In vivo repair of alkylating and oxidative DNA damage in the mitochondrial and nuclear genomes of wild-type and glycosylase-deficient *Caenorhabditis elegans*

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Abstract

Base excision repair (BER) is an evolutionarily conserved DNA repair pathway that is critical for repair of many of the most common types of DNA damage generated both by endogenous metabolic pathways and exposure to exogenous stressors such as pollutants. *C. elegans* is an increasingly important model organism for the study of DNA damage-related processes including DNA repair, genotoxicity, and apoptosis, but BER is not well understood in this organism, and has not previously been measured *in vivo*. We report robust BER in the nuclear genome and slightly slower damage removal from the mitochondrial genome; in both cases the removal rates are comparable to those observed in mammals. However we could detect no deficiency in BER in the *nth-1* strain, which carries a deletion in the only glycosylase yet described in *C. elegans* that repairs oxidative DNA damage. We also failed to detect increased lethality or growth inhibition in *nth-1* nematodes after exposure to oxidative or alkylating damage, suggesting the existence of at least one additional as-yet undetected glycosylase.

Keywords

Caenorhabditis elegans; base excision repair; *nth-1*; hydrogen peroxide; methylmethane sulfonate

1. INTRODUCTION

Nuclear and mitochondrial DNA are constantly exposed to endogenous and exogenous agents that induce DNA damage, which if not repaired can block transcription and replication or result in mutations including point changes, deletions, and chromosomal rearrangements. Base excision repair (BER) is the primary mechanism for the removal of small and common types of DNA damage including oxidative damage, bases with small alkyl adducts, single strand breaks, deaminated bases, and abasic sites. This is a critical pathway because a large amount of such damage is generated endogenously. The amount of damage can be increased by exposure to xenobiotics, and faulty repair of such damage has been linked to cancer and aging [1–4].

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BER repairs damage to both the nuclear and mitochondrial genomes. The initial step of BER involves the recognition and removal of a damaged or misincorporated base by DNA glycosylases. Base removal from the DNA strand generates an abasic site which serves as a substrate for an AP endonuclease. In the second step of BER, the AP endonuclease cleaves the phosphate backbone next to the abasic site, creating a single strand break. The remaining abasic sugar is excised by a deoxyribo-phosphodiesterase (dRPase). This cleavage and excision leaves a single nucleotide gap that is filled in by a polymerase and the remaining nick is sealed by a DNA ligase. BER operates similarly in the mitochondria, largely via alternatively-targeted BER proteins and in part via mitochondria-specific proteins [5–7]. Mitochondria and nuclear DNA also have a distinct long patch BER pathway that involves the insertion of a short nucleotide sequence rather than a single nucleotide (short-patch BER) extending from the incision site [8].

The multicellular nematode species *Caenorhabditis elegans* (*C. elegans*) is emerging as a powerful model system for the investigation of the DNA damage response, including mutagenesis [9–11], cell cycle arrest and apoptosis [12, 13], and DNA repair [14–17]. However, there are relatively few publications on BER in this organism. Two glycosylases [18–20] and two endonucleases [21, 22] have been identified, and *in vitro* biochemical analyses have revealed BER activities [20, 23] but a direct analysis of the *in vivo* rate of removal of DNA damage by BER has not been reported in either the nuclear or mitochondrial genomes. Interestingly, a mutant strain (RB877) carrying a deletion in the only identified *C. elegans* glycosylase that would handle oxidative DNA damage, *nth-1*, has had a detectable phenotype in two studies [24, 25] but no phenotype in two other studies [20, 26]. Human NTH-1 is responsible for carrying out the initial recognition step in BER and subsequently excises oxidized pyrimidine bases. Repair *per se* has never been measured in the *nth-1*-deficient *C. elegans* mutant.

To further characterize the critical BER pathway in *C. elegans*, we quantified removal of damage generated by oxidative and alkylating agents in both genomes, in wild-type as well as the *nth-1* mutant strain of *C. elegans*. We also measured the relative sensitivity of the two strains to oxidative and alkylating agents. Using a sensitive and genome-specific QPCR assay, we report robust removal of both types of damage in the nuclear genome and slightly slower removal from the mitochondrial genome, but no detectable difference in removal in the *nth-1* strain compared to wild-type. We also failed to detect a difference in lethality or growth between the strains after exposure to oxidative or alkylating damage.

