



Histone methylation-associated transgenerational inheritance of reproductive defects in *Caenorhabditis elegans* exposed to crude oil under various exposure scenarios



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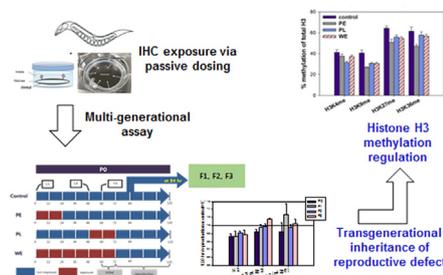
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HIGHLIGHTS

- Transgenerational toxicity of Iranian heavy crude oil (IHC) was investigated in the nematode *Caenorhabditis elegans* in the four consecutive generations under different exposure scenarios.
- *C. elegans* reproduction potential was inhibited by the IHC in the unexposed generations and in the exposed parental generation.
- Whole-life exposure condition exhibited transgenerational inheritance of defective reproduction.
- Decreased methylation of histone H3K9 was found in the exposed generation; however, a heritable diminution in reproduction did not occur in the H3K9 histone methyltransferase defective mutant.
- Reproductive toxicity caused by IHC exposure was found to be transmitted to subsequent unexposed generations, and methylation of histone H3K9 seems to be involved in it.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 21 October 2017

Received in revised form

11 February 2018

Accepted 13 February 2018

Available online 21 February 2018

Handling Editor: A. Gies

ABSTRACT

As part of a study to explore the long-term effects of the Hebei Spirit oil spill accident, transgenerational toxicity and associated epigenetic changes were investigated in the nematode *Caenorhabditis elegans*. Under experimental conditions, worms were exposed to Iranian heavy crude oil (IHC) under three different scenarios: partial early-life exposure (PE), partial late-life exposure (PL), and whole-life exposure (WE). Growth, reproduction, and histone methylation were monitored in the exposed parental worms (P0) and in three consecutive unexposed offspring generations (F₁₋₃). Reproductive potential in the exposed P0 generation in the WE treatment group was reduced; additionally, it was inhibited in the unexposed offspring generations of the P0 worms. This suggests that there was transgenerational inheritance of defective reproduction. Comparison of developmental periods of exposure showed that IHC-

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Keywords:

Iranian heavy crude oil
Caenorhabditis elegans
Transgenerational effects
Differential exposure
Histone methylation

treated worms in the PL group had a greater reduction in reproductive capacity than those in the PE group. Decreased methylation of histone H3 (H3K9) was found in the IHC-exposed parental generation. A heritable reduction in reproductive capacity occurred in wildtype N2 but was not found in a H3K9 histone methyltransferase (HMT) mutant, *met-2(n4256)*, suggesting a potential role for HMT in transgenerational toxicity. Our results suggest that the reproductive toxicity after IHC exposure could be heritable and that histone methylation is associated with the transmission of the inherited phenotype.

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1. Introduction

Epigenetics, the transgenerational transfer of phenotypic characteristics without modification of gene sequences, is a burgeoning area of study in many disciplines of biology (Lim and Brunet, 2013; Kelly, 2014). Known epigenetic mechanisms include DNA methylation, histone modification, and non-coding RNA regulation (Kelly, 2014). Environmental changes may affect the unexposed progeny of exposed generations, with genes showing aberrant silencing or expression, without an associated gene sequence change, for many generations (Lim and Brunet, 2013; Kelly, 2014). A growing body of scientific evidence indicates that exposure to environmental chemicals may contribute to the deregulation of epigenetic mechanisms (Vandegheuchte et al., 2009a, 2009b; Menzel et al., 2011; Manikkam et al., 2012; Lim and Brunet, 2013; Norouzitallab et al., 2014) with reports of multi- and transgenerational effects of environmental chemicals, such as biocides, pesticides, endocrine-disrupting chemicals, and fuel mixtures, in many species (Peterson et al., 2003; Anway et al., 2005; Titus-Ernstoff et al., 2010; Bruner-Tran and Osteen, 2011; Guerrero-Bosagna and Skinner, 2012). However, the relationship between the overarching concept of epigenetics and interesting transgenerational phenomena that alter the physiological phenotype of subsequent generations remains enigmatic and somewhat ill defined.

The nematode *Caenorhabditis elegans* is a valuable model for studying transgenerational effects because of its short life cycle and ease of maintenance under laboratory conditions. Hence, many studies on multi- and transgenerational toxicities of diverse environmental stresses have been performed in this species (Kim et al., 2013; Tauffenberger and Parker, 2014; Zhao et al., 2014, 2015; Jobson et al., 2015; Liu et al., 2015). In addition, the contribution of epigenetic changes to transgenerational effects has been investigated, and potential molecular mechanisms have been suggested (Lim and Brunet, 2013). In *C. elegans*, histone methylation plays a crucial role in epigenetic memory and chromatin structural alterations (Kelly, 2014). Histone methyltransferases (HMTs) regulate the methylation of lysine in histone H3, and differences in the methylation status of lysine can lead to changes in gene transcription activities (Bassett and Barnett, 2014). HMTs of histone H3 are also reported to play a considerable role in epigenetic memory (Kelly, 2014).

