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Original Article

Vitamin A and polyunsaturated fatty acids promote caenorhabditis elegans ^{60}Co radiation resistance associated with expression of cyclogeny genes

Mengyi Liu[1,2], Qianqian Liu[1,2], Youqin Xu[1,2], Xinrui Liu[1,2,5], Chen Yang[1,2], Preet Dhaliwal[3], Yanfang Lu[1,2], Guanchuan Lin[1,2], Xia Li[1,2], Lina Chen[1,2,6], Chao Zhang[1,2,3,4]

[1]Institute of Genetic Engineering, Southern Medical University, Guangzhou, China; [2]Province Key Laboratory of Biochip, School of Basic Medical Science, Southern Medical University, Guangzhou, China; [3]Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, Toronto, Canada; [4]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada; [5]Key Laboratory of Fertility Preservation and Maintenance of Ministry of Education, Ningxia Medical University, Yinchuan, China; [6]Basic Medical College, Xiangnan University, Chenzhou, China

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Abstract: Radiation-induced damage is currently under intensive research. Increasing evidence shows that radiation-induced injury could be resisted by vitamin A (V_A) and polyunsaturated fatty acid (PUFA), however, the mechanisms are yet unknown. This present study explored the effect of V_A and PUFA on radiation damage and the potential mechanisms using the model organism-*Caenorhabditis elegans*. These two radiation protectants were assessed via analysis of survival rate as well as evaluation of cyclogeny-associated genes by qRT-PCR post-radiation. V_A and PUFA did not exert any specific toxic effects on *C. elegans* in the preliminary experiment. The survival rate and fecundity of *C. elegans* decreased after radiation, both of which improved significantly with V_A and PUFA treatment. The morphology of the eggs was altered remarkably by radiation, which could be prevented by V_A and PUFA. Division patterns of the seam cells, cuticle, and molt formation-related genes, *bro-1, cki-1, ceh-16, dpy-2, dpy-8* and *grl-5* were significantly expressed as compared to the V_A and PUFA groups with CK group, post-radiation, respectively. Thus, it can be speculated that radiation caused seam cell hyperplasia, reduced formation of the cuticle, and limited the growth of molt. V_A and PUFA have a protective effect on the division pattern under 200 Gy and arrest the cell cycle checkpoint under 400 Gy. PUFA, not V_A, exhibited radiation resistance by regulating the molt and formation of the cuticle, especially under higher radiation treatment. Moreover, PUFA primarily regulated the seam cell division pattern to resist radiation injury under 200 Gy while controlling the rate of cell division under 400 Gy.

Keywords: Vitamin A, polyunsaturated fatty acid, radiation protection, *Caenorhabditis elegans*, cyclogeny-associated genes

Introduction

Radiation disrupts the proliferation ability of hematopoietic cells. It also damages biofilm and nucleic acids in cells causing DNA strand rupture by generating free radicals [1] and postpones eukaryotic cell division [2] by inhibiting the entry into cell cycle [3]. Therefore, although radiation therapy is one of the three important methods for cancer treatment, the development of radioprotectants for medical and biodefense applications is essential.

Vitamin A (V_A), an unsaturated monobasic alcohol with an alicyclic ring existing in the form of a retinoic ester, plays a major role in radioactive sensitization, protecting normal cells from radiation [4, 5]. The specific underlying mechanisms, however, are yet unclear. Polyunsaturated fatty acids (PUFA) are straight-chain fatty acids containing two or more double bonds and 16-22 carbon atoms. Reportedly, PUFAs play a vital role in inhibiting tumor cell activity, promoting tumor cell apoptosis, and modulating ultraviolet radiation-induced oxidative stress, cell signaling, and gene expression [6, 7].

Nematodes, such as *Caenorhabditis elegans* (*C. elegans*), depend on fatty acid and retinoid-binding protein (FAR), a nematode-specific pro-

tein, to interact with lipid-binding proteins for the uptake of V_A and PUFA to form lipids and other macromolecular structures such as cuticles. These organisms cannot produce fatty acids and retinoids. This mechanism in humans is similar to that of nematodes [8-10]. Herein, we speculate that V_A and PUFAs exert radiation protective effects. Potential mechanisms were tested on the model organism-*C. elegans*.