2. MATERIALS AND METHODS

2.1 C. elegans strains and culture

C. elegans were cultured essentially as described [26]. Briefly, nematodes were subjected to age-synchronization via NaOH-bleach egg isolation and overnight hatch in K^+ medium. *C. elegans* were otherwise cultured in petri dishes on K-agar seeded with OP50 strain *Escherichia coli* or in 96-well plates containing K^+ medium with OP50 food (details for specific experiments provided below). All experiments were carried out at 20° C. In addition to wild-type (N2 Bristol), we used the mutant strain RB877 that carries a 793 bp deletion in the *nth-1* gene, affecting three exons. We outcrossed this strain 3 times with N2 nematodes. Genotyping information, verifying the presence of the *nth-1* deletion, is presented in Supplemental Data File 1. N2 and *nth-1* nematodes were obtained from the *Caenorhabditis* Genetics Center (CGC; Minneapolis, MN, USA).

2.2 Lethality assays

In order to establish a dose response for each chemical, 50–100 age-synchronized L1 worms of both strains were exposed for one hour to varying concentrations of hydrogen peroxide

(H_2O_2) or methyl methanesulfonate (MMS) in 400 μL K medium in 48 well plates, rinsed 3 times in 2 mL K medium, then placed on 60 mm K agar plates seeded with OP50 and observed immediately and every 24 h thereafter for three days. The MMS was dissolved in DMSO. The DMSO concentration in the dosing solutions was <1% in all cases; we previously found that 1% DMSO does not inhibit growth or reproduction in *C. elegans* [27]. Experiments with ~300 nematodes per well resulted in indistinguishable results, indicating that the number of nematodes used was not so high as to deplete the dosing agent (data not shown). At each observation, worms on each plate were scored as unhealthy if they were non-motile unless prodded with a probe, and were considered dead if tapping failed to induce movement. Nematodes showing normal spontaneous movement were scored as healthy.

2.3 Growth assays

In order to test for a possible strain effect on growth in the presence of each chemical, age-synchronized, L1-staged N2 and *nth-1* nematode strains were added to 1.7 ml microcentrifuge tubes and exposed for 1 hour at the following concentrations of chemical in K medium: 1.5 mM H_2O_2 or 2.5 mM MMS. K medium alone was used as a control. After 1 h, the nematodes were centrifuged and the dosing solution was removed and replaced with K medium; this rinse was carried out three times before transferring the nematodes to 96-well plates containing K^+ medium and OP50 bacteria. 100 nematodes were added to each well in 100 μL K^+. uvrA (DNA repair-deficient: [28]) bacteria were added and the extinction coefficient and time of flight were measured at 24, 48, and 72 h using the COPAS BioSort (Union Biometrica), as previously described [29]. The measured extinction coefficient and time of flight values are proxies for nematode size.

2.4 Measurement of DNA damage and repair

Worms were exposed to chemicals as in the growth assay, using 5mM MMS or 5mM H_2O_2, except that exposures were in 200 μL in 96-well plates. After rinsing, the worms were placed on 60 mm K agar plates seeded with OP50. As soon as the plates were dry, worms were picked into PCR tubes containing lysis buffer at a ratio of 1 nematode to 15 μl of buffer, with triplicate tubes for the controls and duplicate tubes for the treatments for each strain. The tubes were then stored at –80°C. Picking was repeated at 3, 6, and 24 h post-treatment. DNA damage and repair were measured using a quantitative PCR (QPCR)-based assay in which we employed previously-described primers to amplify long and short portions of the mitochondrial and nuclear genomes [30]. The *unc-22* target was used as a long nuclear amplicon. This assay works on the principle that DNA damage inhibits the progression of the polymerase used in the QPCR reaction [31, 32]. Measurements of mtDNA amplification were normalized to mtDNA copy number before damage levels were calculated to ensure that amplification was not confounded by biogenesis or degradation of mitochondrial genomes.

2.5 Measurement of mitochondrial DNA copy number

Absolute mitochondrial copy number was measured using aliquots from samples collected for the measurement of DNA damage and repair (Section 2.4). Quantitative, real-time PCR measuring short nuclear and mitochondrial DNA amplicons was carried out as described in Bratic et al [33]. A single real-time PCR run produced three technical replicates for each nematode sample. The resulting values were averaged and the absolute mitochondrial copy number data obtained was normalized to the corresponding nuclear DNA copy number. The relative mitochondrial copy number of each sample was determined compared to the 0h time point.

