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Activation of the nucleotide excision repair pathway by crude oil exposure: A translational study from model organisms to the Hebei Spirit Oil Spill Cohort☆

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ABSTRACT

In order to gain insight into the human health implications of the Hebei Spirit Oil Spill (HSOS), the mechanism of toxicity of the Iranian heavy crude (IHC), the main oil component in the HSOS was investigated in *Caenorhabditis elegans and* zebrafish. The identified mechanism was translated to humans using blood samples from Taean residents, who experienced HSOS with different levels of exposure to the spill. *C. elegans* TF RNAi screening with IHC oil revealed the nucleotide excision repair (NER) pathway as being significantly involved by oil exposure. To identify the main toxicity contributors within the chemical mixture of the crude oil, further studies were conducted on *C. elegans* by exposure to C3-naphthalene, an alkylated polycyclic aromatic hydrocarbon (PAH), which constitutes one of the major components of IHC oil. Increased expression of NER pathway genes was observed following exposure to the IHC oil, C3-naphthalene enriched fraction and C3-naphthalene. As the NER pathway is conserved in fish and humans, the same experiment was conducted in zebrafish, and the data were similar to what was seen in *C. elegans*. Increased expression of NER pathway genes was observed in human samples from the high exposure group, which suggests the involvement of the NER pathway in IHC oil exposure. Overall, the study suggests that IHC oil may cause bulk damage to DNA and activation of the NER system and Alkylated PAHs are the major contributor to DNA damage. Our study provides an innovative approach for studying translational toxicity testing from model organisms to human health.

1. Introduction

The Hebei Spirit Oil Spill (HSOS) accident occurred in Taean, Chungnam, South Korea, on December 7, 2007. At the time of the accident, the amount of spilled oil released to the western coastline of Korea was estimated to be 12,547 kL and this caused a serious concern for human health and ecosystems (Kim et al., 2012). Short- and long-term consequences of oil spills have been thoroughly studied for decades in an ecotoxicological and an epidemiological context (Gwack et al., 2012; Jung et al., 2013; Kang et al., 2016b; Kim et al., 2017a; Kim et al., 2014; Lee et al., 2010; Lee et al., 2013). Ecotoxicity, genotoxicity, and oxidative stress have been reported in various models and from environmental samples, such as sediment, collected in the spilled area. Ji et al. reported that the oil samples in the sediment extracts induced genotoxicity and endocrine-disruption in the DNA repair-deficient chicken DT40 cell line and H295 cells (Ji et al., 2011). Jeong et al. confirmed that the persistence of potential genotoxicity caused by sediments from the severely affected regions as long as five years after the oil spill (Jeong et al., 2015; Liu et al., 2018). Several ecotoxicity studies of crude oils were also conducted under controlled laboratory conditions using marine organisms exposed to crude oils. Jung et al. reported that crude oil induced developmental toxicity through oxidative stress in the olive flounder (*Paralichthys olivaceus*) and

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spotted sea bass (*Lateolabrax maculatus*) (Jung et al., 2017). Han et al. showed that oxidative stress was induced by water accommodated fractions (WAFs) of crude oil in the Antarctic copepod (*Tigriopus kingsejongensis*) and temperate copepod (*Tigriopus japonicus*) (Han et al., 2017). The same group thoroughly investigated the involvement of cytochrome P450 (CYP450) pathways in crude oil toxicity in marine organisms. For instance, they reported increased expression of CYP3024A2, CYP3024A3, and CYP3027C2 by WAF in *Tigriopus japonicas* (Han et al., 2014).

In epidemiology studies, urinary oxidative stress biomarkers were studied in the population that was living either near or far from oil spill site (Kim et al., 2017b; Noh et al., 2015b). These studies reported oxidative stress biomarkers were positively associated with urinary PAH metabolites and were negatively associated with distance from the accident site (Kim et al., 2017b; Noh et al., 2015b). Importantly, this association was found in the population six years after the accident, which suggests HSOS might lead long-term health consequences (Kim et al., 2017b). To this end, it is necessary to thoroughly investigate the mechanism of toxicity of crude oil and to identify the main oil components related to the toxicity in the HSOS. However, because of the complex nature of crude oil, which is a mixture of numerous chemicals, identifying the toxic mechanism has not been fully understood, especially with regards to its implication to human health.

