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Gamma radiation induces life stage-dependent reprotoxicity in *Caenorhabditis elegans via* impairment of spermatogenesis

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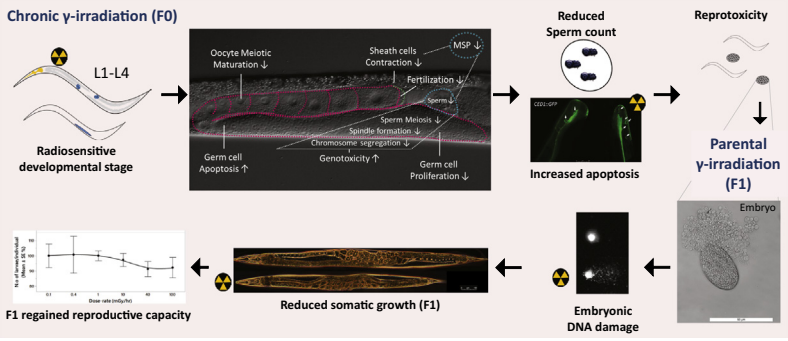
HIGHLIGHTS

- Radiosensitivity of *C. elegans* developmental stage L1-Young L4 was demonstrated following chronic gamma-irradiation.
- Reprotoxic effects were a consequence of sperm meiosis and spermatogenesis impairment.
- Genotoxicity persisted in offspring (F1) of irradiated nematodes and was associated with somatic growth impairment.
- A conceptual model for cellular and biological processes affected by gamma radiation in *C. elegans* was developed based on RNAseq analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

The current study investigated life stage, tissue and cell dependent sensitivity to ionizing radiation of the nematode *Caenorhabditis elegans*. Results showed that irradiation of post mitotic L4 stage larvae induced no significant effects with respect to mortality, morbidity or reproduction at either acute dose ≤6 Gy (1500 mGy·h^{-1}) or chronic exposure ≤15 Gy (≤100 mGy·h^{-1}). In contrast, chronic exposure from the embryo to the L4-young adult stage caused a dose and dose-rate dependent reprotoxicity with 43% reduction in total brood size at 6.7 Gy (108 mGy·h^{-1}). Systematic irradiation of the different developmental stages showed that the most sensitive life stage was L1 to young L4. Exposure during these stages was associated with dose-rate dependent genotoxic effects, resulting in a 1.8 to 2 fold increase in germ cell apoptosis in larvae subjected to 40 or 100 mGy·h^{-1}, respectively. This was accompanied by a dose-rate dependent reduction in the number of spermatids, which was positively correlated to the reprotoxic effect (0.99, PCC). RNAseq analysis of nematodes irradiated from L1 to L4 stage revealed a significant enrichment of differentially expressed genes related to both male and hermaphrodite reproductive processes. Gene network analysis revealed effects related to down-regulation of genes required for spindle formation and sperm meiosis/maturation, including *smz-1*, *smz-2* and *htas-1*. Furthermore, the expression of a subset of 28 *set-17* regulated Major Sperm Proteins (MSP) required for spermatid production was correlated (R^2 0.80) to the reduction in reproduction and the number of spermatids. Collectively these observations corroborate the impairment of spermatogenesis as the major cause of gamma radiation induced life-stage dependent reprotoxic effect.

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Furthermore, the progeny of irradiated nematodes showed significant embryonal DNA damage that was associated with persistent effect on somatic growth. Unexpectedly, these nematodes maintained much of their reproductive capacity in spite of the reduced growth.

1. Introduction

At the cellular level, ionizing radiation is known to inflict damage either indirectly *via* formation of free radicals or by direct interaction with essential molecules including proteins, lipids, RNA and DNA (Reisz et al., 2014), resulting in a complex mixture of adverse effects. While established genotoxic mechanisms include a combination of DSB, SSB (double strand break, single strand break) and oxidative lesions to DNA (Lomax et al., 2013), the adverse effects at an organism level can differ between individual species (Bréchignac et al., 2012; Garnier-Laplace et al., 2013; UNSCEAR, 2006). The biological response to ionizing radiation may also differ between chronic and acute exposure, both in the quality and intensity of effects (Kovalchuk et al., 2000; Pereira et al., 2011; Schwartz et al., 2000; Dubois et al., 2018). Chronic exposure is defined as an exposure of at least 10% of the duration of a species lifespan, and could consequently cover the entire developmental phase of an organism. In this sense, chronic exposure to low doses of ionizing radiation has the potential to produce long-term and hereditary effects. For any species, an assessment of the impacts of chronic radiation on survival, growth, developmental, reproductive and hereditary effects is essential to predict the consequences for a population's sustainability (Adam-Guillermin et al., 2018). Furthermore, certain life stages, tissues or cell types may inherently be more vulnerable to the effects of ionizing radiation, this influencing species radiosensitivity. Reproduction is known to be one of the most radiosensitive biological functions even in tolerant species, as well as being ecologically most relevant (UNSCEAR, 1996). Exposure to chronic ionizing radiation of invertebrates have demonstrated that doses corresponding to <10% of the lethal dose were harmful to reproductive performance, and that the negative effects persisted over multiple generations (Parisot et al., 2015; Hertel-Aas et al., 2011).

The nematode *Caenorhabditis elegans* tolerates acute doses of ionizing radiation >1 kGy without mortality (Johnson and Hartman et al., 1988). This tolerance has been linked to the ability of *C. elegans* to maintain genomic stability following radiation-induced DNA damage by activating checkpoints that induce cell-cycle arrest or apoptosis (Gartner et al., 2000). The majority of studies have been performed using acute high dose X-ray, proton beam or gamma irradiation of post mitotic stage young adult larvae (Gartner et al., 2000; van Haaften et al., 2006; Krisko et al., 2012; Guo et al., 2013; Min et al., 2017). However, in the last decade, more studies have focused on sub-lethal effects on multiple generations as well as on modelling approaches. These have shown that reproduction is a sensitive phenotypical change in nematodes, but there is still little mechanistic understanding of the factors influencing differences between chronic and acute exposures (Buisset-Goussen et al., 2014; Lecomte-Pradines et al., 2017).

The current study utilizes *C. elegans* to compare the effects of acute *versus* chronic gamma irradiation. This includes a systematic investigation of life stage, tissue and cell dependent radiosensitivity during the *C. elegans* development. A combined RNA-sequencing and phenotypic analysis was performed with the aim to elucidate the processes leading to reproduction impairment.

2. Materials and methods

2.1. C. elegans strains and culturing

The N2 Bristol strain was obtained from *Caenorhabditis* Genetic Centre, Minneapolis, MN and used in this study as the wild-type *C. elegans* background for all the irradiation experiments, with the exception of germ cell apoptosis assessment. The GFP (green fluorescent protein) reporter strain bcIs39 [lim-7p::ced-1::GFP + lin-15(+)] was employed to quantify engulfment corpses of apoptotic germ cells as described by Zhou et al. (2001).

Before performing the experiments, worms were maintained for two months at 20 °C in swirling liquid cultures under dark conditions (Brenner, 1974), in order to obtain a healthy stock population. Synchronous populations of nematodes were obtained by alkaline hypochlorite treatment as described by Stiernagle (2006).

2.2. Nematode irradiation and dosimetry

Gamma radiation exposures were conducted at the FIGARO experimental facility at the Norwegian University of Life Sciences (NMBU, Ås, Norway) (Lind et al., 2019). For every experiment performed in this study (Fig. 1), synchronous cohorts of embryos or L1 nematodes were placed on NGM plates (Ø 3 or 6 cm) (1.7% agar, 2.5 mg·mL^{-1} peptone, 25 mM NaCl, 50 mM KH_2PO_4 pH 6.0, 5 μg·mL^{-1} cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$) with fresh *Escherichia coli* OP50 as a food source (cultured overnight at 37 °C in L-Broth medium, Lewis and Fleming (1995)). Experiments were conducted at 20 °C in the dark. For each experiment, three control NGM plates were placed behind lead shielding, and three plates per exposure position were placed at distances equivalent to dose rates from 0.4 to 1490 mGy·h^{-1} (Supporting material S.M. 1, Table S.1).

Field dosimetry (air kerma rates measured with an ionization chamber) was traceable to the Norwegian Secondary Standard Dosimetry Laboratory (Bjerke and Hetland, 2014). Air kerma rates were measured using an Optically Stimulated Luminescence (OSL) based nanoDots dosimetry (Landauer) or Radio Photo Luminescent dosimeters (RPL, GD-301 type, Chiyoda Technol Corporation, Japan) by positioning the dosimeters at the front and back of the plates. Dose rates to water were calculated according to Hansen et al. (2019) and used as a proxy for dose rates to the nematodes (S.M. 1, Table S.1).

2.3. Comparing effects on reproduction by acute and chronic exposure to gamma radiation

To assess the effects of acute irradiation on reproduction, synchronous L4 nematodes were irradiated at 1445 mGy·h^{-1} for 0.75, 2 and 4 h, and total brood size was measured. To assess the effects of chronic irradiation, synchronized nematodes were exposed to 6 dose-rates ranging from 0.9 to 227.9 mGy·h^{-1} from the unhatched embryonic stage until they reached sexual maturity, for a total of 62 h (Fig. 1 and Table S.1 for total doses). Effects on reproduction were assessed by measuring the total number of offspring per adult hermaphrodite (three biological replicates and 5 individuals per replicate).

2.4. Analysis of life stage dependent effects of gamma radiation

To assess life stage dependent adverse effects of ionizing radiation, triplicate samples of synchronized nematodes were irradiated using five dose rates from 0.4 to 100 mGy·h^{-1} plus a control treatment, during selected developmental stages. Four exposure scenarios were designed (see Fig. 1 and Tables S.1–2 for dosimetry) and effects on morphology, growth, fecundity, and total fertility were measured.