2.6 Statistical analysis

All data were analyzed with Statview for Windows (Version 5.0.1, SAS Institute Inc., Cary, NC). DNA damage data were analyzed via multifactorial ANOVA. Initial four-factor ANOVA (chemical, strain, genome, and time point) of all DNA damage data showed main effects for time, treatment, and genome but not strain ($p < 0.0001$, $p < 0.0001$, $p = 0.01$, and $p = 0.73$, respectively). Significant interaction terms were time * treatment ($p < 0.0001$), treatment * genome ($p < 0.0001$), and time * treatment * genome ($p = 0.008$). Subsequent analyses described in Results were carried out using lower-order ANOVA or Fisher's Protected Least Significant Differences (FPLSD) post hoc test. Growth data were not normally distributed (as assessed by the Kolmogorov-Smirnov Normality test) and so were analyzed using Mann-Whitney U or Kruskal-Wallis tests with Bonferroni corrections for multiple tests (chemical and time: H_2O_2 and MMS results were analyzed separately, as were the different timepoints). p values > 0.05 were considered insignificant. Box plots indicate 10th, 25th, 50th, 75th, and 90th percentiles, plus outliers.

3. RESULTS

3.1 Toxicity and Lethality of MMS and H_2O_2

To identify appropriate doses of MMS and H_2O_2 for our DNA damage and repair studies, we first treated the N2 (wild-type) *C. elegans* with increasing concentrations of each toxicant and monitored the nematodes for viability and lethality (Fig. S1). This experiment was replicated five times, with between 36 and 144 nematodes counted per dose and per chemical at 0, 24, 48, and 72 h post-exposure. As molecular damage is expected to precede and produce observable phenotypic changes, and because we did not want DNA repair to be inhibited by frank organismal toxicity, we chose concentrations for each toxicant that resulted in a mild phenotypic response in order to study DNA repair.

3.2 Characterization of BER in Wild-Type C. elegans

Nuclear (nucDNA) and mitochondrial DNA (mtDNA) damage was quantified using QPCR following exposure of N2 nematodes to 5mM MMS or 5mM H_2O_2. The QPCR assay allows the simultaneous measurement of DNA damage in the mitochondrial and nuclear genomes after *in vivo* exposures, without differential extraction of mitochondrial and nuclear DNA [32]. Repair can be measured by ending the chemical exposure and permitting time for removal of damage [31].

In agreement with previous studies, we observed more H_2O_2-induced damage in mtDNA than nucDNA (Fig. 1 and 2; $p < 0.0001$ for treatment by genome interaction). Immediately after treatment, we measured 1.4 lesions/10 kb in mtDNA, as compared to 0.7 lesions/10 kb in the nuclear genome. On the other hand, treating with MMS resulted in greater nucDNA damage (1.7 lesions/10 kb) than mtDNA damage (0.7 lesions/10 kb).

We measured DNA repair in the nuclear and mitochondrial genomes following acute exposure to MMS and H_2O_2. We treated age-synchronized L1 nematodes with MMS and H_2O_2 in the absence of food. Following treatment, *C. elegans* were placed on plates with bacterial food and picked for analysis immediately after the exposure (0 h) and 3, 6, or 24h post-exposure to permit repair. Results reflect at least three experiments replicated in time for each strain and treatment group, with n = 2–3 tubes per experiment for a total n = 6–11 per strain per dose per time point.

Little to no repair of nuclear or mitochondrial DNA damage was observed 3h after exposure ($p > 0.05$ by FPLSD for all 0 to 3 h comparisons for both chemicals and both genomes). In the nuclear genome, repair of MMS-induced damage was detectable after 6 h, resulting in

removal of 22% of the accumulated damage (Fig. 1; p = 0.008, FPLSD). H_2O_2-induced lesions were reduced by 50% after 6 h (p = 0.03, FPLSD). By 24h the levels of H_2O_2-induced nucDNA damage were comparable to baseline levels (97% reduction in lesions) and those of MMS were reduced by 60 percent (p < 0.0001 for 24 vs. 0h in both cases).

As opposed to nucDNA, repair of the lesions induced by MMS and H_2O_2 in the mitochondrial genome was not detectable 6h after exposure (Fig. 2; p = 0.18 for both H_2O_2 and MMS, 0 vs. 6h). However, we measured significant repair of MMS and H_2O_2-induced mtDNA damage 24 h after treatment (78 and 75% repair, respectively, and p < 0.0001 for both). Thus, *C. elegans* has detectable BER in both genomes, but repair is more efficient in the nuclear genome, at least for removal of oxidative and alkylating damage.