In December 2007, the oil tanker Hebei Spirit spilled about 10,800 tons of oil into the Yellow Sea off Taejeon, South Korea. This historical oil spill accident had both short-term and long-term effects on human and ecological health (Peterson et al., 2003; Zock et al., 2007; Guterman, 2009; Hong et al., 2012; Incardona et al., 2012; Jiang et al., 2012; Noh et al., 2015). However, there have been little studies on whether the oil spill had the potential to cause multi- and/or transgenerational effects on exposed organisms.

This study was initiated to gain insights into the long-term consequences of the Hebei Spirit oil spill by investigating the transgenerational inheritance of toxicity after the exposure of *C. elegans* to Iranian heavy crude oil (IHC) and to analyze the

associated epigenetic changes. IHC was chosen as the model crude oil because it was one of the main components of the Hebei Spirit oil spill (Yim et al., 2012). *C. elegans* specimens were exposed to IHC by a passive dosing method, in which the dissolved aqueous concentration of hydrophobic hydrocarbons from IHC was maintained at a constant level throughout the experiment. The effects of early-life chemical exposure on health in later life have recently been receiving much attention (Head et al., 2012). In this context, the responses of the worms to exposure during different periods of their life cycle were compared. Transgenerational toxicity was also investigated in the IHC-exposed parental (P0) generation and in their unexposed progeny (F₁₋₃). Finally, to identify the epigenetic mechanism in IHC-induced transgenerational toxicity, methylation of histone H3 was investigated in the *C. elegans* exposed generation (P0). The role of histone H3 methylation in transgenerational toxicity was further investigated by the analysis of *C. elegans* with loss-of-function mutations of genes encoding HMTs and histone demethylase (HDM).

2. Materials and methods

2.1. *C. elegans* maintenance

C. elegans was cultured in Petri dishes on nematode growth medium (NGM) at 20 °C and fed OP50 strain *Escherichia coli* according to a standard protocol (Brenner, 1974). The life cycle of *C. elegans* comprises the embryonic stage, four larval stages (L1–L4), and adulthood. A synchronously developing population of worms was used in the various experiments to eliminate any variation due to age differences. To produce age-synchronized cultures, eggs from mature adults were isolated using 10% hypochlorite solution, followed by rinsing with M9 buffer (4.2 mM Na₂HPO₄, 2.2 mM KH₂PO₄, 86 mM NaCl, and 1 mM MgSO₄·7H₂O). Synchronized L1 stage worms were then allowed to hatch from eggs in S-basal (0.1 M NaCl, 0.05 M KH₂PO₄, pH 6.0) without a food source, resulting in a synchronized population. For multigenerational analyses, *C. elegans* L1 larvae were cultured in complete K-media (0.032 M KCl, 0.051 M NaCl, 1 mM CaCl₂, 1 mM MgSO₄, and 13 mM cholesterol). Wildtype (N2) and mutant strains were provided by the *Caenorhabditis* Genetics Center (MN, USA). A list of the mutant strains employed in this study is presented in Table S1.

2.2. Iranian heavy crude oil (IHC)

The IHC used as the model crude oil in this study was provided by Taejeon Environmental Health Center (Taejeon, Korea). A detailed analysis of the chemical composition of IHC has been reported previously (Yim et al., 2011; Kang et al., 2014). The polydimethylsiloxane (PDMS) tubing used for the passive dosing exposures was purchased from Dong-Bang Silicone Inc. (Seoul, Korea).

2.3. Exposure of *C. elegans* to IHC by a passive dosing method

Passive dosing is a reliable method to ensure exposure to a constant concentration of hydrophobic organic chemicals from mixtures (Kang et al., 2014; Mustajarvi et al., 2017). The sufficiently high dosing phase to water volume ratio used in passive dosing makes it possible to compensate for any losses of hydrophobic organic chemicals due to absorption by worms, sorption to wall surfaces, and potential volatilization. This method has been shown to be robust for maintaining constant exposure conditions for hydrophobic organic chemicals in many previous studies (Smith et al., 2010; Roh et al., 2014; Fischer et al., 2016). Toxic responses in *C. elegans* were observed at the maximum water-soluble concentration of hydrophobic organic chemicals from IHC maintained by a passive dosing method slightly modified from that described by Kang et al. (2014). PDMS tubes (ID = 2 mm, OD = 3 mm) loaded with approximately 0.9 and 0.4 g of IHC were placed in 12- and 6-well plates with 2 and 5 mL of K-media (0.032 M KCl and 0.051 M NaCl), respectively. The volume ratio of IHC to K-media is greater than that of Kang et al. (2014). Thus, it is reasonable to assume that the aqueous concentration of hydrophobic organic chemicals from IHC was maintained at a constant level throughout the tests without chemical analysis. This preparation was maintained for 24 h in the dark to allow sufficient time for a dissolution equilibrium to be established between the K-media and the IHC in the PDMS tube. After equilibration, worms and *E. coli* OP50 were put into each well. The volume of K-media was set to 10 μ L per worm.