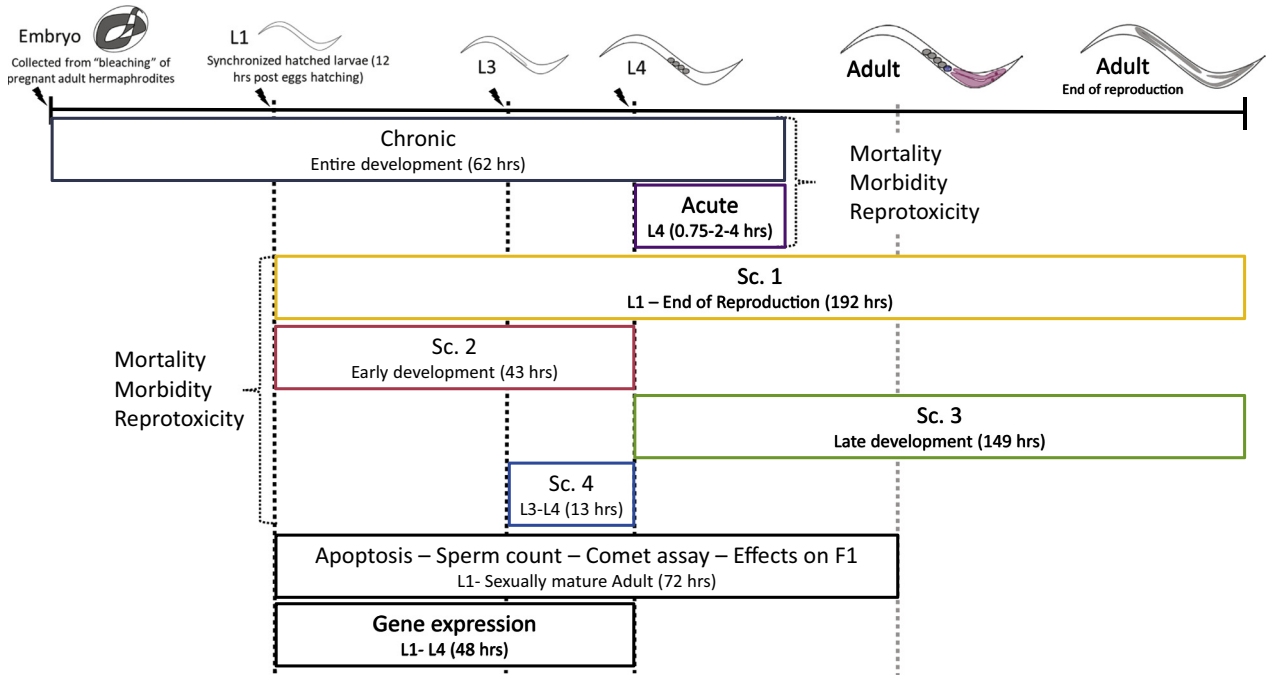


Fig. 1. Experimental design for the gamma irradiation exposures performed in the current study. The irradiation time (hours) is given in parenthesis for each scenario.

2.4.1. Reprotoxic effect assessment

Reproduction effects were evaluated by measuring the cumulative number of larvae (hatched eggs and L1) produced by five nematodes (3 biological replicates, $n = 15$ per treatment) (Table S.1 for dosimetry). From 48 h onwards from L1 stage, the adult worms were transferred to fresh NGM plates every two days for a total of 8 days, and offspring were stained with 1 mL Rose Bengal (0.3 g/L) in an oven at 80 °C for 10 min. NGM plates were then stored at 4 °C and the larvae counted, using a Leica stereo microscope (Leica M205C, 16× magnification).

2.5. Assessment of germline apoptosis

CED1::*GFP* nematodes were exposed in duplicates ($n = 100$) on NGM agar plates (Ø 3 cm) from L1 molt for 72 h (Fig. 1) to either 10.8, 40.8 or 99.9 mGy·h^{-1} of gamma radiation plus control (Table S.3 for dosimetry). After irradiation, ten worms per treatment were mounted onto 2% agarose pads, anesthetized with 30 mM NaN_3 in M9 buffer, and apoptotic germ cells identified as previously described by Lu et al. (2009). Images of one gonadal arm in each adult hermaphrodite ($n = 20$), 16 h post L4 molt, were captured as ~10 serial *Z*-sections of 1.0 μm interval using Nomarski optics in combination with fluorescence signal under a semi-automated research light microscope (Upright Microscope Leica DM6 B) equipped with a GFP ET filter system (512 nm emission and 40× objective). The frequency of *CED1*::*GFP* clustering around cell corpses was successively quantified as described by Zhou et al. (2001).

2.6. Spermatids quantification

After 72 h of irradiation (Fig. 1 and Table S.3 for dosimetry), worms were mounted on glass microscope slides pre-coated with Poly-Lysine (1 mg·mL^{-1}), dissected using a 0.5 × 16 mm gouge needle in M9 buffer to expose the spermatheca, fixed with Paraformaldehyde (2%) and permeabilized by freeze cracking (Sadler and Shakes, 2000). For this purpose, fifteen to twenty hermaphrodites per slide were dissected (three slides per treatment, $n > 45$) under a Leica stereo microscope (Leica M205C, 16× magnification). Slides were then stained with 10 μl DAPI DNA staining (10 μg·mL^{-1}) for 20 min, before proceeding with the spermatids count, under a semi-automated research light microscope (Upright Microscope Leica DM6 B) equipped with a DAPI filter system (461 nm emission and 40× objective).

For each analyzed spermatheca, images were captured as a ~20 serial *Z*-sections of ~5.0 μm interval.

2.7. Gene expression analysis

2.7.1. Transcriptomic analysis

RNA sequencing was performed in order to obtain gene expression profiles of triplicate nematode populations exposed to 10.8 or 99.9 mGy·h^{-1} compared to control nematodes (see Table S.3 for dosimetry). For this purpose, total RNA was extracted from samples snap-frozen immediately after 48 h of exposure from L1 stage on L4-young adult nematodes ($n = 1000$ per replicate) with Direct-zol Reagent (Nordic Biosite) and purified with RNeasy Mini Kit (Zymo Research) according to manufacture instruction. In brief, 100 μL of RNase-free Water and 600 μL of Direct-zol were added to each thawed sample, consisting of ~1000 nematodes, prior to homogenization with bead beating (0.1–0.5 mm Ø) using FastPrep (20 m/s per 10 s). The homogenate was transferred to a new Eppendorf tube, mixed with 700 μL of absolute ethanol (96% EtOH) and treated with DNase I and DNA digestion buffer on Zymo-spin mini Column, before further purification on column. RNA purity and yield (A260/A280 > 1.8, A260/A230 > 2, yield >100 ng/μL) was determined using NanoDrop-1000 Spectrophotometer (NanoDrop Technology, Wilmington, DE) and quality (RIN > 7) was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using RNA Nano LabChip Kit (Agilent Technologies). Photometric parameters and RNA integrity number determined the quality of the RNA sequenced samples. Strand-specific TruSeq™ RNA-seq pair-end libraries with 350 bp fragment size were prepared for each treatment (three biological replicates). For each sample ca 30×10^6 reads (read length 150 bp) were sequenced using two lanes of Illumina HiSeq 4000 (Norwegian High Throughput Sequencing Centre in Oslo, Norway), and made available on ArrayExpress (accession E-MTAB-8004).

Sequenced reads were mapped to the Ensemble reference genome WBcel235 using STAR (Dobin et al., 2013). Statistical analysis for detection of differentially expressed genes (DEGs) was done in R using Deseq2 package (rlog, variance Stabilizing Transformation)

transformed data (Love et al., 2015), with FDR ≤0.05 and $0.3 \leq \log_2 fc \leq -0.3$ as cut off.

2.7.2. Gene ontology and gene set enrichment analysis

In order to obtain information about processes affected by gamma radiation with respect to anatomical, phenotypical and functional processes down to the single-cell level, the DEGs were subjected to gene ontology(GEA), tissue(TEA) and phenotype(PEA) enrichment analyses using the WormBase Enrichment tool (Angeles-Albores et al., 2016; Lee et al., 2017). Analysis was performed using hypergeometric probability distribution with Benjamini-Hochberg step-up algorithm FDR correction (Angeles-Albores et al., 2017).

2.7.3. Pathway and network analysis

For predicted pathway and biological function analyses of DEGs, SimpleMine (Lee et al., 2017), Reactome Knowledgbase (Fabregat et al., 2017) and KEGG Pathways (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al., 2018) tools were used. The analysis was performed on the total number of DEGs for each of the exposure groups and the most significant categories found in each of the databases were compiled and subsequently manually curated in order to obtain annotations of the cellular and molecular processes affected by exposure to gamma radiation.

Gene interaction analysis was performed using GeneMANIA 3.5.1 (Warde-Farley et al., 2010; Franz et al., 2018) within Cytoscape 3.7.1 to identify predicted networks based on the total DEGs resulting from the 100 $mGy \cdot h^{-1}$ exposure.

2.8. Effects of parental irradiation on F1 nematodes

2.8.1. DNA damage analysis on nematode embryonic cells with comet assay

Triplicate samples of synchronous L1 stage larvae (>2500 per replicate) were irradiated for 72 h (Fig. 1) using dose rates from 0.43 to 99.9 $mGy \cdot h^{-1}$ (see Table S.3 for dosimetry). Embryos of irradiated parents were then sampled and DNA damage immediately assessed using the Comet assay. The method detects single strand breaks and alkali-labile DNA lesions using GelBond® films, for a high throughput single cell gel electrophoresis (Gutzkow et al., 2013) was adapted to the conditions of the present experiment. At the end of the irradiation, adult nematodes were removed from NGM plates with 3 × 2 mL of ice-cold Merchant's buffer (0.14 M NaCl, 0.00147 M KH_2PO_4, 0.0027 M KCl, 0.0081 M Na_2HPO_4, 0.01 M Na_2EDTA, pH 7.4). Embryos were gently dislodged from the agar surface by using the tip of a Pasteur pipette. The collected volume (6 mL), containing embryos was filtered using a cell-strainer (Ø 15 μm mesh) to remove the *E. coli* cells. Retained embryos were further rinsed with 6 mL of ice-cold Merchant's buffer. Nematodes embryos were then collected from the cell-strainer in 6 mL of ice-cold Merchant's buffer, and centrifuged at 3000*g* for 2 min.