3.3 Characterization of BER in nth-1 mutant C. elegans

To further characterize the genetic components of BER in *C. elegans*, we utilized the RB877 strain that carries a mutation in the *nth-1* gene in which exons 2 through 4 have been deleted. The glycosylase NTH-1 is responsible for locating damaged bases and carrying out the initial step of BER [20], and the *nth-1* strain accumulates mutations in the nuclear genome [25]. The *nth-1* gene product has a 0.60 likelihood of mitochondrial localization based on the full-length protein sequence reported by Morinaga et al. [20]. Therefore, we expected to see an abrogation of nuclear and mitochondrial BER in *nth-1* nematodes. We also hypothesized that we would observe an elevated level of lesions either before or immediately after treatment, due to accumulation of damage caused by endogenous processes. However, despite the elevated levels of mutations reported in the *nth-1* genome, the quantity of lesions measured in unexposed nematodes or following acute exposure of the *C. elegans nth-1* strain to the genotoxic stress was similar to that of wild-type N2 *C. elegans* (Figs. 1 and 2) (p > 0.05 for main effect of strain and all interaction terms including "strain" as an independent factor).

Surprisingly, DNA repair in the *nth-1* nematodes was also statistically indistinguishable from that of the wild-type N2 strain (Figs. 1 and 2) (p > 0.05 for all N2 vs. *nth-1* comparisons). Lesion frequencies decreased significantly in both nuclear and mitochondrial genomes. The apparent trend of slower repair of H_2O_2-induced nucDNA damage in *nth-1* nematodes as compared to similarly treated N2 *C. elegans* (Fig. 1) was not statistically significant. Thus, the *nth-1* deficiency did not detectably reduce repair in either genome.

We measured the mitochondrial copy number in nematode samples obtained for the detection of DNA damage and repair. This measurement is important to the measurement of damage itself due to the need for normalization to mtDNA copy number. However, we also considered the possibility that mtDNA copy number would change due to either biogenesis or degradation of damaged genomes. However, as indicated in Figure 3 the mtDNA copy number values remain relatively stable throughout the duration of the repair time course (p > 0.05 for all main and interactive effects).

3.4 Effect of nth-1 deficiency on viability and growth after exposure to DNA damaging agents

To test whether *nth-1*-deficient nematodes would show a phenotypic sensitivity to DNA damaging agents, we analyzed the viability of the *nth-1* strain following exposure to MMS and H_2O_2, as we did for N2 nematodes (Fig. S1). The viability of the *nth-1* strain following toxicant exposure was indistinguishable from that of N2s (Fig. S2). Reasoning that a mild repair deficiency might manifest as an alteration in a sublethal rather than lethal phenotype, we then compared growth in N2 and *nth-1* nematodes following MMS and H_2O_2 exposure. We measured larval growth after two and three days (at which point the nematodes had

reached adulthood), to allow any biological effects of unrepaired damage to integrate over time. Nematodes were treated with low amounts of toxicant, designed to produce mild growth inhibition such that potential exacerbation of that inhibition due to the *nth-1* deletion would be detectable. After exposure, nematodes were placed on food plates so as to resume their development, and growth was quantified by measuring size daily. Results reflect three or four distinct experiments with 50 to 100 individuals measured per strain and treatment group each day (approximately 2700 nematodes measured total). The deficiency of *nth-1* in *C. elegans* did not affect growth following treatment of MMS or H_2O_2 (Fig. 4; $p > 0.05$ for all comparisons). Thus, although exposure to MMS and H_2O_2 measurably stunted the growth of nematodes, *nth-1* deficient *C. elegans* did not show an additional sensitivity to oxidative stress or alkylation damage.

4. DISCUSSION

4.1 C. elegans has robust BER capacity in vivo

We challenged *C. elegans* with exposure to the oxidative DNA damaging agent H_2O_2 and the DNA alkylating agent MMS. BER is the primary mechanism in both nuclear and mitochondrial DNA for the removal of both oxidative lesions and small alkyl DNA adducts. We observed robust removal of such damage in both the nuclear and mitochondrial genomes. Thus, BER, like NER [31, 34], appears to be functionally (as determined by rate of damage repair) conserved between *C. elegans* and higher eukaryotes. The rates of removal of H_2O_2- and MMS-induced nuclear and mitochondrial DNA damage are roughly comparable to those reported for oxidative-induced damage in post-mitotic mammalian cells in culture [35]. Similar to our observations of the *C. elegans* nuclear genome, previous studies have shown that human nuclear DNA displays rapid removal of oxidative lesions. Additionally, analogous to human mtDNA, the *C. elegans* mitochondrial genome shows less efficient repair of oxidative stress-induced damage compared to nucDNA. Mitochondria are constantly challenged by superoxide radicals released during oxidative phosphorylation and repair of mitochondrial DNA damage is increasingly recognized as being particularly important [36, 37].