2.4. Exposure scenario and multigenerational assay

Three exposure conditions were examined. First, early-development exposure was tested by exposing L1 synchronized worms for 24 h (PE, partial early-life exposure); this treatment schedule results in exposure of L1 and L2 larval stages. Second, later development exposure was tested using worms at 48 h after L1 synchronization; these were exposed for 24 h (PL, partial late-life exposure) and resulted in treatment of L4 and young adult stages. Third, the effect of whole-life cycle exposure was investigated by exposing L1 synchronized worms for 72 h (WE, whole-life cycle exposure). The responses in the PE and PL treatment groups were then compared. The effect of the duration of exposure was also examined by comparing the responses of these groups to that of the WE group. For the multigenerational assay, worms were similarly exposed to IHC; offspring from exposed worms were used as the F₁ generation and maintained in normal *C. elegans* culture media. Development was monitored over three consecutive generations (P0, exposed parental generation, F₁₋₃, unexposed offspring generations). At 72 and 120 h after the beginning of each new generation, the developmental status (growth and reproduction) of the worms was monitored. The schedules for the life cycle-dependent exposure and multigenerational assay are described in Fig. 1.

2.5. Measurement of growth and reproduction using COPAS

C. elegans growth and reproduction assays were conducted using the Complex Object Parametric Analyzer and Sorter (COPASTM SELECT, Union Biometrica, Holliston, MA, USA) by a slight modification of the method described previously by Boyd et al. (2012). The COPAS instrument is based on flow cytometry and automates the analysis, sorting, and dispensing of *C. elegans* using the axial length of the object (time of flight, TOF), optical density (extinction, EXT), and the intensity of fluorescent markers (Pulak, 2006). For the growth assay, 20 L1 stage *C. elegans* were dispensed into each well of a 96-well microtiter plate and were incubated with or without IHC. After incubation, the worms were aspirated from each well

using the REFLX option of the COPAS, and the TOF was measured for individual worms. To analyze the recorded data, a grid was imposed on the log(TOF) range of all observations, dividing the range into a fixed number of equally sized bins (Smith et al., 2009). The grid was chosen so that the resolution of the histograms of observations was sufficiently high to distinguish growth stages. For the reproduction assay, one young adult stage *C. elegans* was dispensed into each well of a 96-well microtiter plate and incubated with or without IHC. After incubation, the number of worms (offspring) in each well was counted. For growth experiment, about 400 worms were used, while for reproduction experiment, about 30 worms were used at each treatment.

2.6. Morphological observation

To investigate whether IHC exposure causes morphological anomalies, the pharynx, germline, vulva, and tail parts of worms in different treatment groups were analyzed at 72 h after exposure to IHC. Worms were treated with 4 mM levamisole (Sigma-Aldrich) and mounted onto cover slips. The morphology of each worm was assessed under an optical microscope (Leica DM2500, Wetzlar, Germany).

2.7. Quantification of histone modification markers

Histone H3 methylation (i.e., H3K4, H3K9, H3K27, and H3K36) was used to determine changes in epigenetic modification in *C. elegans* due to IHC exposure. Approximately 500 age-synchronized young adults from each generation were collected; total histones were extracted from each group using an EpiQuick total histone extraction kit (OP-0006, EpiGentek, Farmingdale, NY, USA). For the assay, modified fractions of histone H3 were detected using specific antibodies against histone H3 with a modified lysine site, and these were then quantified colorimetrically by reading absorbance in a micro-plate spectrophotometer. Two technical replicates were performed for each sample.

2.8. Gene expression

Total RNA was extracted from the treated and control groups using an NucleoSpin RNA extraction kit (Macherey-Nagel GmbH & Co., Düren, Germany), and the quantity and quality of the RNA was assessed using a NanoDrop instrument (ASP-2680, ACTGene, Piscataway, USA). Synthesis of cDNA was carried out by reverse transcription (RT), and PCR amplification was carried out using a thermal cycler (Bio-Rad, Seoul, Korea). Quantitative real-time PCR (qRT-PCR) analysis was performed using a CFX manager (Bio-Rad) with IQTMSYBR Green SuperMix (Bio-Rad Laboratories, Hercules, CA). Expression of HMT (set-2, met-2, set-25, and mes-4) and HDM (*utx-1*) genes was investigated. The primers were constructed using the sequences available at NCBI (Supplementary Table S2). Gene expression was normalized using *pmp-3* as a housekeeping gene.