Three biological replicates, each comprising >12,000 embryos, were placed in 0.5 mL ice-cold Merchant's buffer (pH 7.4) and cells extracted by mechanical dissociation using a 2 mL glass Dounce tissue grinder and piston B (Sigma-Aldrich®, Germany). After extraction, the resulting cell suspension was transferred into a new Eppendorf tube with 0.5 mL of ice-cold Merchant's buffer and settle by gravity on ice for 10 min. A volume of ~400 μL was then gently removed from the supernatant, and a sample from the suspension close to the pellet was taken in order to check for cell viability by using Trypan blue exclusion assay ($10\ mg \cdot mL^{-1}$) (Sigma-Aldrich®, Germany) (Strober, 2015). The cell-suspension was adjusted to 1×10^6 $cells \cdot mL^{-1}$ and resuspended in 1:1 low melting point agarose (1.35%, LMP) at 37 °C. By using a multichannel pipette, four technical replicates (4 × 4 μL), from each biological replicate were immediately dispensed onto a cold GelBond® film. Cell lysis was performed overnight in lysis buffer at 4 °C (2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris-base, 0.2 M NaOH, 0.034 M N-Laurylsarcosine, 10% DMSO, 1% Triton X-100, pH 10). The unwinding was performed by immersing the films in cold electrophoresis solution (0.3 M NaOH, 0.001 M Na_2EDTA, pH 13) for 40 min. Electrophoresis was performed in cold, freshly prepared electrophoresis solution for 20 min at 4 °C, 25 V and 0.8 V/cm, with circulation of the solution kept over time.

Immediately after the electrophoresis, the films were immersed in neutralization buffer (0.4 M Tris-HCL, pH 7.5) 2 × 5 min, fixed in ethanol (>90 min in 96% EtOH) and dried overnight.

SYBR®Gold Nucleic Acid Gel Stain (Life Technologies, Paisley, UK) in TE-buffer (1:10,000) (1 mM Na_2EDTA, 10 mM Tris–HCl, pH 8) was used to stain the nuclei before scoring of films, once the drying process was accomplished. Comets' scoring was performed at 40× magnification under an Olympus BX51microscope (light source: Olympus BH2-RFL-T3, Olympus Optical Co., Ltd.; camera: A312f-VIS, BASLER, Ahrensburg, Germany). Forty randomly chosen cells per replicate (160 cells per biological replicate, total of 480 cells per dose rate) were scored using the Comet IV analysis software (Perceptive Instruments Ltd., Bury St. Edmunds, UK). Tail intensity (% Tail DNA), defined as the percentage of DNA migrated from the head of the comet into the tail, was used as a measure of DNA damage induced by gamma radiation. Mean percentage (%) of DNA in the tail per exposure group was calculated using the median values of % Tail DNA from the 40 comets from each technical replicate (total of 12 median values per exposure group).

2.8.2. Developmental and reprotoxic effects assessment in progeny (F1) of exposed (F0) nematodes

The effect of ionizing radiation was evaluated on the progeny (F1) of nematodes (F0) exposed for 72 h from L1 stage to reproducing adult hermaphrodites (Fig. 1). Adults were washed off the NGM plates using 2 × 3 mL of M9-buffer. Subsequently, embryos were gently dislodged from the agar surface using the tip of a Pasteur pipette. M9 buffer was added to the plates and the collected volume (6 mL), containing embryos was filtered throughout a cell-strainer (Ø 15 μm mesh) in order to remove *E. coli* cells. Embryos were washed off the cell-strainer with 6 mL of M9 buffer, centrifuged at 3000*g* for 2 min, and incubated on non-seeded NGM plates overnight. The following day, synchronous L1 nematodes were transferred to seeded NGM plates (three biological replicates and 5 individuals per replicate) and kept under control conditions. Effects on morphology, growth, development and reproduction were assessed as previously described (Sections 2.4.1 and S.1).

2.9. Statistical analysis

Statistical analysis was performed using Minitab® 18 (Minitab Statistical Software (2010). [Computer software]. State College, PA: Minitab, Inc. (www.minitab.com)), JMP Pro v14 (SAS institute, Cary, NC, USA) and SigmaPlot 10.0 (Systat Software, San Jose, CA). Significant differences between different treatments were calculated using one-way analysis of variance (ANOVA) and, when significance was found, the Tukey pairwise comparisons method was applied. For ANOVA analysis, normality and homogeneity assumption were assessed on residuals by using Anderson-Darling normality test and visually on residuals *vs*. fitted value plot, respectively. Statistical significance was considered when *p*-value was lower than 0.05, unless differently stated.

The Effective Dose-Rate estimations were obtained on 10 and 50% of the population (EDR10 and EDR50) for reproduction and DNA damage on embryonic cells, by using the free software RegTox developed by Eric Vindimian (http://www.normalesup.org/~vindimian/en_download.html). For this purpose, the Hill model was used with corresponding confidence intervals of 95%.

Principal Component Analysis (PCA) was performed in order to find possible correlation between selected endpoints.

3. Results

3.1. Chronic exposure to ionizing radiation exacerbates reprotoxic effects compared to acute irradiation

In order to compare toxic effects of acute and chronic irradiation on nematodes, synchronous populations of *C. elegans* were exposed to similar total doses, but at different dose-rates of gamma radiation (S.1 Table for dosimetry). The chronic exposure from egg stage to young adult stage (62 h) was performed with dose-rates ranging from 0.9 to 227 mGy·h^{-1}, while acute exposure of young adult nematodes was conducted at 1445 mGy·h^{-1}. Neither exposure resulted in any mortality nor in any obvious morbid effects. However, while acute exposure did not induce any significant effect in terms of reproduction, the total number of hatched larvae per adult hermaphrodite was significantly affected in chronically exposed nematodes. The number of offspring was significantly reduced (Tukey *post hoc*, *p*-value <0.05) by 43% and 61%, when nematodes were chronically exposed from embryos to adult stage to 108 mGy·h^{-1} (total dose 6.7 Gy) and 228 mGy·h^{-1} (total dose of 14 Gy), respectively (Fig. 2). The calculated EDR50 (*i.e.*, the dose rate able to inflict a 50% effect on reproduction) was 160 mGy·h^{-1} (equivalent total dose 9.9 Gy), with the 95% confidence interval ranging from 134 to 192 mGy·h^{-1}. The corresponding EDR10 was estimated to 31.3 mGy·h^{-1} (95% CI 15.9 to 49.3 mGy·h^{-1}), with ED10 total dose of 1.9 Gy.

In contrast, the acute exposure of L4 nematodes (total dose up to 6.0 Gy) did not show any significant effect on reproduction (Tukey *post hoc*, *p*-value >0.05) (Fig. 2). This indicated that radiosensitivity of *C. elegans* could be linked to vulnerable life stage(s) or processes during larval development.

3.2. Exposure to gamma radiation during early larval development is detrimental to reproduction

Life-stage dependent radiosensitvitiy was assessed with respect to development, morbidity, fecundity and the cumulative number of hatched larvae per adult hermaphrodite by targeted irradiation of selected developmental stages (Fig. 1).

This revealed a significant contribution of life-stage dependent sensitivity with respect to reprotoxic effects (Fig. 3). As expected, no significant morbidity or effect on fecundity was seen, while a minor reduction of the total body length was measured (SM.1, Section S.1). A dose-rate dependent effect on reproduction was seen in nematodes exposed

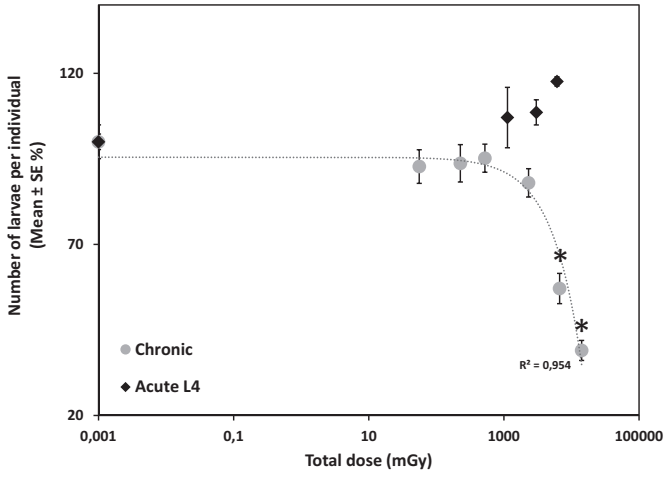


Fig. 2. Total number of offspring per adult hermaphrodite (Mean ± SE in %) measured after chronic or acute exposure to ionizing gamma radiation. Adults were placed on fresh plates every 24 h from onset of egg laying for a total of 6 days. Asterisk indicates significant difference from control treatment (*p*-value <0.05).