4.2 Differential sensitivities of the nuclear and mitochondrial genomes

We observed more mtDNA than nucDNA damage after H_2O_2 exposure, as has been observed in other systems [38]. It is interesting to note that MMS caused more nucDNA than mtDNA damage; this same pattern was observed previously in cell culture [39]. Thus, while many genotoxins cause more mtDNA than nucDNA damage, this pattern is not universal.

4.3 The role of nth-1 in C. elegans BER

The relative importance of the *nth-1* gene in BER in *C. elegans* is not clear. On the one hand, it has been shown biochemically to have glycosylase activity [20], and the RB877 strain with a deletion in the gene is somewhat mutation-prone [25] and shows transcriptional evidence of genotoxicity [24]. On the other hand, we [26] and others [20] detected no genotoxicant sensitivity phenotype, and we now report that we could not detect any difference in BER in *nth-1* deficient nematodes compared to wild-type. If NTH-1 were the sole glycosylase responsible for BER then the loss-of-function mutation in *nth-1* nematodes would be expected to inhibit repair. Thus, perhaps the most likely possibility is that there is another as-yet unidentified glycosylase present in *C. elegans* that complements the activity of NTH-1. This is commonly the case in bacteria [40, 41], yeast [42, 43] and mammals [44, 45] and Morinaga et al. presented evidence for such activity in *C. elegans* [20].

In a previous study, mouse models lacking functional NTH-1 show neither phenotypic nor life span abnormalities; nor did they display enhanced sensitivity following exposure to oxidizing agents such as H_2O_2 due to redundancy in substrate specificity provided by thymine glycol glycosylases (TGG1 and TGG2) which putatively replace NTH-1 [44]. Additionally, bacterial NEI and their human homologs (NEIL 1 and NEIL 2) have been shown to excise oxidized pyrimidine bases [46]. In previous studies, *nei* and *nth* double mutant *E. coli* strains demonstrated extreme sensitivity to exogenous oxidative damage while cells lacking either single enzyme activity were similar to wild type [41]. Although NEI serves as a complementary glycosylase to NTH-1 in *E. coli* and mammalian cells, a homolog of NEI in C. elegans was not detected by BLAST search [20]. To date, only one nth homologue has been identified in *C. elegans*. Further studies need to be carried out to identify other potential *C. elegans* glycosylases that may be functioning in BER in NTH-1 deficient nematodes.

It has also been suggested that NER may serve as an important back-up pathway for BER in this species [24, 47]. In support of this theory, BER-deficient yeast cells only show partial sensitivity when exposed to the alkylating agent MMS while cells deficient in both the BER and NER pathways are severely compromised [48]. However, compensation by NER would not explain the robust removal of oxidative and alkylating damage in mtDNA, as NER is not operative in mitochondria [14, 22, 31]. Autophagy-mediated removal of mtDNA damage could play a role, but the kinetics of that process appear to be slower than what we observed here [49]. Alternatively, *C. elegans* appears to lack any homologue to DNA polymerase β, the polymerase required for the gap-filling step during BER in humans [23]. Additionally, *C. elegans* has been shown to express DNA polymerase θ, a polymerase with lesion bypass activity [50, 51]. Pol θ is also active in the repair of oxidative damage in vertebrate cells, having redundant function with pol β in BER [52]. It is possible that pol θ is similarly involved in BER in *C. elegans*. The absence of polymerase β along with the lack of the requirement for NTH-1 suggests that *C. elegans* may have an alternative molecular pathway involving pol θ that mediates the cellular response to oxidative and alkylating DNA damage.

5. CONCLUSIONS

Ours is the first study to directly measure BER in *C. elegans*. While we have provided evidence for general similarity for BER processes in *C. elegans* and higher eukaryotes, much remains to be discovered. For example, is BER developmentally regulated, as transcriptional profiling [30]would suggest? What is the DNA polymerase involved in BER in *C. elegans*, since it appears to lack DNA polymerase β [23]? What is the full complement of glycosylases in this species? Ultimately, the complete BER system needs to be elucidated for *C. elegans*. The ability to confidently extrapolate DNA damage-related results obtained in this model organism to humans awaits a fuller understanding of this organism's response to endogenous and exogenous genotoxins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We measured repair of oxidative and alkylating DNA damage *in vivo* in the mitochondrial and nuclear genomes of *C. elegans*.