2.9. Statistical analyses

The significance of differences among and between treatments was tested statistically using a one-way analysis of variance (ANOVA). This was followed by a post-hoc test (Tukey, $p < 0.05$). All statistical analyses were carried out using IBM SPSS 20.0 (SPSS Inc.), and graphs were prepared in SigmaPlot (Version 12.0).

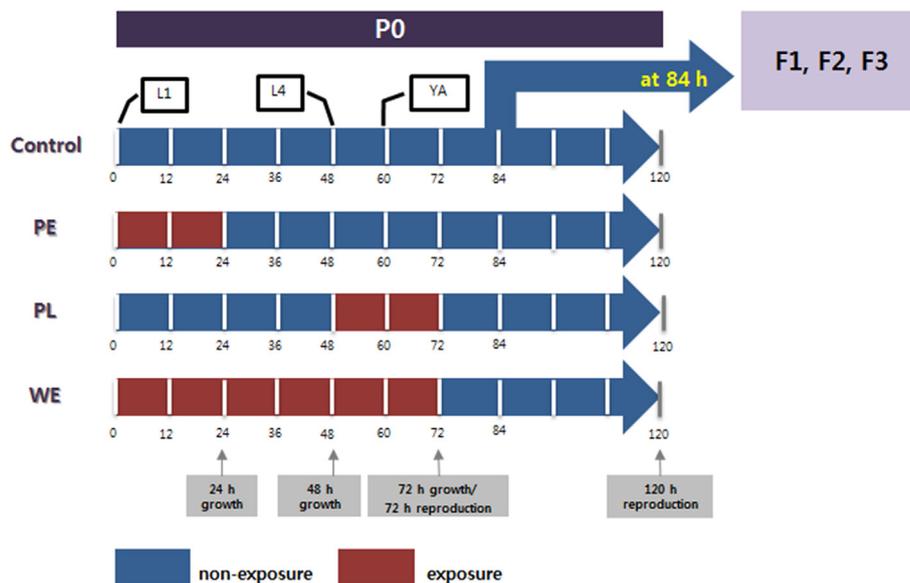


Fig. 1. Exposure schedule for the multigenerational assay of *C. elegans* exposed to IHC; only the P0 generation was exposed to IHC. The passive dosing method was used to treat the animals for three different periods: PE, partial early-life exposure (24 h exposure from L1); PL, partial late-life exposure (24 h exposure from L4); WE, whole-life exposure (72 h exposure). L1 worms were selected from the previous generation after 84 h using COPAS and were cultured in normal media for multigenerational assay. Growth and early reproduction were measured at 72 h when synchronized L1 growth begins, and full reproduction was quantified at 120 h in each generation.

3. Results and discussion

3.1. Effect of different exposure conditions on *C. elegans* development

The development of worms in control and IHC exposure groups was assessed (Fig. 2). In the PE and PL groups, the development was delayed in IHC-exposed worms compared to that in controls; this effect was greater in the WE group (Fig. 2A–B). At the 72-h sampling interval, reproductive capacity was significantly reduced by IHC exposure. Reproductive failure at 72 h was more pronounced in the PE treatment group than those in the PL and WE groups (Fig. 2C). This result suggests that *C. elegans* may be more vulnerable to IHC exposure at an early life stage than later in life. Our results confirmed differential sensitivity to IHC with respect to developmental stages (PE vs. PL) and exposure duration (PE, PL vs. WE). Defects such as reproductive failure are often linked to abnormalities in related organs. To investigate whether this was the case here, the morphologies of the pharynx, germline, vulva, and tail parts of worms were analyzed at 72 h (Fig. S1). Microscopic analyses revealed that worms from all treatment groups were morphologically similar to untreated controls, except for reproductive capacity, for which worms in the WE group did not have any eggs. This result suggests that delayed or defective egg formation may occur because of the exposure to IHC and result in a decreased rate of reproduction (Fig. 2C).

3.2. Transgenerational toxicity of IHC in *C. elegans*

Next, we investigated whether differential sensitivities to IHC, depending on exposure time and duration, cause any effect on unexposed progeny. A multigenerational assay showed that exposure to IHC affected *C. elegans* reproductive capacity beyond the exposed generation. A statistically significant decrease in growth (~28%) was observed in exposed worms (P0) in the WE treatment group but not in either the PE or PL groups. However, this effect was reversed in the subsequent unexposed F_{1–3} generations (Fig. 3A). This suggests that the effect of IHC exposure on growth was not

transmissible to the next generation in *C. elegans*.