C. elegans stem cell-like lateral hypodermal (seam) cells, a type of epidermal germinal cells, symmetrically arranged on either side of the body, influence growth and development of *C. elegans*. Seam cells undergo asymmetric cell division in every larval period and the three symmetric cell divisions in the L2 period that maintain a specific number of seam cells. In the late L4 period, all seam cells cease to divide permanently and fuse together to form a symbolical corneous layer structure in adults-alae. *C. elegans bro-1*, an ortholog of human CBFβ, is required for normal proliferation and differentiation of seam cells [11]. Inactivation of *cki-1* CIP homologs could substantially rescue the defects observed in seam cell division in *bro-1* mutants [11]. *C. elegans cki-1* encodes a homolog of the mammalian cyclin-dependent kinase inhibitor p27/KIP1 that is required for the arrest of cell division in larval blast lineages, dauer larvae (a specific larval stage that *C. elegans* enter into in hostile environments), and starved L1 larvae. *cki-1* plays a negative regulatory role in the transition of cells from G1 to S phase [12]. *ceh-16*, a gene encoding the *C. elegans* engrailed homolog, is expressed in seam cells during embryogenesis and is required for adequate specification and differentiation of seam cells. Moreover, *ceh-16* is critical for specifying the fate of seam cells by preventing their fusion with neighboring hypodermal cells and controlling migration during embryogenesis [13].

Collagen provides a barrier between organisms and environment and is vital in morphogenesis, sports, and signal transduction. It also acts as a cell matrix protein [14]. The cuticle of *C. elegans* is synthesized five times during development: once in an embryo before hatching and the other four times at the end of each larval stage, before molting. *dpy-2* encodes a rare cuticular collagen that is required for maintaining the normal body length of *C. elegans* in later larval stages and proper formation of circumferential furrows on the surface of the cuticle of *C. elegans*, which is termed as annuli [15]. *dpy-8* encodes a collagen with a nematode-specific N-terminal domain that is required for normal body morphology and putatively for a normal rate of embryonic cell division.

Molting is the formation and degradation of the protective outer cuticle. Moreover, depending on the period, it is also defined as the synthesis and secretion of proteins such as collagens and proteolytic enzymes, which play an essential role in growth and proliferation of *C. elegans* [16]. During development into an adult, the larvae of *C. elegans* undergo molting a total of four times. As a regulation gene of molting, the *glr*-5 gene of *C. elegans* also regulates the developmental cycle by participating in Hedgehog signaling pathways in *C. elegans* [17].

Based on the above description, *bro-1* and *ceh-16* might promote seam cell proliferation and/or self-renewal, with *cki-1* as a probable downstream target. Reportedly, radiation arrests cell cycle and inhibits cell proliferation and migration as V_A and PUFA exert a potent effect on cell growth and differentiation [18-20]. Additionally, *dpy-2* and *dpy-8* altered the body shape and affected germline proliferation and fertility under the regulation of *grl-5*. Radiation accelerates the development of *C. elegans*, which is associated with expression level of *dpy* [21]. Therefore, in the current study, we attempted to explore the mechanism of V_A and PUFAs in protecting irradiated *C. elegans* on the gene level. The six genes of *C. elegans*, *ceh-16, cki-1*, *bro-1*, *dpy-2, dpy-8* and *grl-5* were found to be influenced by V_A and/or PUFA treatment on the function of radiation protection on the cell cycle and development cycle. In order to observe the effects of V_A and PUFAs on radiation resistance and repair in *C. elegans*, we assessed the growth and morphology of *C. elegans* as well as the expression of the above genes after irradiation, with or without V_A and PUFA treatment.

Materials and methods

C. elegans strains

Wild-type C. elegans strain, N2, was provided by South China Agricultural University, Guangzhou, Guangdong, China. The strains were maintained according to standard protocols [22].

Table 1. Primer sequences of housekeeping genes and the genes of interest for qRT-PCR

cdc-42	Forward	5'TGTTTGCTTCTCCGTGGTTGCT3'
	Reverse	5'CGTTGACACTGGTTTCTGCTTG3'
ceh-16	Forward	5'CCAGAACAAGCGTGCCAAACT3'
	Reverse	5'CTGAACCTTTGCCAACTGAGC3'
bro-1	Forward	5'GACGGGAGCGGTTTAGGGTT3'
	Reverse	5'GGACCATTCCAACTTGACAGG3'
cki-1	Forward	5'GATTCGTTTATGAAGTTATTCCAGA3'
	Reverse	5'GCTCCTCCTTATCAGATGTGCT3'
dpy-8	Forward	5'CTTCTGATTCTGCCGCTGCTT3'
	Reverse	5'ATGATGATGTGCTCTGTGACTT3'
dpy-2	Forward	5'ATGAAATCGCAAACGAGTGGG3'
	Reverse	5'TTGAGAACCGTGAAATGTTATCG3'
grl-5	Forward	5'GTTGTTGATTGTCGGATTTGCC3'
	Reverse	5'CCAGCTCCTCCAGCGGTGAA3'

Synchronization of worms

Worms were collected and washed by M9 buffer (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, and 0.25 g $MgSO_4 \cdot 7H_2O$). 2 mL lysis solution (6 mL stock solution containing 2 mL 10% sodium hypochlorite solution, 1 mL 10 M sodium hydroxide solution, and 3 mL distilled water) was used to decompose the worms in 4 mL M9 buffer. After agitating for 9 minutes, the worm eggs were washed by M9 buffer 4 and observed by fluorescence microscopy.