- Base excision repair of both types of damage in both genomes was comparable to what has been observed in mammals.

- H_2O_2 caused more mitochondrial than nuclear DNA damage, but the reverse occurred with methylmethanesulfonate (MMS).

- A deletion in the *nth-1* glycosylase gene did not reduce DNA repair or sensitize *C. elegans* to exposure to H_2O_2 or MMS.
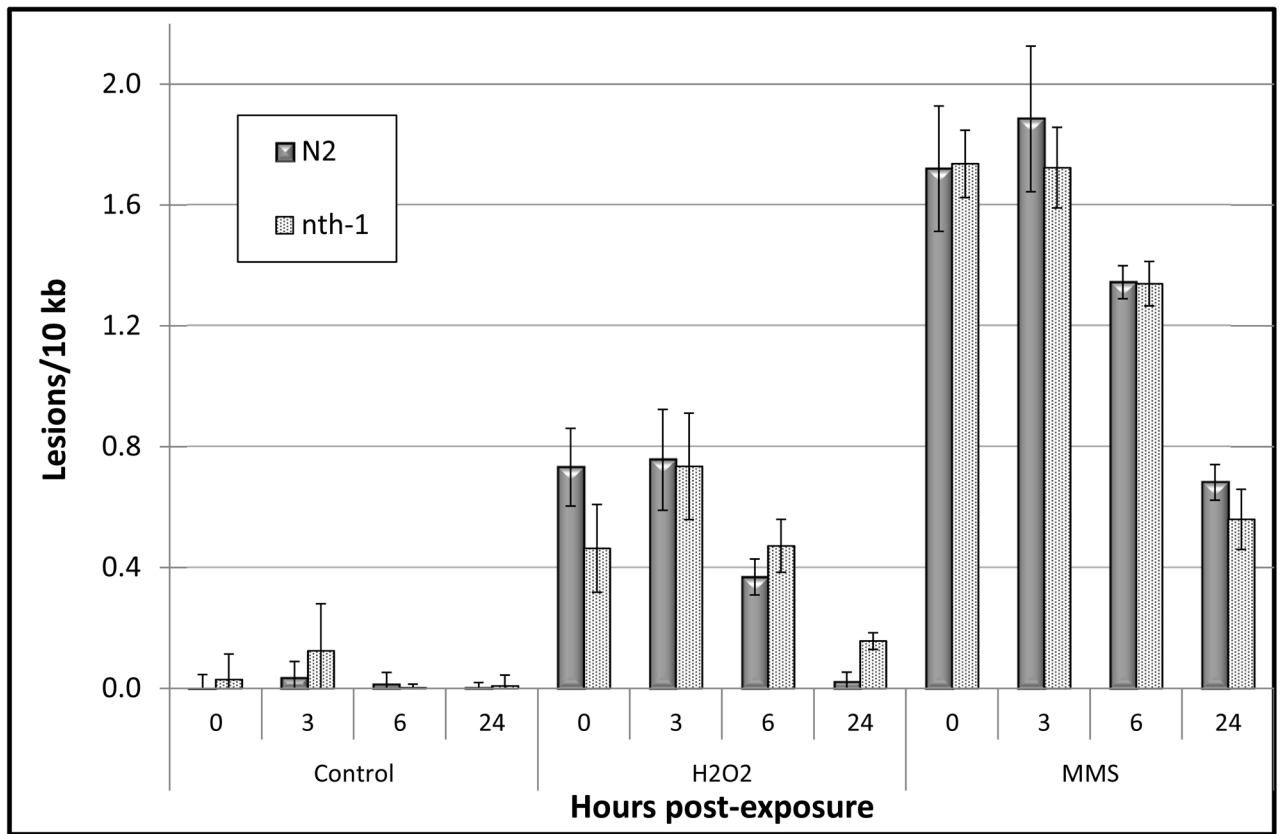
Figure 1.
N2 and *nth-1 C. elegans* show robust nuclear DNA repair following damage induced by 5mM H_2O_2 or 5 mM MMS. Nuclear DNA damage was analyzed following acute exposure of L1 larvae to H_2O_2 and MMS (0h). Subsequent repair was measured 3, 6, or 24h post-exposure. n = 6–11 nematodes per toxin per time point. Error bars represent the standard error of the mean.