A significant decrease in reproductive potential (40–65%) was observed in the P0 worms exposed to the IHC in all three treatment groups, especially in the PL and WE groups. Similarly, F₁ progeny from all three treatment groups showed an approximately 40% decrease in reproductive capacity; this effect was not observed in the F₂ and F₃ generations (Fig. 3B). To investigate this effect further, the numbers of offspring were counted at 72, 84, 96, and 120 h in the three treatment groups in the P0 and F_{1–3} generations (Fig. S2). The effect of exposure period and duration was clearly seen at 120 h in the P0 generation (Fig. S2A); however, in the F₁ generation, the differences among treatment groups were less evident than in the P0 generation (Fig. S2B). In the F₂ generation, reproductive toxicity was rescued in the PL treatment group, which was not observed in PE and WE groups (Fig. S2C). No effects were seen in progeny in the F₃ generation of the PE and PL treatment groups; however, an approximately 40% lower reproductive capacity was observed in the F₃ progeny in the WE group compared to that in the control (Fig. S2D).

Our analyses demonstrated that reproduction was more significantly affected than growth after exposure to IHC (Fig. 3). The decrease in reproductive capacity was more significant in the PL than in the PE group (Fig. S2), suggesting the possibility of a delayed reproductive development effect of IHC exposure on *C. elegans*. The most interesting result was that the reduced reproductive capacity was present until the F₂ generation in the PE and WE groups and remained until the F₃ generation in the WE group (Fig. S2D). The reduction in reproductive capacity in the F₁ generation seems to be due to parental effects, while that in the F₂ is probably due to the impairment of germ cell development in the F₁ generation of exposed P0 worms. The change in the F₃ generation of the WE group can be considered a true transgenerational effect, indicating that IHC exposure can lead to heritable retardation of reproductive development in *C. elegans* following the whole-life exposure in the P0 generation.

In *C. elegans*, transgenerational studies have mainly been conducted on dietary and starvation effects. For example, Tauffenberger and Parker (2014) reported the transmission of

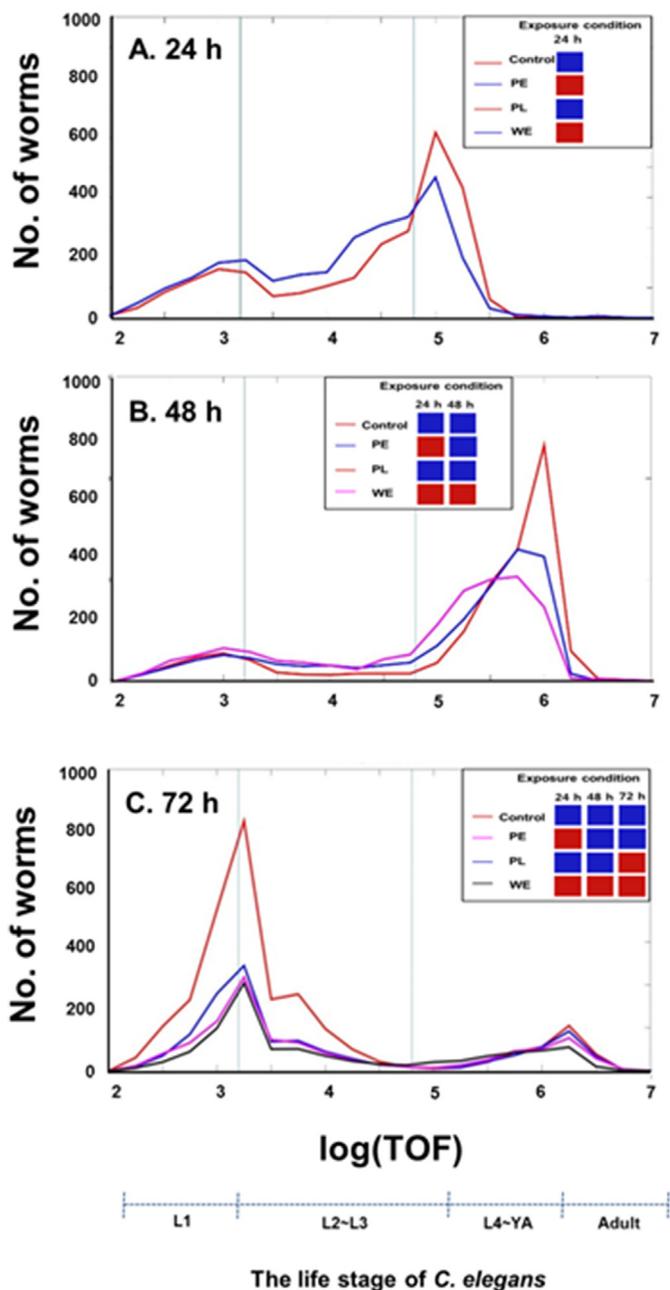


Fig. 2. Development of *C. elegans* exposed to IHC for different periods (PE, PL, and WE) measured at 24 (A), 48 (B), and 72 h (C) after exposure using COPAS. The effect of IHC on growth was investigated using log(TOF), which reflects the length of the worms (i.e., growth), after 24 and 48 h of exposure. The effect of IHC on reproduction was investigated at 72 h after exposure using frequency values (the number of offspring) to assess the reproductive potential. For each experiment, at least 400 worms were assessed. (■: unexposed, ■: exposed).