Radiation of C. elegans strains

Five thousand synchronized *C. elegans* cells were cultured on NGM for 48 hours, followed by exposure to 0 (without radiation), 200 and 400 Gy of ^{60}Co γ-radiation using GMII ^{60}Co radiation machine (Beijing Gamma High and New Technology Co., Ltd.) for 0, 66 and 133 minutes, respectively. The number of living worms was enumerated post-radiation using the dilution method [23]. Moreover, 5000 worms treated with 0, 200 and 400 Gy were transferred to fresh NGM medium [3 g NaC1, 2.5 bactopeptone, 17 g bacto-agar, 1 mL cholesterol in ethanol (5 mg/mL), 1 mL M CaCl, 1 mL M $MgSO_4$, 25 mL M potassium phosphate buffer (pH 6.0), 975 mL distilled water] [22] and the number of living worms was enumerated after 48 hours to evaluate effects of radiation on the fecundity of *C. elegans*.

V_A and PUFA treatment and toxicity trials

In order to assess the effect of V_A (Shanghai Yuanye Bio-Technology Co., Ltd, purity ≥ 89%) and PUFA (Sigma-Aldrich, catalog# 47033) on radiation resistance, both were solubilized in 95% ethanol at a concentration of 62.5 μg/mL and 250 μg/mL, respectively. Finally, each V_A-containing NGM (V_A^+) plate contained 6.25 μg V_A and each PUFA-containing NGM ($PUFA^+$) plate contained 25 μg PUFA. 1000 *C. elegans* were maintained on each of NGM (V_A^+) and NGM ($PUFA^+$) plates. To ensure that V_A or PUFA were absorbed, the worms were cultured for five generations in V_A or PUFA medium and the medium was refreshed after 72 hours. *C. elegans* of the V_A group (*C. elegans* cultured on NGM medium with V_A), PUFA group (*C. elegans* cultured on NGM medium with PUFA), and CK group (*C. elegans* cultured on NGM medium without V_A and PUFA) were each irradiated by ^{60}Co with 0, 200 and 400 Gy dose. The number of surviving worms was counted after 4 hours post-radiation.

Morphological analysis

C. elegans has been reported to present high selectivity and sensitivity to 200 Gy [24]. Thus, this dose was used for examining radiation-induced phenotypic changes in the organism as well as the protective effects of V_A and PUFA. Three experimental groups were set up: CK, V_A and PUFA receiving 200 Gy radiation dose. The CK group without radiation served as the control group. The eggs, obtained from worms by synchronization, were observed after 3 hours post-radiation treatment by fluorescence microscopy (×100 magnification).

RNA extraction

Worms were separated from each NGM plate using the M9 buffer and then subjected to three rounds of freeze cracking by alternating between liquid nitrogen and room temperature. Total RNA was extracted using TRIzol (Invitrogen, TaKaRa) Reagent, following the manufacturer's instructions. The concentration and purity (OD260/280) of RNA was measured by Nanodrop 2000 (Thermo Scientific).

Quantitative real-time reverse transcription PCR

One μg total RNA was reverse-transcribed into cDNA on MJ Mini™ Personal Thermal Cycler (Bio-Rad), using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa) according to the manufacturer's instructions. Real-time PCR was performed on the ABI Prism

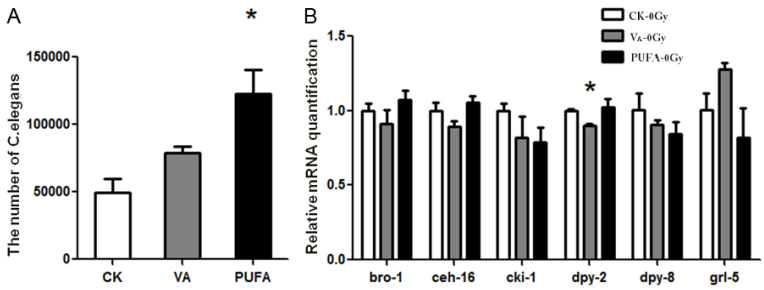

Figure 1. Toxicity trials showed that apparent toxicity was not observed when treated with V_A and PUFA. In order to estimate whether V_A and PUFA, at a concentration of 6.25 μg/25 mL NGM and 25 μg/25 mL NGM, respectively, were detrimental to *C. elegans*, the amount of *C. elegans* (A) and the expression of the six radiation-related genes (B) in CK, V_A and PUFA groups without radiation were analyzed. The value of *cdc-42* (a housekeeping gene) mRNA expression in the CK group was arbitrarily set to 1. Error bars indicate standard error. An average of three representative experiments is shown. Data were analyzed by ANOVA followed by Dunnett's t-test or two-tailed Student's t-test. **represents $P < 0.01$ when comparing the V_A and PUFA groups with the CK group, respectively.