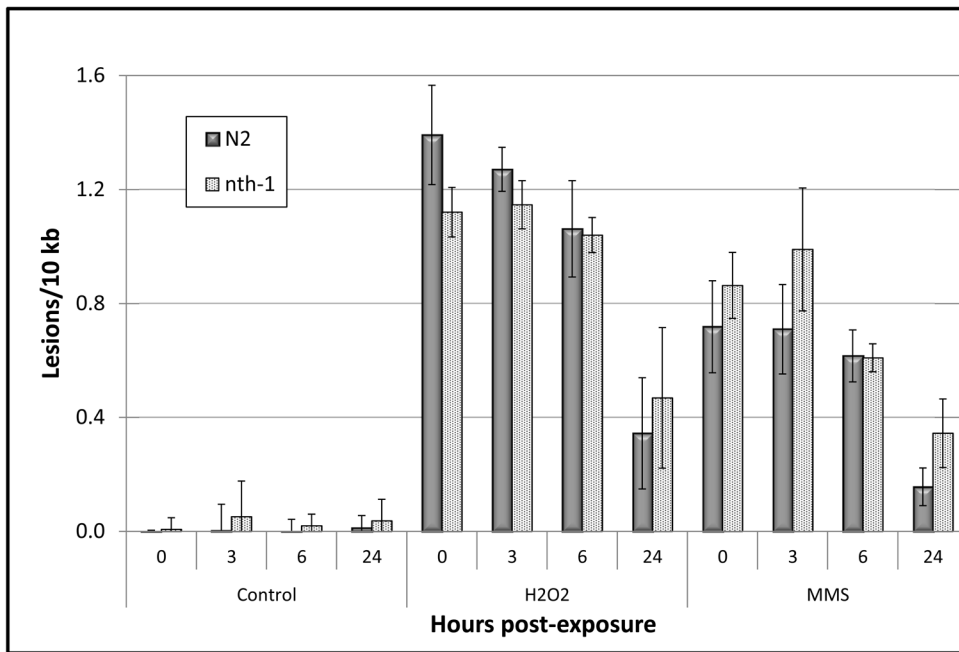
Figure 2.
N2 and *nth-1 C. elegans* show efficient repair of H_2O_2 and MMS-induced mitochondrial DNA damage. Mitochondrial DNA damage was measured in N2 (wild-type) and *nth-1 C. elegans* using QPCR following exposure of L1 larvae to 5mM H_2O_2 or 5mM MMS (time 0h). Repair of mitochondrial DNA was analyzed 3, 6, or 24h post-exposure. n = 6–11 nematodes per toxin per time point. Error bars represent the standard error of the mean.
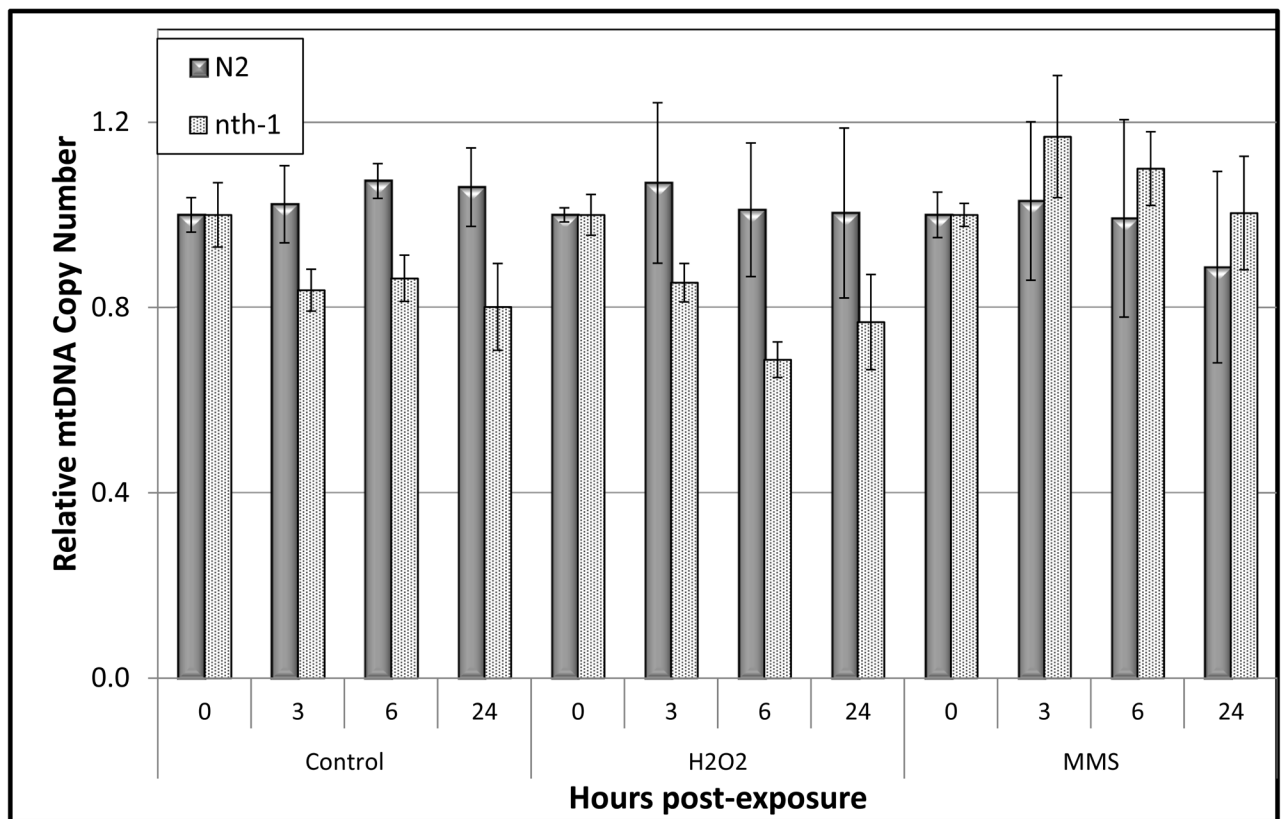
Figure 3.