reduced reproductive capacity after high dietary glucose in *C. elegans* until the F_2 generation, followed by a gradual recovery. Jobson et al. (2015) demonstrated changes in diverse variables, such as fecundity, feeding behavior, embryo quality, male incidence, and stress resistance, over several generations following starvation of the P0 generation. Our results indicate that exposure to IHC leads to transgenerational reproductive toxicity in *C. elegans*. Further observations of other traits over more generations will be needed to obtain a better understanding of the long-term and transgenerational effects of IHC exposure.

We anticipated that the PE treatment would result in greater toxicity than the PL treatment; however, there was no clear difference between the two groups (Fig. 3). Exposure duration was found to be a more important factor than exposure period because, for all endpoints tested, the highest toxicity was observed in the WE treatment group. Although we could not discern any clear relationship between early-life chemical exposure and later-life health effects, it is possible that analyses of broader health outcomes and more sensitive endpoints, in combination with more sophisticated exposure treatments, might clarify the potential transgenerational toxicity of IHC.

3.3. Histone methylation status in *C. elegans* exposed to IHC

To elucidate the underlying mechanism of this transgenerational toxicity, we investigated whether epigenetic modifications were involved. We focused on histone methylation as it has been suggested to be the underlying mechanism for epigenetic transgenerational effects in model organisms such as *Drosophila melanogaster* and *C. elegans* (Lim and Brunet, 2013). In *C. elegans*, methylation of histone H3 (H3K4me, H3K9me, H3K27me, and H3K36me) plays a crucial role in epigenetic memory and chromatin structural alterations (Kelly, 2014).

In the exposed generation (P0), the overall global histone methylation, including mono-, di-, and tri-methylation, was lower in the WE treatment group by 8%, 23%, 14%, and 6% for H3K4me, H3K9me, H3K27me, and H3K36me, respectively, than that in the control group (Fig. 4). A statistically significant decrease in methylation was observed in H3K9 in all treatment groups (PE, PL, and WE). A decrease was also observed for H3K27, although this was not statistically significant. Methylation of H3K9 and H3K27 is usually associated with transcriptional repression (Bassett and Barnett, 2014). Therefore, this result suggests that the changes in histone methylation status might have increased global transcriptional activity in the exposed generation.

Next, we investigated the expression of HMT and HDM genes involved in H3K4, H3K9, H3K27, and H3K36 methylation in the P0 and F_{1-3} generations (Fig. 5). As described in Table S1, the SET-2 class of HMTs catalyzes H3K4 methylation (Xiao et al., 2011); similarly, MET-2 class HMTs regulate H3K9 methylation (Towbin et al., 2012), MES-4 class HMTs regulate H3K36 methylation, and SET-25 class HMTs regulate H3K9 methylation. The *utx-1* gene encodes an HDM for regulating the methylation level of H3K27 (Wenzel et al., 2011). We examined the relative expression of *set-2*, *met-2*, *set-25*, *mes-4*, and *utx-1* genes and found that these were decreased significantly (40%) in the WE treatment group, and to a smaller extent (about 20%) in the PL group. Differential expression of most of the genes was not observed after exposure to IHC in the PE group; the exception was the *mes-4* gene, which showed an approximately 20% reduction. The changes to gene expression did not occur in unexposed F_{1-3} generations. Although the status of the methylated histones was not measured in F_{1-3} worms, our results suggest that methylated histone marks might not be heritable through successive generations. This suggestion is consistent with the results of recent studies that examined glucose phenotypes (Greer et al., 2014; Tauffenberger and Parker, 2014). Nevertheless, the inheritance of altered levels of histone H3 methylation might occur, but this might be limited to specific genes rather than to all H3 histone molecules. One other notable result was the response of the PL group, which was found to be comparable to that of the WE rather than the PE group, in terms of both reproduction response (Fig. 3B) and histone methylation (Fig. 4). However, we did not see any difference between the PE and PL groups at the organismal level with respect to growth and reproduction (Fig. 3), indicating that histone methylation gene expression was more sensitive to the