7500 Real-Time PCR System (Applied Biosystems) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus, TaKaRa) according to manufacturer's protocol. 20 μL PCR reactions contained 2 μL cDNA and the following cycling conditions were utilized: pre-denaturation at 95°C for 30 seconds, followed by 40 cycles at 95°C for 5 seconds, and 60°C for 34 seconds. The primers were synthesized by Sangon Biotech (Shenggong Biotechnology Co., Ltd, **Table 1**). *cdc-42* was used as a housekeeping gene [25]. A no-template control was included in the reaction for each primer pair and was found to be consistently negative. All experiments were carried out using three biological and three technical replicates. The amount of relative RNA expression was calculated according to $2^{-\Delta\Delta Ct}$ method [26].

Statistical analysis

All values are expressed as mean ± standard deviation. Significant differences at $P < 0.05$ were tested using one-way ANOVA followed by Dunnett's t-test or two-tailed Student's t-test. The difference between groups was considered to be significant at *$P < 0.05$ and **$P < 0.01$.

Results

V_A and PUFA had no toxic effect on C. elegans

In order to estimate whether V_A and PUFA, at a concentration of 6.25 μg/25 mL NGM and 25 μg/25 mL NGM, respectively, were detrimental to *C. elegans*, the number of organisms in CK, V_A and PUFA groups were enumerated directly and expression of six radiation-related genes was tested. The value of *cdc-42* (a housekeeping gene) mRNA expression in the CK group was arbitrarily set to 1.

The results showed that the number of *C. elegans* treated with V_A (78,900 ± 3,657) and PUFA (122,300 ± 14,421) was higher than those without the drug treatment (49,400 ± 8,260). A significant difference was observed between the PUFA and CK groups ($P < 0.05$), however, none was observed between the V_A and CK groups (**Figure 1A**). Compared to the CK group, only expression of *dpy-2* showed a significant decrease in the V_A group ($P < 0.05$) while the difference in the other five genes in the V_A group and all the six genes in the PUFA group did not differ significantly (**Figure 1B**). Although expression of *dpy-2* in the V_A group was 0.896-fold that of the CK group, the amount of *C. elegans* treated with V_A was 1.59-fold that of the CK group and no significant difference was observed in the expression of *dpy-2* in the V_A group after radiation. Thus, V_A and PUFA were not distinctly toxic to *C. elegans*.

^{60}Co radiation suppressed the survival rate and fecundity of C. elegans that could be prevented by V_A and PUFA treatment

In order to evaluate the effects of ^{60}Co radiation doses on the growth of *C. elegans*, the number of cells was counted directly after radiation treatment and the culture continued after 48 hours. The results showed that 200 and 400 Gy radiation induced 74.691% and 70.838% death, respectively, in worms (**Figure 2A**). The average amount of progeny generated by nematodes (after being cultured for 48 hours post-radiation) in the control group (94300 ± 3184) was 3.514-fold than that in the 200 Gy group (26833 ± 1170) and 10.964-fold that of the 400 Gy group (8066 ± 339) (**Figure 2B**). These results indicate that ^{60}Co radiation treatment led to mortality of *C. elegans* and decreased

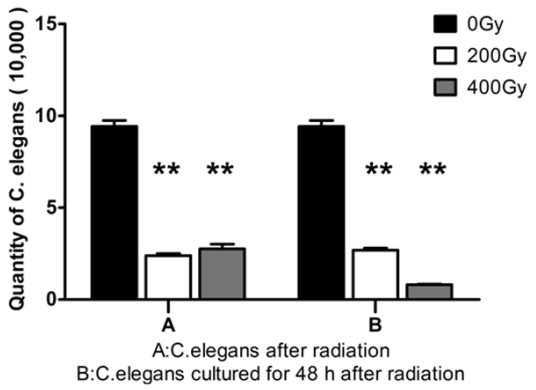

Figure 2. ^{60}Co radiation causes lower survival rate and fecundity of *C. elegans*. Analysis of survival rate (A) and fecundity (B) of *C. elegans* receiving 0, 200 or 400 Gy. The survival rate and fecundity were determined as the number of *C. elegans* and cultured for 48 hours post-radiation, respectively. Error bars indicate standard error. An average of three representative experiments is shown. Data were analyzed by ANOVA. **represents $P < 0.01$ when comparing the V_A and PUFA groups with the CK group, respectively.