Iranian heavy crude (IHC) oil, one of spill sources of the HSOS, is a complex mixture of hydrocarbons. Among aromatics which are known to be responsible for baseline toxicity, benzene, toluene, ethylbenzene, and xylene (BTEX), along with PAHs and alkylated PAHs account for 76%, 2%, and 21%, respectively (Kang et al., 2014). Although BTEX is at the highest level, C3- and C4-naphthalenes and other alkylated PAHs are expected to be the most predominate aromatic hydrocarbons, contributing to the toxicity of IHC oil (Kang et al., 2014). As more volatile compounds like BTEX are rapidly evaporated right after the spill, more persistent aromatics, such as alkylated PAHs, are thought to contribute to major toxicity (French-McCay, 2002; McGrath et al., 2005). Due to solubility limit of high molecular weight PAHs, C3-naphthalene was suggested as one of main component to exhibit toxicities (Kang et al., 2014).

The nematode, *Caenorhabditis elegans,* and the zebrafish, *Danio rerio* are the most popular model organisms for various aspects of human health studies, including identification of toxic mechanism of chemicals. They both have many advantages as model organisms, due to small size, high fecundity, fully sequenced and highly conserved genomes. *C. elegans* offer special advantages in mechanistic toxicology, due to easiness of genetic manipulations, such as, the RNA interference and functional mutants. Zebrafish, as a vertebrate model, has unique features in developmental toxicology (Hill et al., 2005; Tanguay, 2018).

In this study, to gain an insight of human health implication of HSOS, we investigated mechanism of toxicity of IHC oil using model organisms, *C. elegans* and *D. rerio.* IHC oil was chosen as the model crude oil because it was one of the spill sources of the HSOS and C3-naphtalene in IHC oil was suggested as one of the main component to exhibit toxicities (Yim et al., 2012). *C. elegans* were exposed to IHC oil using a passive dosing method in which the dissolved aqueous concentration of hydrophobic hydrocarbons from IHC was maintained at a constant level throughout the experiment. Our strategy was to begin with a *C. elegans* transcription factor RNAi library screening to gain insight into toxicity-related pathways, followed by validation in the selected pathways in *C. elegans* and zebrafish. *C. elegans* RNAi screening was followed by bioinformatics analysis revealing that the nuclear excision repair (NER) pathway is involved in toxicity due to IHC oil exposure. To identify the main contributors within the crude oil chemical mixture, we looked further into compound group, such as C3-naphthalenes, one of the major components of IHC oils. As the NER pathway is conserved in fish and humans, expression of NER pathway genes was investigated in zebrafish exposed to IHC oil. Finally the result from model organisms was translated to human by verifying the same pathways using the blood samples from Taean residents, who experienced HSOS with different levels of exposure to the spill.

2. Materials & methods

2.1. Maintenance of C. elegans

C. elegans were cultured in Petri dishes on nematode growth medium (NGM) and fed the OP50 strain of *Escherichia coli* at 20 °C, according to a standard protocol (Brenner, 1974). Worms were incubated at 20 °C and young adults (three days old) from an age-synchronized culture were used in all experiments. To produce age-synchronized cultures, eggs from mature adults were isolated using a 10% hypochlorite solution, followed by rinsing with M9 buffer (4.2 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 86 mM NaCl, and 1 mM $MgSO_4 \cdot 7H_2O$).

2.2. Maintenance of zebrafish

Wild-type zebrafish of the AB strain were maintained at 26 ± 1 °C under a 14/10 h (light/dark) photoperiod cycle. They were maintained in a housing system where the chemicals, biologicals, aeration, and water filtration were constantly controlled (Genomic Design Bioengineering, Daejeon, South Korea). Animals were fed three times a day, two times with commercial flakes (Vipagran; Sera, Loessnitz, Germany) and once with brine shrimp (Artemia franciscana, INVE Aquaculture, Belgium). For obtaining embryos, male and female adult zebrafish were placed in a spawning tank at a ratio of 2:1, respectively. Spawning was triggered at the beginning of the light cycle. After 1 h, eggs were collected and fertilized eggs were separated under the microscope. For mating, all fish were randomly chosen from different clusters.