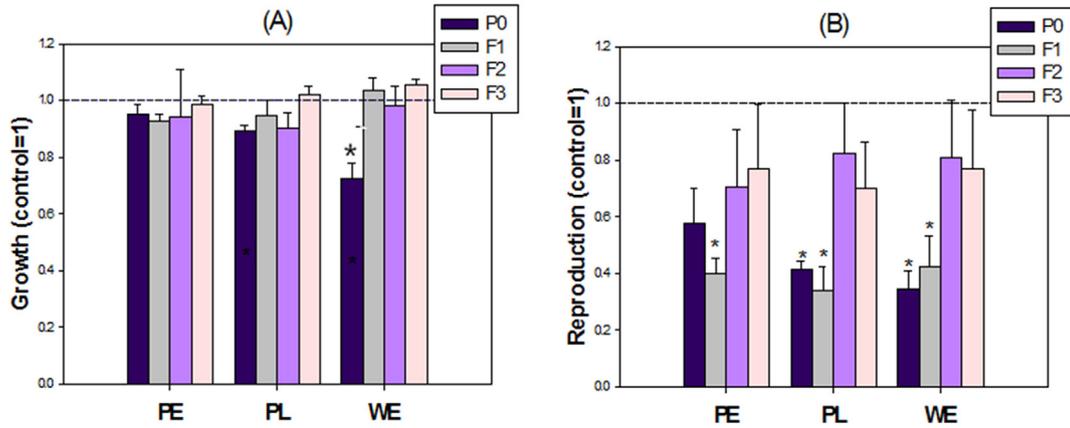


Fig. 3. Effect of IHC exposure on growth (A) and reproduction (B) of *C. elegans* over four consecutive generations (P0 - F3) in the three treatment groups (PE, PL, and WE). *C. elegans* worms (P0) were exposed to IHC, and the offspring of these exposed adults were maintained in normal *C. elegans* culture media, and their development was monitored for three consecutive generations (P0: exposed generation; F1-3: unexposed generations) using COPAS. Growth potential was measured using log(TOF) values at 72 h (A), while reproduction potential was measured using frequency values at 72 h (B) (Control = 1, n = 8; mean ± standard error of the mean; *p < 0.05).

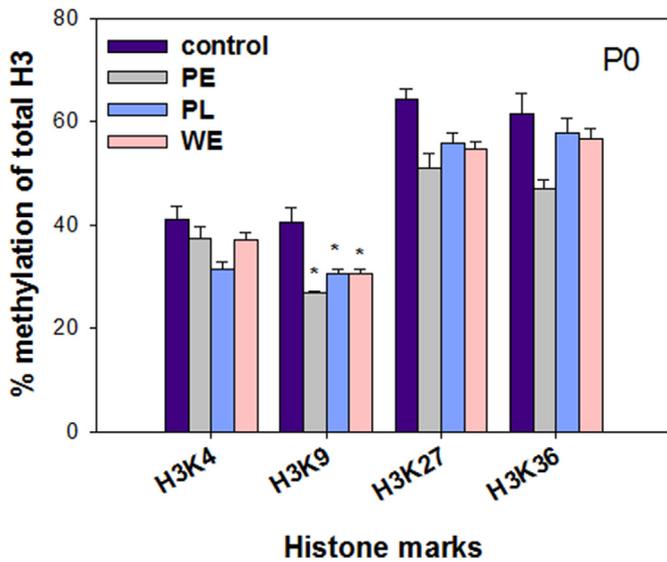


Fig. 4. Total methylation status of histone H3 (H3K4, H3K9, H3K27, and H3K36) in *C. elegans* exposed to IHC in the three treatment groups (PE, PL, and WE). Methylation of each histone residue was normalized according to the total histone 3 value (total H3 = 1, n = 3; mean ± standard error of the mean; *p < 0.05).

PL treatment (Fig. 5). In the PL treatment, worms were exposed to IHC for 24 h from 48 h after L1 synchronization; this treatment

regime exposes L4 and young adult stages, during which germ cell proliferation occurs. Therefore, this period seems to be critical for reproductive and transgenerational toxicity, as reflected in our results (Figs. 3–5).

3.4. Role of histone methylation in transgenerational effects of IHC toxicity

In 2011, Greer and colleagues reported that the inheritance of longevity in *C. elegans* was related to H3K4me3 (Greer et al., 2011). Later, Tauffenberger and Parker (2014) confirmed the involvement of H3K4me in transgenerational inheritance of glucose phenotypes (reproductive defects). Though the role of H3K9 in transgenerational inheritance in *C. elegans* has not yet been reported, the importance of the balance between H3K4me and H3K9me in epigenetic inheritance has previously been noted (Greer et al., 2014), suggesting that H3K4me and H3K9me are involved in the molecular mechanism of transgenerational inheritance. It was also previously demonstrated that the MET-2 class of HMTs, which act on H3K9, is involved in germline reprogramming and is critical for preserving epigenetic memory in combination with spr-5 in *C. elegans* (Kerr et al., 2014). Considering our results (Figs. 4 and 5) and previous reports, we performed a functional study of H3K4 and H3K9 methylation using loss-of-function mutants for the *set-2* and *met-2* genes (Fig. 6). To elucidate their roles in transgenerational effects of IHC toxicity, reproduction in worms carrying these mutations was monitored for four consecutive generations (exposed P0 and unexposed F1-3 generations). The effect of transgenerational