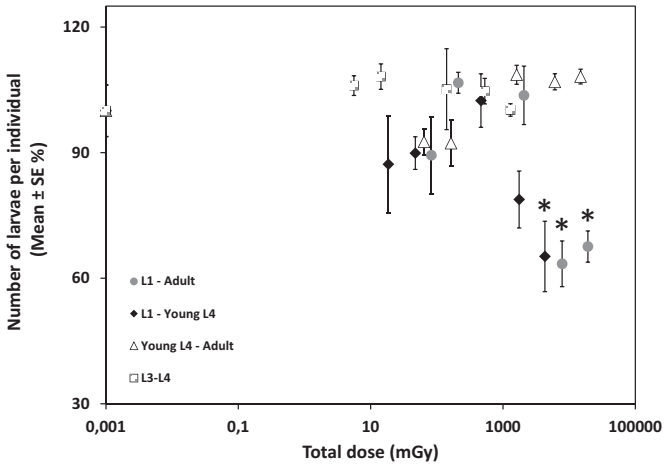


Fig. 3. Total number of offspring per adult hermaphrodite (Mean ± SE in %) measured after four different scenarios of exposure to chronic gamma radiation. Asterisk indicates significant difference from control treatment (*p*-value <0.05).

from the L1 stage throughout the reproductive period of adult hermaphrodite (192 h) as well as those exposed from L1 up to the Young L4 stage (43 h) (Fig. 3). At the two highest dose-rates of exposure (40.8 and 99.9 mGy·h^{-1}), nematodes irradiated from L1 molt to end of reproduction (total doses 7.8 and 19 Gy, respectively) showed a significant decrease in the cumulative number of hatched larvae (37% and 34% reduction respectively) compared to controls (Tukey *post hoc*, *p*-value < 0.05). Nematodes irradiated at 99.9 mGy·h^{-1} from L1 to young L4 molt (total dose 4.3 Gy) showed a 35% reduction (Tukey *post hoc*, *p*-value < 0.05), while no significant decrease, compared to controls, was seen at 40.8 mGy·h^{-1} (total dose 1.8 Gy) (Tukey *post hoc*, *p*-value > 0.05). This demonstrates that despite the differences in exposure times and total dose, the detrimental effects on reproduction were similar when these two scenarios were compared.

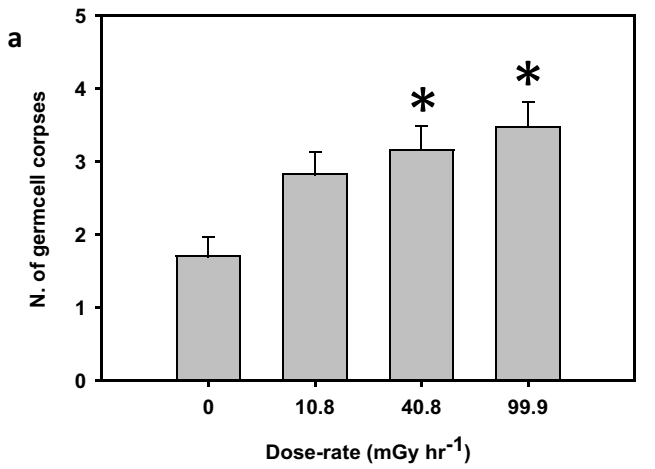
In contrast, neither nematodes irradiated from L4 molt throughout the reproductive period (143 h), nor the nematodes exposed from L3 to early L4 molt showed any significant reprotoxic effect (Tukey *post hoc*, *p*-value > 0.05), even when the total dose reached 14.9 Gy.

3.3. Enhanced germ cell apoptosis in chronically irradiated young adult nematodes

Assessment of apoptosis after 72 h of exposure to gamma radiation revealed a dose-rate dependent increase in the number of germ cell corpses in the *C. elegans* reporter strain *CED1*::*GFP* (MD701) (Fig. 4a–c). A significantly increased number of apoptotic germ cells was found when nematodes were exposed to the two highest dose-rates (40.8 and 99.9 mGy·h^{-1}) compared to control nematodes (Tukey *post hoc*, *p*-value < 0.05). At these dose-rates we observed an average of 3.1 and 3.4 apoptotic germ cells per gonadal arm respectively (Fig. 4a,b). This corresponds to a 2-fold increase in apoptosis compared to the control treatment (1.7 apoptotic germ cells per gonadal arm). We also noted a slight (1.6-fold higher), but not significant effect on germ cell apoptosis in nematodes exposed to 10.8 mGy·h^{-1} (Tukey *post hoc*, *p*-value > 0.05).

3.4. Chronic irradiation reduces the number of spermatids

In order to identify the cause of the reprotoxicity shown after irradiation during the early development, effects induced by chronic gamma irradiation on spermatogenesis were assessed in adult hermaphrodites at 72 h of exposure from L1 stage (Fig. 5). Nematodes exposed to total doses equal or >2.8 Gy showed a significant reduction in the number of spermatids compared to control nematodes, with dose-rates of 38.9



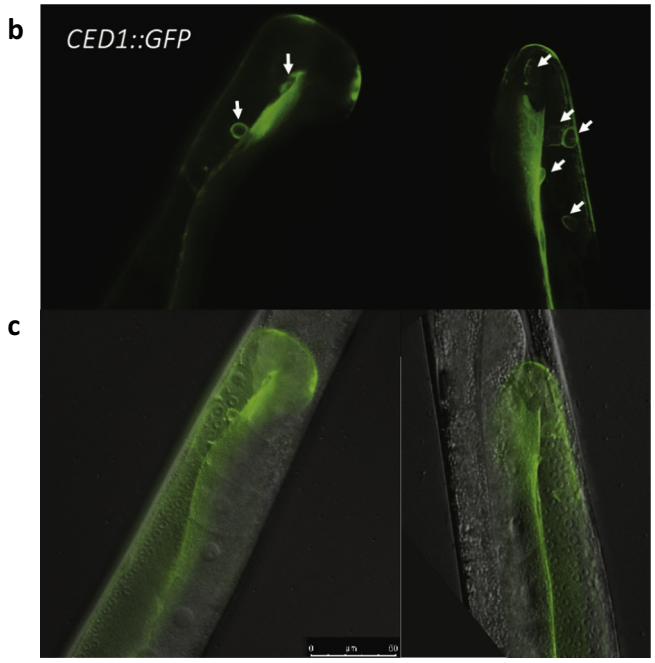


Fig. 4. a) Effect of chronic exposure to gamma radiation (72 h) on germ-cell apoptosis (number of germ cell corpses ± CI) pr gonadal arm in young adult *CED1::GFP* hermaphrodites ($n = 20$). Asterisks indicate significant difference compared to control treatment (p-value <0.05). b) Epifluorescence photomicrographs of gonadal arms in control hermaphrodite (left) and hermaphrodite irradiated at 100 mGy·h^{-1} (right). White arrows indicate apoptotic germ cells expressing the *CED1::GFP*. Scale bar: 50 μm. c) Nomarski and epifluorescence photomicrographs of gonadal arms from the same nematodes shown in Fig. 5b. Scale bar: 50 μm.

and 101 mGy·h^{-1} showing a 34% and 23% of reduction, respectively (Tukey *post hoc*, p-value < 0.05).

3.5. Gene expression analysis

In order to identify changes in the gene expression profiles during critical stages of gonadal development, a transcriptome analysis was performed on nematodes exposed to 10 and 100 mGy·h^{-1} for 48 h from L1 stage (S.M. 1). A total number of 1.75×10^3 genes was expressed in all samples, while the number of differentially expressed genes (DEGs) was 359 at the highest dose-rate of exposure (100 mGy·h^{-1}) compared to 540 resulting from the 10 mGy·h^{-1} exposure group (FDR < 0.05, log2FC ≤ −0.3 or ≥0.3) (Figs. S.2a-b and S.3a).

Among the DEGs a group of 54 genes was found to be in common between nematodes exposed to 10 and 100 mGy·h^{-1} (Fig. S.3b).

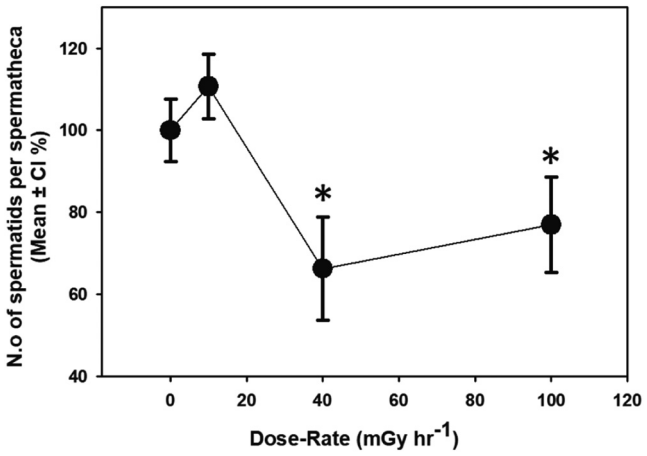


Fig. 5. Effect of chronic gamma irradiation on the number of spermatids per spermatheca (Mean % relative to control ± Confidence Interval, n = 20) counted in young adult hermaphrodites (72 h from L1 stage). Asterisk indicates significant difference compared to control treatment (p-value <0.05).

3.5.1. Functional enrichment analysis of DEGs

Gene function analysis of DEGs assessed by Gene Ontology (GOTERM) enrichment showed distinct differences in functionally enriched categories between the 10 and 100 mGy·h^{-1} exposures.

A total of 21 significantly over-represented Biological Functions were identified for the 10 mGy·h^{-1} group (Fig. S.4). Integrated pathway analysis combining the outputs from Simplemine, Reactome and KEGG databases corroborated the enrichment analysis from the 10 mGy·h^{-1} exposed group with respect to cuticle-collagen, protein and lipid metabolism (Table S.4). In addition, we found 10 genes with functions related to biological oxidation and Glutathione metabolism and 45 genes related to Immune system, Signal transduction, Peroxisome and Response to pathogens.

A total of 18 GOTERMs were significantly over-represented among the down-regulated genes in the 100 mGy·h^{-1} exposure group (Fig. 6a), while no significant GOTERM resulted from the list of up-regulated genes. The GOTERMs were related to cellular components such as organelle, cytoplasm, nucleus, nucleolus, cytoskeleton, mitochondrion, and structural constituent of ribosome. Biological and molecular functions included multicellular organism reproductive process, rRNA metabolic process, RNA splicing, peptide biosynthetic process and macromolecule biosynthetic process (Fig. 6 and Table S.5). From the 100 mGy·h^{-1} group 159 of 174 down-regulated genes had an annotation in the Tissue Enrichment Analysis tool (TEA, Fig. 6b). The significantly enriched terms were mostly related to reproduction, and included Reproductive system, Male, Spermatheca, Oocyte and Amphid sheath cell. The Phenotype Enrichment Analysis (PEA, Fig. 6c) showed that the Linker-cell migration variant, Cytoplasmic processing body (P-granule) variant, and Spindle position variant were the most significant terms. Pathway analysis identified 7 biological functions related to reproduction (Table S.5). These comprised exclusively down-regulated genes (101) related to spermatogenesis, 28 of them being Major Sperm Proteins, 3 genes related to sperm meiosis and maturation. Fifteen of these genes also participate in germline proliferation, spindle formation and oogenesis.