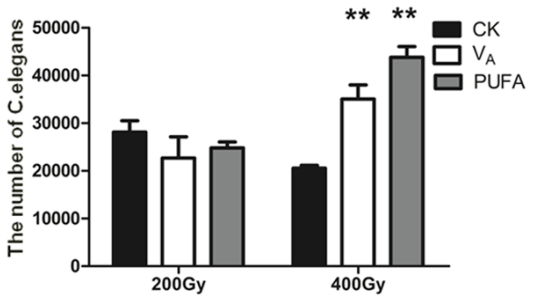

Figure 3. V_A and PUFA pre-administration cause higher survival rate of *C. elegans* after 400 Gy radiation dose. Analysis of survival rate of *C. elegans* receiving 0, 200 or 400 Gy. Error bars indicate standard error. An average of three representative experiments is shown. Data were analyzed by ANOVA. **represents $P < 0.01$ when comparing the V_A and PUFA groups with the CK group, respectively.

fecundity, which was dose-dependent. These results were similar to previous reports [27].

V_A and PUFA were added into NGM medium, respectively. Compared to the CK group, both V_A and PUFA groups showed a significantly higher survival rate ($P < 0.01$) after 400 Gy radiation treatment (**Figure 3**). Additionally, the PUFA group (43825 ± 2235) presented an overall higher survival rate than the V_A group (35050 ± 2952). However, no significant difference was observed among the CK, V_A and PUFA groups receiving 200 Gy radiation dose. These results indicate that PUFA exerted a greater protective effect than V_A at 400 Gy dose.

Morphology of C. elegans eggs

Eggs of the CK and control groups were sleekly elliptical with a normal eggshell (**Figure 4A**). However, circular (**Figure 4B**), rough (**Figure 4D**), short macro-axis (**Figure 4D**, **4E**), and small caves (**Figure 4C**, **4E**) were noted when 200 Gy radiation dose was administered. Although eggs of the V_A (**Figure 4F**) and PUFA groups (**Figure 4G**) receiving 200 Gy dose appeared rough, they still maintained their eggshells while some eggs had no eggshell at all in the CK group receiving 200 Gy (**Figure 4D**, **4E**).

^{60}Co radiation affects expression of multiple C. elegans genes

ceh-16, *bro-1*, *chi-1*, *dpy-2, dpy-8* and *grl-5* were selected for assessing the degree of radiation-induced damage. The six radiation-related genes can be grouped into two categories: cell cycle-related genes (*ceh-16*, *bro-1*, *chi-1*) and developmental cycle-related genes (*dpy-2*, *dpy-8*, *grl-5*). Expression of these genes in *C. elegans* were quantified by qRT-PCR.

The results showed significant differences in expression of five genes between the 200 Gy and 0 Gy groups ($P < 0.05$). Two genes (*bro-1* and *ceh-16*) were highly expressed in the 200 Gy group while the other three genes (*cki-1*, *dpy-2*, *grl-5*) were poorly expressed compared to the 0 Gy group. Significant differences were observed in the expression of five genes between the 400 Gy and 0 Gy groups ($P < 0.05$). One gene (*bro-1*) was highly expressed in the 400 Gy group while the other four genes (*cki-1*, *dpy-2*, *dpy-8*, *grl-5*) were expressed at lower levels compared to the 0 Gy group (**Figure 5**).

V_A and PUFA affect expression of multiple selected C. elegans genes post-radiation

Expression of the selected IR-related *C. elegans* genes was tested in different groups treated with V_A or PUFA, respectively, and analyzed by quantitative RT-PCR, as described above.

When exposed to 200 Gy radiation, lower expression of the three genes (*bro-1*, *ceh-16 and cki-1*) was observed in the V_A group compared

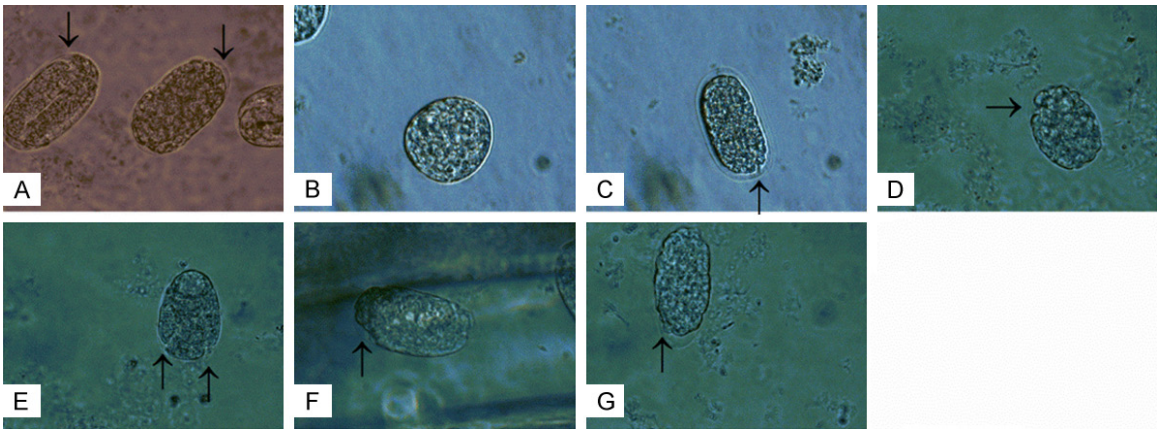

Figure 4. Morphology of several eggs obtained by synchronization post-radiation. (A) Image of the egg without radiation. The egg was sleekly elliptical, had normal eggshell (black arrow). (B-E). The eggs obtained by radiation of *C. elegans* with 200 Gy were circular (B), rough (D), had short macro-axis (D, E), and small caves (C-E, black arrow). (F-G) Image of the eggs obtained by radiation-treated *C. elegans* with 200 Gy in V_A (F) and PUFA groups (G). Although rough in appearance, the eggshells were retained (F-G, black arrow), while some eggs in the CK group lacked eggshells (D, E).