2.3. Human subjects

To investigate the long-term health effects of HSOS, a cohort was established after the accident from the population in Taean, as described previously (Noh et al., 2015b). The adults (aged 18 years or older) who agreed to participate in the survey in the cohort study were 9585 from 2009 to 2017. Within this cohort, subjects from 2014 were used in this study. High and low exposure groups were distinguished based on the distance from the oil band and contaminated coast and their participation in clean-up work (Choi et al., 2016; Noh et al., 2015a). The highly exposed group were people who were less than 15 km from the spill site and participated in clean-up work for more than 200 days. The low-level exposure group consisted of people who were more than 25 km from the spill site and participated in less than 5 days of clean-up work. Characteristics of each cohort were described previously (Kim et al., 2017b). The information on study subjects of high and low exposure groups are shown in Table S1. The residence areas are shown in the map (Fig. S1). Twenty study subjects were used and blood was collected at the Taean Environmental Health Center (Taean, Republic of Korea) and handled according to an IRB protocol at the institution. Blood samples were obtained from participants after a 12 h fast and stored in a freezer at −80 °C.

2.4. IHC oil and C3-naphthalene

The IHC oil used as the model crude oil in this study was provided by the Taean Environmental Health Center (Taean, Korea). A

detailed analysis of the chemical composition of IHC oil has been reported previously (Kang et al., 2014; Yim et al., 2011). Those previous studies revealed that alkylated naphthalenes are the most abundantly found polycyclic aromatic hydrocarbons in IHC. Thus, 1,4,5-trimethylnaphthalene (1,4,5-TMN) (purity 95%) was used as a surrogate for C-3-naphthalene in IHC oil and purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). It is also reasonable to assume that 1,4,5-TMN reflects the characteristics of C3-naphthalene because octanol-water partition coefficients (Kow) of C3-naphthalenes is not significantly different (Kang et al., 2016a).

2.5. Preparation of the C3-naphthalene-enriched fraction

IHC was fractionated according to true boiling point using distillation apparatus (i-Fischer DIST TD-2892 CC, Waldbuttelbrunn Germany). Thirty-five distilled fractions (labeled from F1 to F35) were collected from 15 °C to 400 °C by 10 °C increments. Chemical compositions such as BTEX and PAHs for all fractions were measured using gas chromatography−mass spectrometry (GC-MS) (Agilent 7890 GC with 5975 MS, Santa Clara, CA US). Each fraction had dominant aromatics according to its boiling point, among which F22 was enriched with C3-naphthalene (74.1%) followed by C4-naphthalene (19.0%) and C2-naphthalene (5%). F22 was selected for further toxicity test focused on C3-naphthalene-enriched fraction.

2.6. Preparation of the water-soluble fraction

To investigate the toxicity of IHC oil, a water-soluble fraction (WSF) of IHC was prepared using a polydimethylsiloxane (PDMS) tube purchased from Dong-Bang silicone Inc., Korea. This contained IHC, as described previously (Kang et al., 2014). Briefly, 0.9 g IHC was inserted into the PDMS tube (inner diameter = 2 mm, outer diameter = 3 mm) with 100 mL of K-medium (0.032 M KCl and 0.051 M NaCl in distilled water). The solution was stirred at 200 rpm in the dark for 24 h to attain dissolution equilibrium. For zebrafish exposure, K-medium was replaced with fish water. The WSF solution was used for toxicity tests without any further treatment. To prepare a WSF of a C3-naphthalene-enriched IHC fraction, approximately 0.7 g of C3-naphthalene-enriched IHC fraction was loaded into the PDMS tube and instructions were followed as described above.

2.7. Exposure of C. elegans and zebrafish to IHC oil

The organisms were exposed to the prepared WSF of IHC oil and C3-naphthalene-enriched IHC fraction at 25, 50, and 100% of final volume (v/v). 1, 4, 5-TMN was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution. 1, 4, 5-TMN was used at 50, 100, and 200 μM. The final concentration of DMSO in the solution was 0.1%. No effect was observed with 0.1% DMSO in young to adult stages of *C. elegans* or 96 h post fertilization (hpf) zebrafish embryos.