In addition, a significant effect was identified on Cell-cycle, Programmed cell death, Chromatin organization and DNA repair, Cellular stress response, Immune system modulation, and Signal transduction. A further 24 DEGs were related to Protein Metabolism, Macroautophagy and Peroxisome. Among these, we found up-regulation of stress-activated protein kinases (*jnk-1* and *mak-1*) (Kawasaki et al., 1999), a target of ERK kinase MPK-1 (*toe-4*) (Miller and Chin-Sang, 2012), ferritin (*ftn-1*) (Kim et al., 2004), Ubiquitin conjugating enzymes (*ubc-3* and

ubc-8) (Dove et al., 2017; Jones et al., 2001) and Ubiquitin carboxyl-terminal hydrolase (*ubh-4*), which are hallmarks of cell response to damage to proteins, mitochondria and lipids.

3.5.2. Network analysis

In order to identify operational gene interactions, a Genemania (Franz et al., 2018) network analysis was performed on the complete list of DEGs resulting from the 100 mGy·h^{-1} exposure group. Out of 359 genes, 331 clustered into three distinct groups, connected by co-expression, shared protein domain and physical or predicted interaction (Fig. S.5). One of these clusters corresponded to the genes involved in reproduction identified by Tissue Enrichment and Pathway analysis. Within this cluster, we identified a common attribute in the Cytosolic Motility Protein (Fig. S.5). This included a total of 71 genes, 64 of these were spermatogenic (assigned according to Ortiz et al. (2014)), including *ssp-10*, *ssp-35* and *sss-1* as well as 28 MSP class genes. In addition, nearest neighbors included *htas-1* (sperm specific histone H2A) *smz-1* and *smz-2* (involved in spermatid meiosis chromosome segregation) (Samson et al., 2014; Chu et al., 2006).

The second cluster was defined by 11 Serine/Threonine protein kinase genes (Figs. S.5, S.6) related to stress response, cell-cycle control and meiosis. Among these genes, *mak-1*, *jnk-1* and *air-1* were identified by the first neighbor analysis as main inter-nodes connecting 157 genes. Specifically, the Aurora/Ipl1 Related kinase *air-1* represented a major node, showing co-expression with two subsets of genes (Fig. S.6), one interconnecting two of the major clusters and containing 8 genes with protein kinase activity (*W02B12.12*, *Y38H8A.3*, *C39H7.1*, *T05A7.6*, *mak-1*, *T07F12.4*, *F32B6.10* and *ZC123.4*). In addition, *air-1*, which is required for the assembly/stabilization of female meiotic spindle microtubules (Sumiyoshi et al., 2015), physically interacts with *spd-5* and *ran-1* (Boxem et al., 2008), also involved in spindle formation (Hamill et al., 2002; Cheng et al., 2008).

The third cluster comprised genes related to gene regulation and chromatin remodeling, such as *cec-5* gene, predicted to have methylated histone binding activity, *rpb-5*, *Y54H5A.1* and *ruvb-2* with DNA binding activity (Poulin et al., 2005) and the major sperm protein *vpr-1*, which is required for proper distal tip cell migration during somatic gonad development (Cottee et al., 2017). The latter was also identified as a major node, sharing the same protein domain with 30 spermatogenic genes and co-expression with 9 non-spermatogenic genes. The *cec-5 and let-418* genes, involved in the negative regulation of germline transcription and vulva development (Käser-Pébernard et al., 2014; Turcotte et al., 2018), were connected to 26 genes, including *air-1* and *vpr-1* (targets of *cec-5*). Furthermore *let-418* targets were *ima-3* involved in meiosis I (Weber and Brangwynne, 2015), *emb-4* required for regulation of the transcription in the germ line (Tyc et al., 2017), and *his-24* involved in epigenetic regulation of heterochromatin (Jedrusik-Bode, 2013).

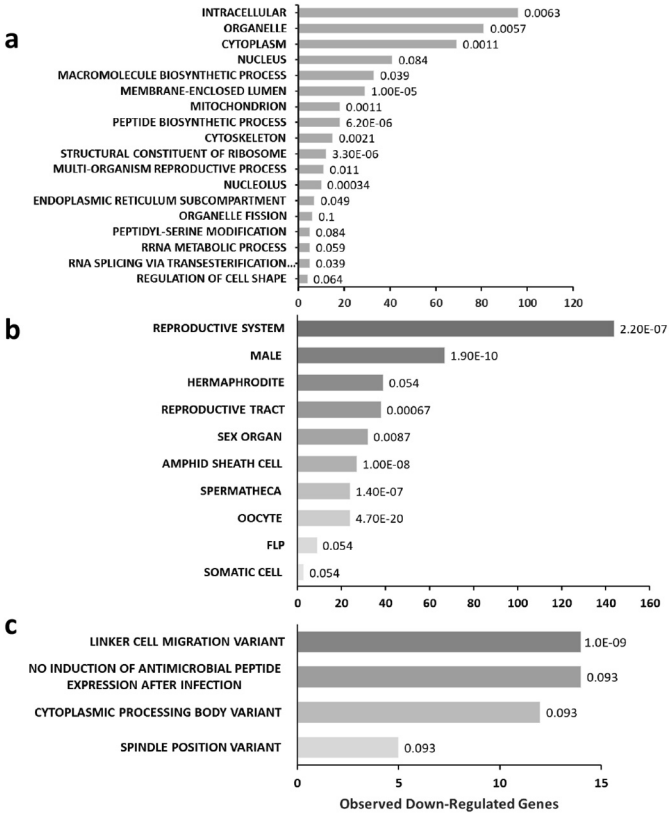


Fig. 6. a) Functional categories of over-represented Gene Ontology (GO) terms, b) Tissue Enrichment Analysis (TEA) and c) Phenotype Enrichment Analysis (PEA) of down regulated genes resulting from *C. elegans* exposed for 48 h to 100 mGy·h^{-1} of gamma radiation. Hypergeometric probability distribution was adopted to calculate the enrichment of down-regulated genes observed in each specific function. (Data labels indicate *q*-values).

3.6. Adverse effects on the progeny (F1) of irradiated nematodes

3.6.1. Radiation induced DNA damage in C. elegans embryonic cells

In order to assess DNA damage on the progeny of irradiated parents, a protocol for performing Comet Assay on *C. elegans* embryonic cells was developed (see Section 2.8.1). The Comet assay was performed using embryos to extract homogeneous essentially undifferentiated cell populations that were mitotically active (Fig. 7a) (Ehrenstein and Schierenberg, 1980; Wood, 1988). The established protocol produced high numbers of viable cells (assessed using trypan blue staining), with low level background comet tail in control cell populations (2.2–5.8%) compared to a previous study done by Ng et al. (2019).

Comet assay on embryonic cells showed a tendency of increased DNA damage (Mean % tail intensity and frequency of cells with significant DNA damage) after exposure of parents to dose-rates ranging from 0.43 to 10.8 mGy·h^{-1} although this was not statistically significant (Tukey *post hoc*, $p > 0.05$) (see Figs. 7c and S.7). However, exposure to dose-rates of 40.8 and 99.9 mGy·h^{-1} caused significant DNA damage, with a 3.9 and 4.4 fold increase of tail intensity, compared to non-irradiated embryonic cells (Tukey *post hoc*, $p < 0.05$, Fig. 7b,c).

The EDR50 value calculated for the DNA damage was 38.4 mGy·h^{-1}, with the 95% confidence interval ranging from 13.9 to 39.2 mGy·h^{-1}.

Moreover, the proportion of damaged cells increased in a dose rate dependent manner, where all cells from the 40.8 and 99.9 mGy·h^{-1} (2.94 and 7.19 Gy total dose) treatments showed DNA damage significantly higher than control level (6% tail intensity) (Fig. S.7).

3.6.2. Significant size reduction accompanied by low reprotoxic effects on parentally irradiated F1 nematodes

To investigate the late effects on the parentally irradiated (F1) embryos, the F1 generation was followed during development and effects were measured with respect to mortality, morphology, growth, and reproduction.

No effect was observed with respect to mortality, but a clear dose/dose rate-dependent reduction on the total body length was measured at 96 h post L1 molt (see Fig. 8a–c). This reduction was statistically significant already at the lowest dose-rate of exposure 0.43 mGy·h^{-1} (Tukey *post hoc*, *p*-value < 0.05). The reduction in body length was not associated with other visible anatomical morbid changes as formation of pharynx, gastrointestinal tract, and reproductive systems appeared intact, but were smaller in size (Fig. 9c). We also observed a trend towards reduced total brood size for the parentally irradiated F1 nematodes, (Fig. 8b), but the effect was not significant compared to control nematodes (Tukey *post hoc*, *p*-value > 0.05).