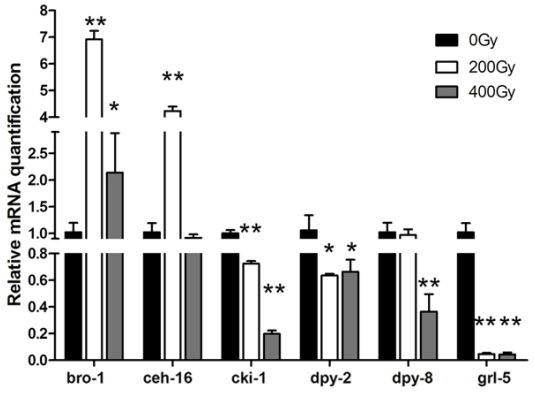

Figure 5. ^{60}Co irradiation affects the expression of several *C. elegans* genes. Expression of several genes was measured by qRT-PCR in *C. elegans* that received a radiation dose of 0, 200 or 400 Gy. Data are reported as fold-induction relative to the expression in untreated worms and normalized to *cdc-42*. The average of three representative experiments is shown. Error bars indicate standard error. The mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. *represents $P < 0.05$ and **represents $P < 0.01$ when comparing the V_A and PUFA groups with the CK group, respectively, as analyzed by ANOVA.

to the CK group. Significant differences in the expression of four genes between the PUFA and CK groups was observed ($P < 0.05$), whereas three genes (*bro-1, ceh-16, cki-1*) expressed lower in the PUFA group and *dpy-2* gene expressed higher compared to the CK group. Four hundred Gy treatment displayed significant differences in the expression of two genes between the V_A and CK groups ($P < 0.05$), one gene (*cki-1*) expressed higher in the V_A group while another gene (*grl-5*) expressed lower than the control. The fives genes (c*eh-16, cki-1, dpy-2, dpy-8* and *grl-5*) showed higher expression with 400 Gy in the PUFA group compared to the CK group (**Figure 6B**).

Discussion

In the present study, we compared survival rate, morphology, and expression of six genes of *C. elegans* under various treatments. According to the results described above, V_A and PUFA, at a concentration of 6.25 μg/25 mL NGM and 25 μg/25 mL NGM, respectively, did not exert specific toxic effects on *C. elegans* in the preliminary experiment. Thus, it was speculated that radiation caused seam cell hyperplasia, reduced formation of the cuticle, and limited growth as well as molt. V_A and PUFA had a protective effect on the division pattern at 200 Gy and cell cycle checkpoint arrest under 400 Gy. PUFA, rather than VA, displayed radiation resistance by regulating molt and formation of the cuticle, especially under higher radiation treatment. Moreover, PUFA primarily regulated seam cell division pattern to resist radiation injury under 200 Gy while it controlled the rate of cell division under 400 Gy.

Reportedly, *bro-1* and *ceh-16* play critical roles in promoting seam cell proliferation and/or self-renewal, with *cki-1* as a probable downstream target. Overexpression of *bro-1* and