mtDNA copy number is relatively stable over 24h in N2 and *nth-1 C. elegans* strains exposed to 5mM H_2O_2 or 5mM MMS. Mitochondrial DNA copy number was measured in N2 (wild-type) and *nth-1 C. elegans* using quantitative, real-time PCR following exposure of L1 larvae to H_2O_2 and MMS. Data represents the relative copy number in samples analyzed 3, 6, or 24h post-exposure as compared to the 0h time point. n = 6–11 nematodes per toxin per time point. Error bars represent the standard error of the mean.
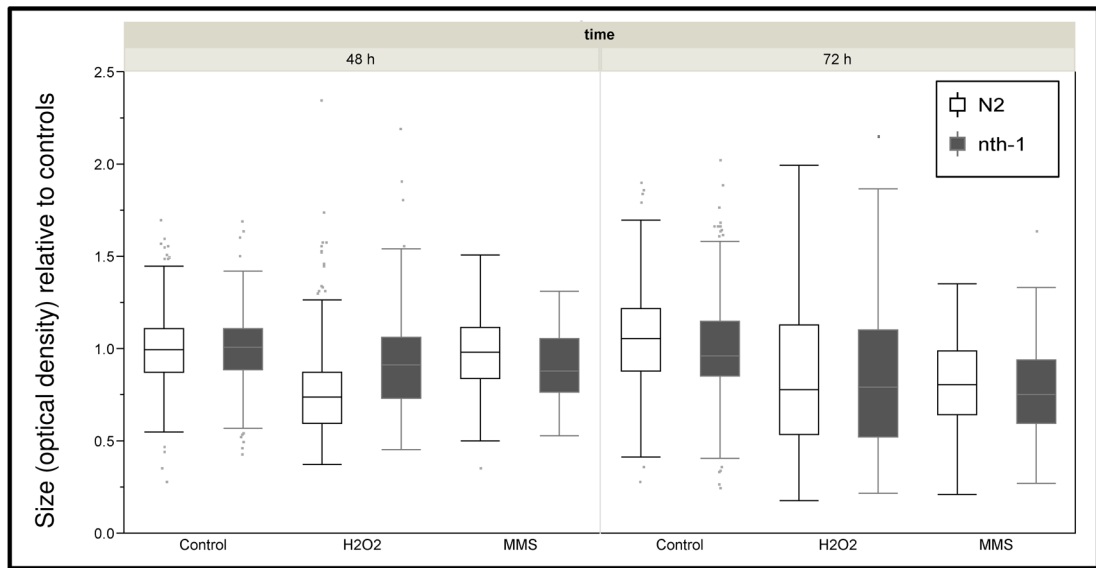
Figure 4.
Growth inhibition by hydrogen peroxide (1.5 mM) and MMS (2.5 mM) is indistinguishable in the N2 and *nth-1* strains. n = 74–334 nematodes per time point per strain per chemical; results include four separate (pooled) experiments.