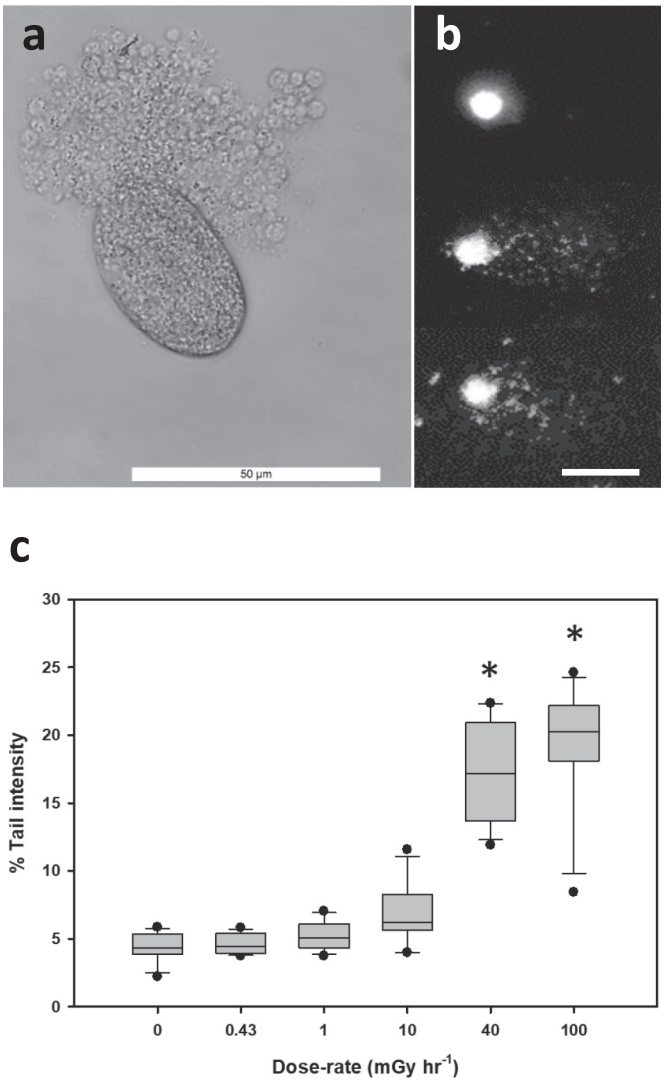


Fig. 7. a) Undifferentiated mitotically active embryonic cells harvested by mechanical disruption of gastrula stage embryos of irradiated parents. Micrograph from a semi-automated research light microscope at 40×, bright field optics. Scale bar: 50 µm. b) Comet micrographs taken at 40× magnification under an Olympus BX51 microscope (light source: Olympus BH2-RFL-T3, Olympus Optical Co.). From Top to Bottom: Control, 40 and 100 mGy·h^{-1}. Scale bar: 10 µm. c) DNA damage (Mean of Tail intensity in %) assessed on embryonal cells from parentally irradiated embryos, using the Comet assay. Asterisks indicate significant difference from control treatment (p-value <0.05).

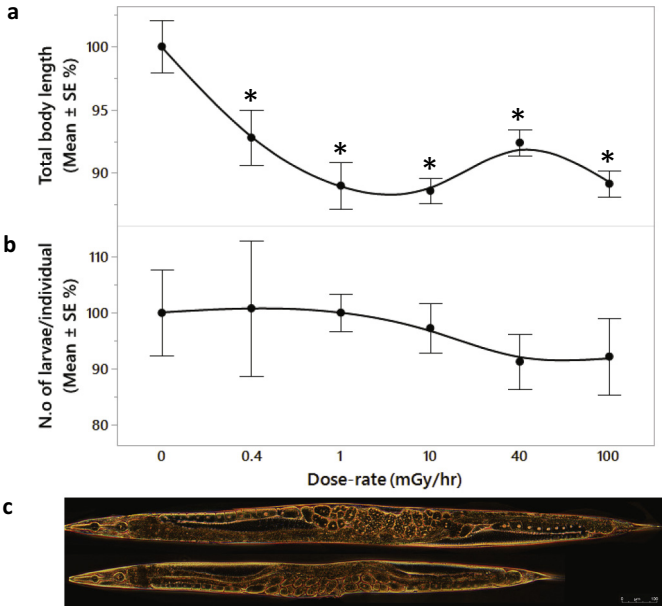


Fig. 8. Effects on somatic growth in offspring of nematodes exposed to gamma radiation. a) Total body length relative to control ± SE in % measured at 96 h of development using a stereo microscope (Leica M205C, 10× magnification) coupled with a computer-connected camera. Asterisks indicate significant difference compared to control treatment (p-value <0.05). b) Total number of offspring per adult hermaphrodite (Mean % relative to control nematodes ± SE), produced by nematodes parentally exposed to chronic gamma radiation. Adults were placed on fresh plates every 48 h from onset of egg laying for a total of 6 days. c) Physiological appearance of F1 adult hermaphrodites (96 h post L1), resulting from parental (F0) exposure to chronic gamma radiation (UP: Control, Bottom: 100 mGy·h^{-1}). Micrographs from a semi-automated research light microscope at 10×, phase-contrast optics, Scale bar: 100 µm.

4. Discussion

4.1. Chronic irradiation induces life-stage dependent reprotoxic effects in C. elegans

Caenorhabditis elegans is considered among the most radioresistant of organisms, tolerating >1 kGy dose of ionizing gamma radiation (Hartman and Herman, 1982, Hartman et al., 1988, Johnson and Hartman, 1988, Gartner et al., 2000, Bailly and Gartner, 2013, Guo et al., 2013). In contrast, recent studies have revealed that chronic exposure may cause adverse cellular and reproductive effects at much lower doses (Hartman and Herman, 1982; Hartman et al., 1988; Johnson and Hartman, 1988; Gartner et al., 2000; Bailly and Gartner, 2013; Guo et al., 2013; Buisset-Goussen et al., 2014; Lecomte-Pradines et al., 2017; Dubois et al., 2018). We therefore hypothesized that the apparent differences in effect may either be caused by different efficacy of acute *versus* chronic irradiation. Alternatively, the discrepancy in effects may be related to radiosensitivity of individual life stages, cell types or molecular functions in *C. elegans*.

In the present study, exposure of L4 young adults *C. elegans* to acute and chronic gamma irradiation (~6 Gy) did not cause any significant effect with respect to mortality, morbidity, or any of the reproductive endpoints, confirming that nematodes can tolerate high acute doses of radiation without mortality (Hartman and Herman, 1982; Krisko et al., 2012) (Fig. 2). Results are also consistent with previous studies where significant effects on hatchability and fecundity appeared only at doses >50 Gy (Krisko et al., 2012 and Dubois et al., 2018). In comparison, subjecting nematodes during development (embryos to L4 young adults) to chronic irradiation at a similar cumulative dose (>4 Gy), did not affect mortality or morbidity, but caused significant reprotoxic effects (Figs. 2 and 3). This demonstrates that the pre-L4 young adult stage is more sensitive to ionizing gamma radiation compared to the post mitotic stage. However, it was not evident whether the observed reprotoxic effects were related to a specific developmental stage, tissue or vulnerable cell type.

The results from the four exposure scenarios further support the differences in radiosensitivity between early and late larval development in this nematode. A dose-dependent reprotoxic effect was observed when larvae were exposed during their early development (L1-Young L4), while no effects were seen when adult stages were irradiated (Fig. 3). Furthermore, our results showed that extending the irradiation to include the embryonal stage did not enhance the reprotoxic effect compared to exposure during larval stage only. In *C. elegans* DNA repair is particularly robust during early embryogenesis (Clejan et al., 2006), and somatic cells in larvae are more tolerant to DNA damage than germ cells (Vermezovic et al., 2012; Lans and Vermeulen, 2015). Based on the observed reprotoxic effects (Figs. 2 and 3), it appears that the post-embryonic development is the phase where the critical damage occurred. During this phase, cell proliferation resumes and