2.8. C. elegans RNAi screening using a transcription factor library

A 384 transcription factor (TF)-containing RNAi feeding library was purchased from Source Bioscience Inc. UK, which covers 41% of the predicted TFs in *C. elegans* (Reece-Hoyes et al., 2005). The full TF list in the library is presented in Table S2. RNAi screening was conducted based on the protocol described previously, with some modifications (Squiban et al., 2012). Briefly, RNAi bacterial clones were incubated overnight in 96-deep well plates containing Luria-Bertani broth (LB) media with 100 μg ml^{-1} ampicillin and then inoculated with 5 mM isopropyl-β-D-1-thiogalactoside (IPTG) for 1 h at room temperature. RNAi bacterial pellets were prepared by centrifuging and dissolving in complete-K medium (0.032 M KCl, 0.051 M NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 13 mM cholesterol) with 100 μg ml^{-1} ampicillin and 5 mM IPTG (Lehner et al., 2006). For the screening, 100 μl of dissolved RNAi feeding bacteria were dispensed in each 96-well plate using a multi-channel pipet. Age-synchronized L1 stage worms were dispensed into each well using a Complex Object Parametric Analyzer and Sorter (COPAS™ SELECT, Union Biometrica, Holliston, MA, USA) and were allowed to grow into adults in the prepared RNAi bacteria-containing wells at 20 °C for 60 h. Next, the control group exposed to 100 μl of K-medium and the treatment group was exposed to 100 μl of IHC oil at 20 °C for 72 h at a final concentration of 50% (v/v). After exposure, the number of offspring from RNAi-fed worms was counted using COPAS. The screening was performed on the N2 strain using 5-well replicates with independent duplicates. After the analysis, the toxicity of each RNAi clone was normalized using the response of the empty vector (EV), L440, as a control RNAi. The EV was reported to have no effect on the worm phenotype (Sonnichsen et al., 2005). Upon exposure to 50% (v/v) WSF, reproduction of EV-fed worms decreased by about 20%.

After bioinformatics analysis, RNAi clones, with a significantly altered response following exposure to IHC oil, were selected for individual evaluation. For validating the results, RNAi bacteria agar plates were prepared as described above (Kamath et al., 2001). The N2 progeny were allowed to grow to adults on RNAi bacteria plates containing 1 mM IPTG and 100 μg mL^{-1} ampicillin at 20 °C. RNAi fed adult worms were dispensed into 96-well plates and exposed to IHC oils. When they reached to the young adult (YA) stage, which is 72 h after dispensing, RNAi-fed YA stage worms were exposed to 25, 50, and 100% IHC (v/v) for another 72 h and the reproduction was investigated by counting the number of offspring using COPAS (Pulak, 2006).

2.9. qRT-PCR

Young adult *C. elegans*, 96 hpf zebrafish larva, and human blood samples were prepared for PCR analysis. The morphologically unproblematic model organisms were selected for quantitative real time polymerase chain reaction (qRT-PCR). Total RNA was extracted from *C. elegans,* 96 hpf zebrafish larva and Human blood using an RNA extraction kit (NucleoSpin, Macherey-Nagel). The quantity and purity of the RNA were assessed using a Nanodrop (ASP-2680, ACTGene, Piscataway, USA). cDNA was synthesized using an oligo (dT) primer (Bio-Rad Laboratories, Hercules, CA). Quantitative real time-PCR (qRT-PCR) analysis was accomplished with a CFX manager (Bio-Rad) using an IQ™SYBR Green SuperMix (Bio-Rad Laboratories, Hercules, CA). The qPCR reaction was performed using the two-step RT-PCR method with the IQ™ SYBR Green SuperMix (Bio-Rad) and analyzed according to the $^{\triangle\triangle}$CT normalized expression method. Control samples and housekeeping genes (*pmp-3*; *C. elegans*, *β-actin*; zebrafish, *gapdh*; Human) were used for normalization. Expression of the *C. elegans* NER genes (*xpc-1, xpa-1,* T16H12.4, and *ercc-1*), zebrafish NER genes (*xpc, xpa, gtf2h2,* and *ercc1*), and human NER genes (*xpc, xpa, gtf2h2,* and *ercc1*) were investigated. The primers were constructed based on sequences available at NCBI (Table S3).