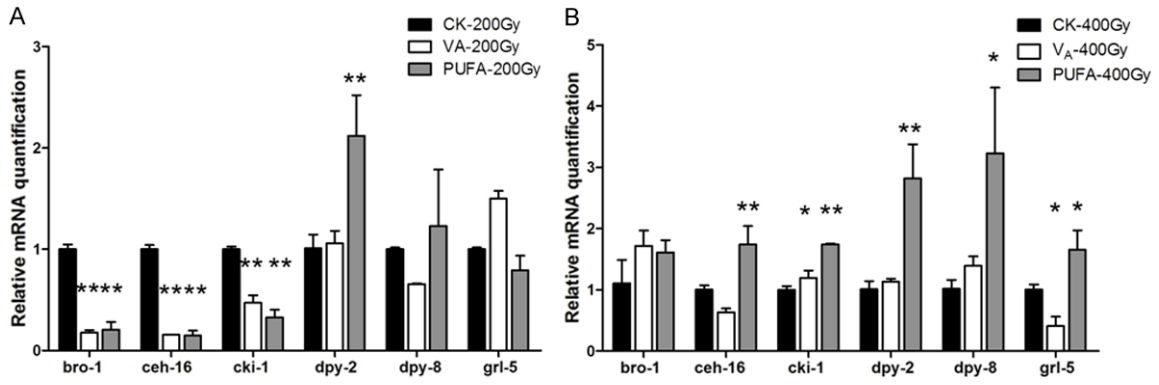

Figure 6. Altered gene expression in *C. elegans* with V_A and PUFA pre-administration. Expression of several genes was measured by qRT-PCR in *C. elegans* exposed to 200 Gy (A) and 400 Gy (B) in the CK, V_A and PUFA groups. Data are reported as fold-induction as compared to expression in the CK group and normalized to *cdc-42*. An average of three representative experiments is shown. Error bars indicate standard error. The mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. *represents $P < 0.05$ and **represents $P < 0.01$ when comparing the V_A and PUFA groups with the CK group, respectively, as analyzed by ANOVA.

ceh-16 causes seam cell hyperplasia, while the loss of *cki-1* function also results in an increased number of seam cells [28, 29]. Moreover, seam cell hyperplasia could also be caused by an increased number of seam cells undergoing symmetrical cell division and/or by changes in fate due to the loss of division asymmetrically. Thus, it was speculated that radiation induces death of seam cells, which leads to generation of additional seam cells. The number of seam cells was found to be less with radiation than those without radiation and the eggs were smaller (**Figure 4B**). Expression of *ceh-16* did not change in *C. elegans* that received 400 Gy radiation. *bro-1* and *ceh-16* were more highly expressed when cells were treated with 200 Gy than 400 Gy, which indicates that 400 Gy caused severe damage to *C. elegans* and the homeostatic control was limited.

When function of *dpy-2* was suppressed, seam cells stacked up adjacent to one another, thereby disrupting normal cell contact and leading to formation of an unusual stiff cuticle that altered the shape of the body as short and fat. They only made left-turns and the stiff body in adults caused them to roll helically during movement [22, 30]. The loss of *dpy-8* function inhibited cell divisions due to the reduction of early embryonic cell divisions that could be observed in *dpy-8* mutations among *emb-5* (temperature-sensitive) embryos. In addition, abnormal cell division was corrected while abnormal rate of cell division was observed in *emb-5* embryos [31]. The subsequent 10 seam cells divided to produce daughter cells that fused with hyp7 (the largest syncytium of hypodermis to cover the worm maximally), thereby indicating that *dpy-8* reduced the formation of cuticles that otherwise grew with the hypodermis. The cross-sectional area of the buccal cavity, increasing gradually at each molt, limited the rate of resource acquisition and growth rate of the larval worm. Inability of the buccal cavity without molting exhibited unusual properties of the cuticle [30]. With respect to molting, the developmental cycle of *C. elegans* was regulated by *grl-5* via participating in Hedgehog signaling pathways. Additionally, *ceh-16* was crucial for specifying seam cell fate by preventing fusion of seam cells with neighboring hypodermal cells and controlling migration of seam cells during embryogenesis. As an effect of radiation treatment, downregulation of *dpy-2, dpy-8* and *grl-5* and the upregulation of *ceh-16* implied that radiation prevented the fusion of seam cells with hyp7, promoting the stack of seam cells, reducing the formation of cuticle, forming abnormal cuticles, limiting growth by inhibiting molt, and causing inability of the buccal cavity that affects feeding. Thus, radiation may have prevented fusion of seam cells with hyp7 and reduced formation of the cuticle. Moreover, collagen served as a vital part of the eggshell. Down-regulation of *dpy-8* and *dpy-2* could not encode adequate collagen for the formation of appropriate egg-shell, hence, the eggs displayed defects in shells post-radiation and appeared rough (**Figure 4**).

Retinoic acid, the most potent natural form of vitamin A, directs P19 stem cells to differentiate into cells displaying an endodermal phenotype at low concentrations and induces differentiation to neuroectoderm at higher concentrations [20]. Omega-3 and -6 PUFAs and their metabolites can act through multiple mechanisms to promote proliferation and differentiation of various stem cell types [19]. In *C. elegans*, radiation-treatment with 200 Gy decreased expression of *bro-1* and *ceh-16* in the V_A and PUFA groups compared to the CK group. The loss of *bro-1*' function suppresses symmetrical division and the loss of *ceh-16*' function may cause specific seam cells to undergo asymmetrical fission, which otherwise should have been symmetrical self-renewal expansion [11, 29]. V_A and PUFA relieve seam cell hyperplasia by regulating the division pattern. Additionally, symmetrical division maintains homeostatic control at the population level instead of the individual cell level and can replenish the seam cell pool in the case of injury. Asymmetric division cannot display these characteristics. Downregulation of expression of *cki-1* under 200 Gy could rescue reduction in the number of seam cells caused by low expression of *bro-1*. The interaction of lower expression of *bro-1*, *ceh-16*, and *cki-1* is speculated to maintain the number of seam cells as well as normal development and growth of *C. elegans*. Normal development corresponding to size and long axis of eggs in the V_A and PUFA groups were similar to those without radiation compared to being smaller in the CK group at 200 Gy (**Figure 4**). This result was in agreement with a previous study that showed blocking of downstream negative regulatory factors, such as *cki-1* and *bro-1,* where these could partially or completely rescue the mutation effect [32]. Therefore, radiation injury induced symmetrical seam cell division to increase the number of seam cells, however, *C. elegans* treated with V_A and PUFA did not require an excess of symmetrical division to repair the injury. Thus, V_A and PUFA had a protective effect on radiation resistance, suppressing excessively symmetrical self-renewal expansion division.