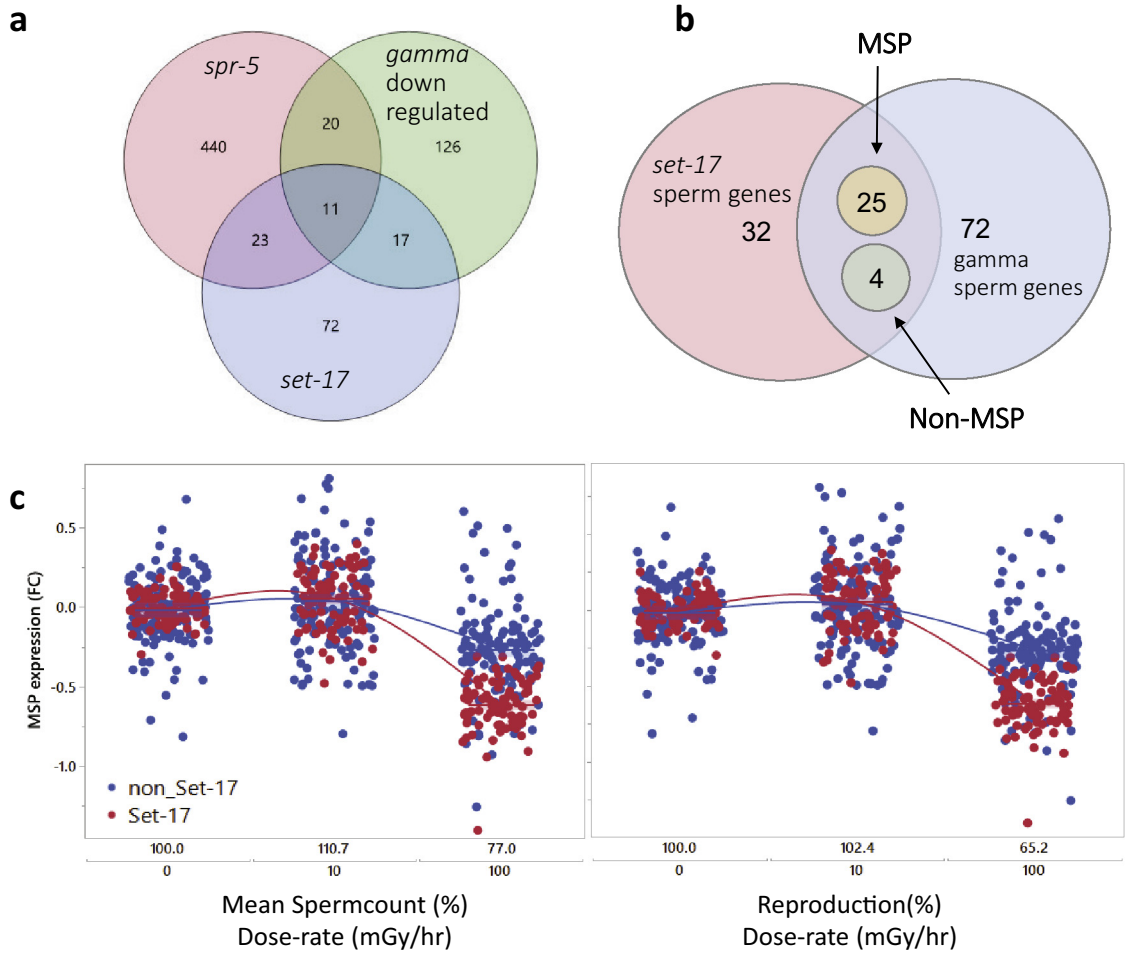


Fig. 9. a) Venn diagram of down-regulated genes resulting after chronic exposure to gamma radiation (4.8 Gy) or regulated by *spr-5* or *set-17* (gene expression data from Katz et al. (2009) and Engert et al. (2018), respectively). b) Venn diagram of spermatogenic genes regulated by chronic exposure to 4.8 Gy of gamma radiation and by *set-17* (Engert et al., 2018). c) MSP expression (Fold Change) plotted as a function of fertility (No. offspring/individual %), No. of spermatids (%) and dose-rate of exposure ($mGy \cdot h^{-1}$) to gamma radiation ($R^2 = 0.8$). In red 25 MSP genes found significantly down-regulated (FDR <0.05) after chronic exposure to 4.8 Gy of gamma radiation and in common with *set-17* regulated spermatogenic genes found by Engert et al. (2018). In blue MSP genes not regulated by *set-17*. Spermatogenic genes were assigned according to Ortiz et al. (2014).

the reproductive tract is generated, with the establishment of Z1-Z4 gonad (Pazdernik and Schedl, 2013) and Z2 and Z3 germline precursor cells to initiate gonadogenesis (Kimble and Hirsh, 1979).

The reduction in number of hatched larvae per adult caused by irradiation of L1- Young L4 to a total dose of 4.3 Gy was similar to that following irradiation of the L1 to the end of reproduction to a total dose of 7.8 Gy. Furthermore, since no effects were seen when the L4-adults were irradiated to total doses of up to 15 Gy, it would appear that the L1 to the young L4 stage are the most critical radiosensitive stages with respect to reprotoxicity (Figs. 2 and 3). The results thus suggest that post L4 stage larvae are able to effectively ameliorate genotoxic effects, at least up to doses of 15 Gy.

4.2. Effect of ionizing radiation on the C. elegans germline: Enhanced apoptosis and impaired sperm production

In order to investigate the mechanisms behind the observed reprotoxicity we assessed adverse effects on the germline of irradiated nematodes with respect to DNA damage, by measuring the number of apoptotic cells and the number of produced spermatids. The apoptosis assessment was carried out using a reporter strain (*CED1::GFP*), while the N2 Bristol strain was used for the spermatid measurement. In both cases, irradiation covered the radiosensitive L1-L4 developmental stage.

Germ cell death in *C. elegans* is known to be a natural physiological event, where half of the potential oocytes are removed (Gumienny et al., 1999; Lettre and Hengartner, 2006). Apoptosis is as an important surveillance mechanisms that ensures quality control in the germline (Bailly and Gartner, 2013), which may be enhanced by genotoxic insult like high doses of ionizing radiation *via* a series of DNA damage response mechanisms including cell-cycle arrest and programmed cell death (Gartner et al., 2000).

Strikingly, our results showed that, in comparison to the reprotoxic effects and to previous studies where germ cell apoptosis was only identified after acute doses of exposure, exerted on L4 nematodes, (>60 Gy) (Schumacher et al., 2001; Schumacher et al., 2005), already a dose as low as 2.9 Gy during L1 to L4 stages effectively enhanced the number of apoptotic germ cells (Fig. 4a). Thus showing that proliferating oocytes are very vulnerable to the effects of ionizing radiation, but also that germ cell apoptosis in *C. elegans* is a highly responsive protective mechanism that removes damaged cells and reduces the probability of misrepair at such low doses. The enhanced germ cell apoptosis observed in the present study may therefore be considered as a defense mechanism activated to obtain an efficient removal of non-salvageable oocytes (Andux and Ellis, 2008), preserving the embryos genome integrity (Lans and Vermeulen, 2015) and viability of the progeny (Bailly and Gartner, 2013).

While oocytes are continuously produced and can be replenished, each hermaphrodite produces a limited amount (~300) of spermatocytes during the L3/L4 stage (Chu and Shakes, 2013). The internal fertilization of *C. elegans* is extremely efficient. An unmated hermaphrodite

will use all of its sperm to produce offspring (Singson, 2001). Spermatogenesis has been reported to be affected by chronic irradiation in other invertebrate species (Hertel-Aas et al., 2011). We therefore hypothesized that spermatogenesis might also be a vulnerable process in *C. elegans*. Accordingly, we found a significant reduction on the number of spermatids at 2.8 Gy (Fig. 5), which is similar to the dose causing enhanced germ cell apoptosis (Fig. 4). In terms of the dose rates, in both cases dose-rates of 8–10 $mGy \cdot h^{-1}$ showed non-significant effects from controls, while significant changes were seen at higher dose-rates such as 40 and 100 $mGy \cdot h^{-1}$. The Pearson correlation analysis identified a positive correlation between the reduction in spermatids and the observed reprotoxic effect (PCC: 0.99 for L1-End of reproduction, PCC: 0.86 for L1-Young L4 exposure) (Figs. 2–4, S.8). Consistently, the limiting factor for self-fertility in *C. elegans* is not the number of oocytes, but rather the amount of self-sperm produced by the hermaphrodite (Hodgkin and Barnes, 1991).

Our results therefore suggest that the defective spermatogenesis induced by chronic exposure to ionizing radiation is the most plausible cause of the life stage-dependent reprotoxic effects in *C. elegans*.

4.3. Chronic exposure to gamma radiation impairs expression of genes required for spermatogenesis, oogenesis and embryogenesis

Ionizing gamma radiation is able to exert adverse effects on genes and proteins directly, through DNA damage (single and double strand breaks as well as DNA oxidation), or indirectly *via* formation of free radicals, recombination and induction of ROS (National Research Council, 2006). Consistent with these known effects, the transcriptomic analysis revealed that chronic exposure to gamma radiation induced differential regulation of genes involved in Cell-cycle control, Programmed cell death, Chromatin organization, DNA repair, Biological oxidation and Cellular stress response (Table S.5). The transcriptomic data also reflected significant differences between exposure to 10 $mGy \cdot h^{-1}$ and 100 $mGy \cdot h^{-1}$ (0.4 and 4.8 Gy total dose) with respect to toxic effects, including reproduction, apoptosis and spermatid production. It is known that the set of genes involved in apoptotic cell clearance in *C. elegans*, also mediates the removal of residual bodies during spermatogenesis. Defective clearance of residual bodies has been proven to reduce the number of spermatids in both males and hermaphrodites, possibly by decreasing sperm transfer efficiency (Huang et al., 2012; Ellis and Stanfield, 2014). Notably, physiological germ-cell death has not been reported in male gonads, and apoptosis appears to be restricted to oogenesis in hermaphrodites (Lettre and Hengartner, 2006).

We therefore hypothesized that other *hitherto* unknown mechanisms could be involved in the impaired spermatogenesis.

In line with the observed adverse phenotypic effects, the gene expression analysis at L4-stage showed that central molecular and cellular processes related to reproduction, and in particular to spermatogenesis, were negatively affected at 100 $mGy \cdot h^{-1}$ (total dose >4 Gy) (Fig. 6a–c, Table S.5). Consistent with the reduction of spermatids (Fig. 5), we found significant down-regulation of genes related to chromosome segregation in sperm meiosis (*smz-1* and *smz-2*) (Chu et al., 2006) and chromatin condensation during sperm maturation (*htas-1*) (Samson et al., 2014). Throughout spermatogenesis, the processes of meiosis, sperm differentiation, and chromatin remodeling are intimately intertwined, RNA inhibition of the gene *smz-1* or *smz-2* has shown to induce the arrest of spermatocytes progression through meiotic division thus affecting male fertility (Chu et al., 2006).

Moreover, down regulation of 28 sperm cytoskeletal structural protein genes (MSP) and 3 sperm-specific genes also suggested a severe defect in spermatogenesis (Table S.5). This family of proteins accounts for >40% of the cytosolic protein in *C. elegans* sperm (Smith, 2006). Several gamete-signaling events are required for high levels of oocyte maturation and ovulation and major sperm proteins (MSPs) play a central role not only in pseudopod motility, but also in promoting oocyte meiotic maturation, sheath contraction and ovulation of the oocyte in the spermatheca (Miller et al., 2001). When we performed a more thorough investigation on the 101 down-regulated genes spermatogenic (assigned according to Ortiz et al. (2014)), a significant correspondence (29 genes) with a previous study from Engert et al. (2018) was found (Fig. 9a,b). In the study from Engert and co-authors, a 50% reduction in terms of fertility was due to down-regulation of 28 MSP genes as a result of the mutation in the gene *set-17*(*n5017*). Furthermore, *let-418*, which was down-regulated in our transcriptomic analysis, interacts physically and genetically with *spr-5* to promote the normal development of germline stem cells (Käser-Pébernard et al., 2014). *Spr-5* is a histone H3K4 demethylase with a role in meiotic double-strand break repair (Nottke et al., 2011). Loss of *spr-5* and *let-418* has shown to induce immediate sterility and aberrant gonad development, demonstrating a collaborative role of these two genes in promoting fertility (Käser-Pébernard et al., 2014). Our network analysis showed interactions *via* co-expression between chromo-domain genes *let-418* and *cec-5* with 26 genes involved in gonad development, regulation of transcription in the germ line and meiosis (Fig. S.5).