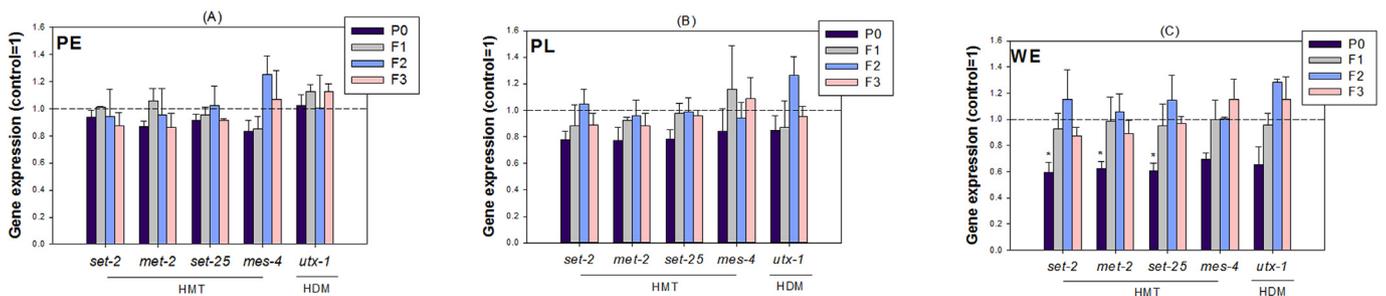


Fig. 5. Relative levels of expression of HMTs and HDM genes for H3K4, H3K9, H3K27, and H3K36 in *C. elegans* exposed to IHC in the PE (A), PL (B), and WE (C) treatment groups. Relative gene expression was normalized against the expression of *pmp-3* gene. The expressions of HAT (*set-2*, *met-2*, *set-25* and *mes-4*) and HDM (*utx-1*) genes in *wildtype* (N2) are expressed as mean values compared to those of the control (Control = 1, n = 3; mean ± standard error of the mean; *p < 0.05).

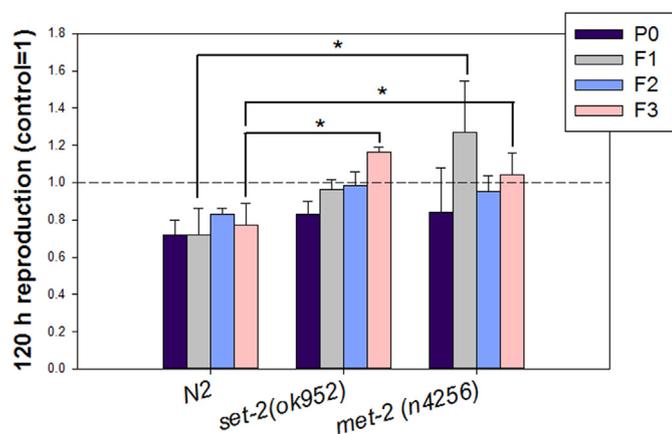


Fig. 6. Reproduction in wildtype and mutant *C. elegans* after exposure to IHC in the three different treatment groups. Measurements were performed at 120 h in each generation in wildtype (N2), *set-2(ok952)*, and *met-2(n4256)* mutants, and all data were normalized against each control group. The statistical difference between wildtype (N2) and mutants was analyzed. (Control = 1, n = 3; mean \pm standard error of the mean; *p < 0.05).

toxicity was investigated only using the WE treatment because transgenerational effects on reproduction potential were found only in offspring of treated wildtype (N2) under WE conditions. The transmissible reproductive toxicity observed in the wildtype was absent in the *set-2(ok952)* and *met-2(n4256)* mutants (Fig. 6). These results suggest that the activation of the HMTs of H3K4 and H3K9 is required for the transgenerational effect of IHC toxicity. IHC-induced reproductive defects might be transmitted to unexposed generations through the deregulation of HMTs by IHC exposure.

Overall, our results indicate that the reproductive toxicity induced by IHC exposure in *C. elegans* was transmitted to subsequent unexposed generations and that methylation of histone H3K9 seemed to be an important component of this effect. Our study provides an insight into transgenerational epigenetics by showing the inheritance of toxic effects of crude oil exposure in the next three generations in nematodes. Epigenetic modification of phenotypes as a result of environmental stress is generally considered to be transient and is expected to quickly revert back to the original phenotype (Burggren, 2016). As epigenetic phenotype switching is dynamic and temporary and thus can help to bridge periods of environmental stress, this study could further provide a basis for developing an epigenetic biomarker of environmental stress.

Acknowledgments

This work was supported by the Mid-career Researcher Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (NRF-2017R1A2B3002242) and through the Taean Environmental Health Center operating grant for the Investigation of Long-term Health Effects of the Hebei Spirit Oil Spill funded by Ministry of Environment of Korea. *C. elegans* strains were provided by the Caenorhabditis Genetics Center (MN, USA), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.chemosphere.2018.02.080>.

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