Expression of *bro-1* and *ceh-16* post 400 Gy did not alter as a consequence of the treatment with V_A and PUFA. Thus, it can be speculated that 400 Gy dose might have been severely hazardous to *C. elegans,* such that the majority of seam cells pathologically underwent apoptosis and symmetrical division was essential to replenish the number of seam cells. *cki-1* played a negative regulatory role on the transition of cells from G1 to S phase. At 400 Gy, expression of *cki-1* increased such that the cells were arrested in S phase in the V_A and PUFA groups and the relative extension of G1/S phase extended the duration for DNA repair, thereby reducing the risk of faulty DNA damage. In addition, resistance to radiation increased in the V_A and PUFA groups with 400 Gy putatively attributable to fewer seam cell divisions post-radiation, resulting in less tissue damage. Thus, it is concluded that the radiation resistance provided by V_A and PUFA is exhibited in cell cycle checkpoint arrest rather than regulation of seam cells division patterns at 400 Gy.

Dietary intake of hempseed meal (HSM) with optimal balanced PUFAs accelerates both body growth and developmental rates in Drosophila via stimulation of cell growth and ecdysone synthesis [33]. Compared to the CK group, significant upregulation of *dpy-2* and *dpy-8* in the PUFA group receiving 400 Gy estimated that additional collagen of *C. elegans* was synthesized to maintain normal body length and normal embryonic cell division rate and to inhibit the molting-defect. Adequate collagen and upregulation of *grl-5* accelerated the molt of the larva. However, expression of *grl-5* in PUFA group with 200 Gy did not present a significant difference, thus, it was speculated that worms with PUFA entered the dauer larva state to confront adversity. During this period, expression of molting-relative genes was not required for regulation of molting; thus, expression of *grl-5* did not alter distinctly. Significant upregulation of *ceh-16* suppressed asymmetrical division of seam cells to produce daughter cells and prevented the fusion of seam cells with neighboring hypodermal cells. Therefore, it was hypothesized that PUFA exhibited radiation resistance by the upregulation of *dpy-2* and *dpy-8* and expression of *ceh-16* reactivity increased to suppress excessive production of collagen and benefit the molt. The results show that PUFAs has a potential radioprotective effect by limiting abnormal formation of the cuticle, promoting molt, and increasing the growth rate of *C. elegans*.

As described above, upregulation of *bro-1* suppressed symmetrical division and upregulation of *ceh-16*, causing specific seam cells to under-

go an asymmetrical fission instead of symmetrical self-renewal expansion. The results show that expression of *bro-1* and *ceh-16* in the 200 Gy-treated PUFA group decreased while the expression of *dpy-8*, which accelerates the rate of seam cell division, did not alter significantly. This indicates that PUFA primarily regulated seam cell division to resist radiation injury by maintaining the balance between asymmetrical and symmetrical cell division rather than controlling the rate of cell division.

When *C. elegans* received 400 Gy, expression of *ceh-16* increased rather than decreasing without any significant change. Thus, it could be speculated that 400 Gy induced severe damage in *C. elegans* such that the cell division pattern could not resist radiation injury. PUFA induced upregulation of *dpy-8* to accelerate the rate of seam cell division to resist radiation injury. Consecutively, expression of *ceh-16* increased as a response in order to prevent hyperplasia.

Expression of *dpy-2* decreased post-radiation, which could be blocked by PUFA treatment. Thus, it can be inferred that seam cells did not stack up in PUFA-treated samples, suggesting that excessive collagen was synthesized that allowed stretching of the body cuticle. Expression of *dpy-2* did not alter significantly, which infers that the cuticle was not V_A target for radiation resistance. The results show that expression of *dpy-2* exhibits a similar variation tendency between 200 and 400 Gy implying that *dpy-2* is a relatively stable target against radiation damage.

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Disclosure of conflict of interest

None.

Address correspondence to: Chao Zhang and Lina Chen, Institute of Genetic Engineering, Southern Medical University, 1838 Guangzhou Boulevard North, Guangzhou, China. Tel: +86-13824447151; E-mail: zhangchao1558@126.com (CZ); smucln@126.com (LNC)

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