in the face of Cd-induced toxicity. For future research, it would be valuable to explore in greater depth the molecular pathways through which SFN improves egg-laying and progeny quality in *C. elegans*, as well as to characterize in detail the developmental alterations caused by cadmium exposure.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/oxygen5030015/s1, Table S1. Summary of experimental parameters and quantitative data associated with Figure 2; Table S2. Percentage of live nematodes in each biological replicate corresponding to Figure 2; Table S3. Summary of experimental parameters and quantitative data associated with Figure 3A; Table S4. Summary of experimental parameters and quantitative data associated with Figure 3B; Table S5. The number of eggs laid in each biological replicate corresponding to Figure 3A,B; Table S6. Summary of experimental parameters and quantitative data associated with Figure 3C; Table S7. The percentage of eggs hatched in each biological replicate corresponding to Figure 3C; Table S8. Summary of experimental parameters and quantitative data associated with Figure 4A; Table S9. Summary of experimental parameters and quantitative data associated with Figure 4B; Table S10. Summary of experimental parameters and quantitative data associated with Figure 4C; Table S11. Summary of experimental parameters and quantitative data associated with Figure 4D; Table S12. Summary of experimental parameters and quantitative data associated with Figure 5A; Table S13. Summary of experimental parameters and quantitative data associated with Figure 5B; Table S14. The percentage of adult nematodes in each biological replicate corresponding to Figure 5A,B; Table S15. Summary of experimental parameters and quantitative data associated with Figure 6; Table S16. Data represents relative of green fluorescent protein (GFP) in the CL2166 strain in each biological replicate corresponding to Figure 6.

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Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	Analysis of Variance
ARE	Antioxidant Response Element
C. elegans	*Caenorhabditis elegans*
CAT	Catalase
Cd	Cadmium
$CdCl_2$	Cadmium chloride
CGC	*Caenorhabditis* Genetics Center
DMSO	Dimethyl sulfoxide
GFP	Green Fluorescent Protein
GST-4	Glutathione S-transferase 4
IIS	Insulin/IGF-like Signaling
KCl	Potassium chloride
K_2HPO_4	Dibasic potassium phosphate
KH_2PO_4	Monobasic potassium phosphate
LB	Lysogeny Broth
LC_{50}	Lethal Concentration 50
$MgSO_4$	Magnesium sulfate heptahydrate
NaCl	Sodium chloride
NaClO	Sodium hypochlorite
Na_2HPO_4	Disodium phosphate
NaOH	Sodium hydroxide
NGM	Nematode Growth Medium
Nrf2	Nuclear factor erythroid 2–related factor 2
OD_{600}	Optical Density at 600 nm
PAIP	Research and Graduate Support Program
ROI	Region of Interest
ROS	Reactive oxygen species
SEM	Standard Error of the Mean
SFN	Sulforaphane
SOD	Superoxide dismutase

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