This may imply that DNA double-strand breaks, resulting from exposure to ionizing radiation, may play a role in the regulation of *spr-5* and *set-17* and thereby inducing defective meiosis, which is consistent with the down-regulation of *smz-1* and *smz-2*, reduction of spermatocytes, fertility and consequently the down-stream regulation of 28 MSP genes (Fig. 9a–c).

We also identified a potential downstream effect of the impaired spermatocyte/MSP expression by the down-regulation of *spd-5* and *air-1*, two genes essential for the centrosome maturation and spindle assembly during the first mitotic division of the *C. elegans* zygote (Hamill et al., 2002). Consistent with this result, *air-1* was also a target of the major sperm protein *vpr-1* in our network analysis (Fig. S.6). This is an essential gene which shares the protein domain with the *MSP*s and whose expression is crucial in neuron and germ cells to induce gonadogenesis (Cottee et al., 2017), suggesting that in *C. elegans* exposure of early life stages to ionizing radiation may also impair this signaling mechanism required for the development of sexual organs. Moreover, prior to fertilization, the major sperm proteins have shown to promote oocyte microtubule reorganization (Harris et al., 2006). This suggests that the down-regulation of Aurora A kinase/AIR-1, shown in our transcriptomic analysis, may play a central role not only for the impairment during the formation of the spindle microtubules in female meiosis, but also for the regulation of mitotic cell cycle, as shown by the physical interaction with the gene *spd-5*. This notion was further supported by the down regulation of 23 genes related to germline proliferation, spindle assembly, oogenesis and embryonic development (Table S.5). In sum these observations substantiate that chronic exposure to ionizing radiation (>4 Gy total dose) in early stage nematodes has a profound effect on the entire *C. elegans* reproductive system (Fig. 10).

4.4. Embryonic DNA damage leads to a significant impairment on somatic growth but minimal effects on reproduction in the progeny (F1) of irradiated nematodes

Although DNA damage like DSB may cause replication problems (Bailly and Gartner, 2013), particularly when cell division rate is high *e.g.* during early embryogenesis, a previous study showed that *C. elegans* embryos are relatively tolerant to high doses of UV or other genotoxic agents (Holway et al., 2006). However, little was known about parental exposure to low doses of the germline and the later effects on the surviving embryos. Therefore, in this study we have investigated the embryonic DNA damage exerted by parental exposure to low doses of ionizing gamma radiation in combination with somatic growth impairment and reprotoxic effects on the F1 progeny. The focus of these experiments was therefore to examine the radiosensitivity in nematodes exposed during the proliferation stage, corresponding to cell divisions from a single cell (prior fertilization) to 558 essentially undifferentiated

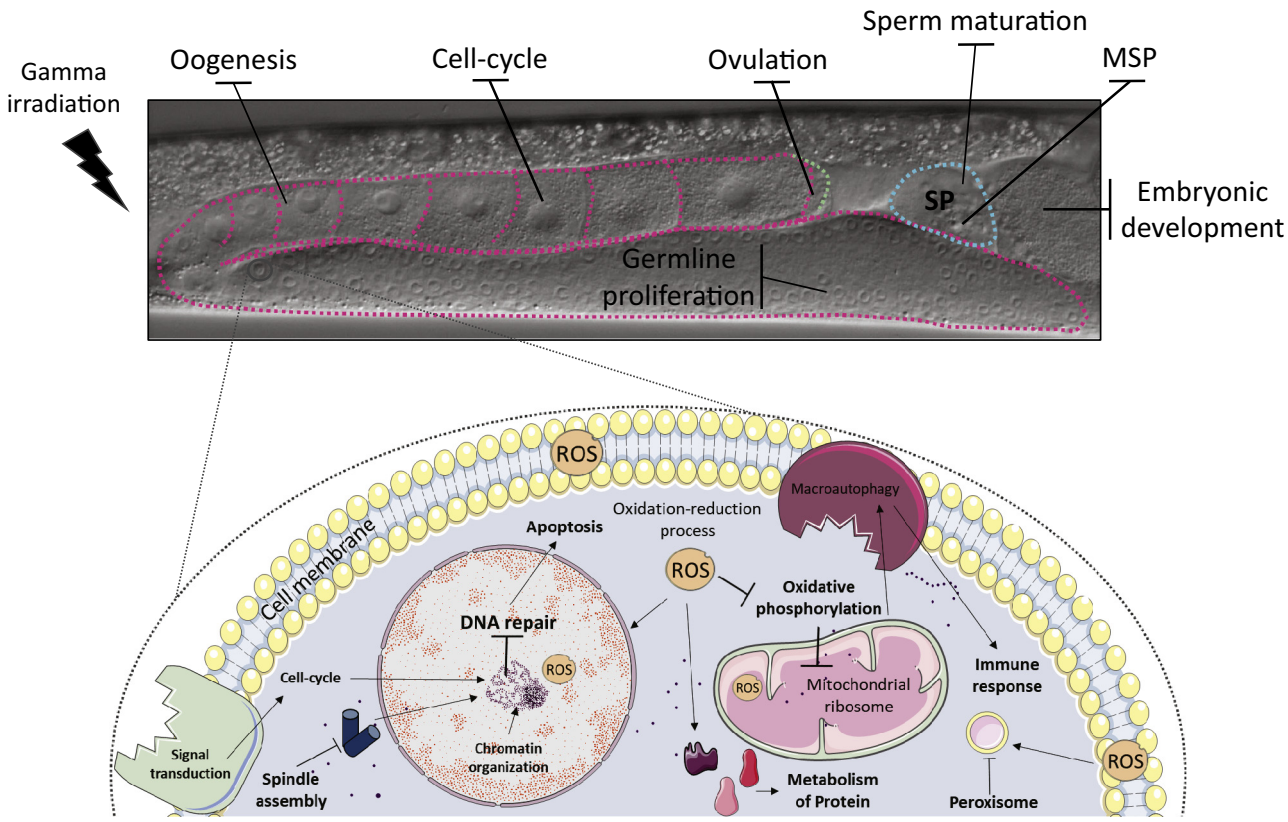


Fig. 10. Conceptual model of cellular and molecular processes induced (↑) or inhibited (T) after chronic exposure to gamma radiation (100 mGy·h^{-1}) in the nematode *C. elegans*.

cells by the end of "16 E stage" (Ehrenstein and Schierenberg, 1980; Wood, 1988). Our results demonstrated a dose-dependent sensitivity of embryonic cells in terms of DNA damage. Specifically, at accumulated doses higher than 2.9 Gy we observed an increased frequency of damaged cells (Fig. S.7) and a significantly higher damage compared to background levels (control treatment) (Fig. 7b,c).

Despite the significant damage seen in these embryonic cells and consistent with Dubois et al. (2018), we could not observe any deleterious effect on hatching and or lethality on embryos parentally exposed at doses up to 7.2 Gy. In a previous study, an acute dose of 50 Gy during early embryonal development was required to induce almost complete embryonic lethality for wild type. This effect was considered to be a consequence of cell proliferation (Clejan et al., 2006). In the same study, no embryonic lethality was observed when late-stage embryos, composed of non-cycling cells, were irradiated with doses up to 140 Gy, even in NHEJ (non-homologous end joining) or HR (homologous recombination) deficient mutant strains. Consistent with these results, we did not observe any lethality or significant effect on the nematodes fertility at much lower doses of exposure, since the total number of offspring showed only a minor and non-significant decrease at doses higher than 2.9 Gy (dose-rate of 40 mGy·h^{-1}) (Fig. 8b). This result showed that nematodes parentally exposed were either able to ameliorate the observed genotoxic effect, or that the doses adopted in our study were not sufficient to induce any impairment during the development of the somatic gonads.

In contrast, parental irradiation was able to induce a clear dose-dependent reduction in terms of somatic growth of the offspring (Fig. 8a), with nematodes being significantly smaller already at the lowest dose of exposure (0.03 Gy, dose-rate of 0.4 mGy·h^{-1}). Although we did not assess DNA damage in somatic cells any further during the nematodes' development, the combination of somatic growth impairment with the high levels of genotoxicity seen in embryonic cells (Figs. 7b,c, S.7) demonstrates the remarkable tolerance of these embryos, but implies a considerable related cost to repair this damage. HR is known to provide error free DSB repair, but this repair mechanism is only active when the sister chromatid template is available, *i.e.* in proliferating somatic cells and germ cells at all embryonic stages (Clejan et al., 2006). In contrast, non-proliferating somatic cells arrest in G1 and perform NHEJ, which is the major pathway for repair of radiation-induced DNA damage in quiescent somatic cells of *C. elegans* embryos, but is an error prone mechanism. Indeed, a mis-segregation of chromosome fragments was found by Clejan et al. (2006) to be the likely trigger for the somatic developmental abnormalities displayed in irradiated late-stage NHEJ mutant embryos.

Thus, parental irradiation of nematodes impairs the somatic growth of embryos significantly, while the negative effects on reproductive performance are less severe. This is probably a result of the different activity of these DNA repair pathways on a mixed population of replicating and quiescent cells that rely on HR and NHEJ.

5. Conclusions

Sensitivity to ionizing gamma radiation in *C. elegans* is highly dependent on life stage. The post-mitotic adult nematodes tolerate both acute and high dose chronic irradiation without adverse effects. In contrast, L1-L4 developmental stages are highly sensitive to gamma radiation induced reprotoxic effects. At the mechanistic level, gamma irradiation induced genotoxic insult, germ cell apoptosis and reduced spermatids production. The decrease in spermatids production was identified as the major cause of the reduced fertility. Parental exposure leads to DNA damage in developing embryos. Surprisingly, these progeny were able to maintain a high reproductive capacity, despite reduced somatic growth.

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CRediT authorship contribution statement

Erica Maremonti: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Dag M. Eide:** Data curation, Formal analysis. **Deborah H. Oughton:** Conceptualization. **Brit Salbu:** Resources. **Fabian Grammes:** Data curation. **Yetneberk A. Kassaye:** Methodology. **Rémi Guédon:** Methodology, Data curation. **Catherine Lecomte-Pradine:** Methodology. **Dag Anders Brede:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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