2.10. Pathway analysis

From the RNAi feeding library screening results, RNAi clones showing more than two-fold reproductive changes (increase or decrease) were identified as potential genes affected by IHC oil exposure. Identified genes were then analyzed using canonical pathway analysis software, such as KEGG (Kanehisa et al., 2012) and

REACTOME (Fabregat et al., 2018), which allow for identification of the biological network. Entities with a p value < 0.05 were considered in REACTOME.

2.11. Statistical analysis

Statistical analyses were conducted using SPSS (SPSS Inc., Chicago, IL, USA), version 13.0. All the experiments were performed with three biological replicates. The statistical significance of differences among groups was determined by one-way ANOVA followed by Tukey post hoc test.

2.12. Statement on the welfare of animals

All procedures were approved by the Institutional Animal Care and Use Committee at University of Seoul, and conducted in accordance with relevant guidelines and regulations in Republic of Korea.

3. Results & discussion

3.1. C. elegans transcription factor RNAi screening reveals potential toxicity pathways for IHC oil

To identify the mechanism of toxicity of IHC oil, *C. elegans* TF RNAi screening was performed using reproduction as the endpoint. IHC exposure leads to an approximately 20% decrease in reproduction for EV-fed *C. elegans* and RNAi feeding resulted in exacerbated or rescued reproductive toxicity compared to EV-fed *C. elegans*. Among 384 TFs, we first identified 19 RNAi candidates that caused exacerbated or rescued reproductive toxicity at more than 50% compared to EV-fed *C. elegans* (See Table 1, with a full list of the responses for each RNAi presented in Table S2).

Most of the selected RNAi candidates corresponded to hormone maintenance-related TFs, such as nuclear hormone receptors (NHR) (i.e. *nhr-121*, *nhr-23*, *nhr-113*, *nhr-217*, *nhr-3*, *nhr-119*, and *nhr-221*). Among them, *nhr-121* and *nhr-23* are known to be orthologues of members of the nuclear receptor family of intracellular transcription factors including RAR-related orphan receptors (ROR) (Table 1). RORs are known to be involved in lipid and glucose metabolism and immune processes which have influence on metabolic syndrome and asthma (Cook et al., 2015; Hamzaoui et al., 2011). Previous epidemiological studies on the Taean cohort reported the prevalence of asthma among the highly exposed population (Jung et al., 2013; Kim et al., 2013). It has been also reported that PAHs effect cytokine regulation via ROR in asthma patients (Ple et al., 2015). These epidemiology results support our finding, on which the NHR family plays an important role in toxicity induced by IHC oil exposure.

IHC-induced toxicity was exacerbated in worms-fed with T16H12.4 more than 50% compared to EV-fed worms. T16H12.4 is an ortholog of human general transcription factor IIH subunit 2 (GTF2H2), which is known to be involved in the NER system (Lans and Vermeulen, 2011). This result suggests a protective role for this gene against bulky DNA damage by IHC oil exposure.

To validate the screening results, we conducted concentration-response analysis using single RNAi clone in Table 1 (T16H12.4). Reproduction of T16H12.4 RNAi-fed worms decreased in IHC-oil exposure concentration-dependent manner compared to EV RNAi fed-worms, where such a tendency was not observed (Fig. 1). This result confirms the effects of IHC oil on the gene originally identified by RNAi screening.

To further understand the mechanism of toxicity, we conducted pathway analysis based on RNAi screening results using the KEGG mapper and REACTOME databases. Using the gene list shown in Table 1, we identified pathways involved in the toxicity of *C. elegans* due to IHC oil exposure. In KEGG pathway analysis, fifteen RNAi results showed rescued toxicity following IHC oil exposure and were not involved in any of the biological pathways, while four RNAi results showed exacerbated toxicity following IHC oil exposure and were involved in NER and baseline gene regulation (Table 2). In REACTOME pathway analysis, fifteen rescued RNAi results were involved in an AKT signaling pathway associated with *egl-43* (Table 2). Four RNAi results were found to be involved in a pathway related to NER as well as transcription processing by T16H12.4 as an entry gene (Table 2). Human ortholog analysis of individual genes and pathway analysis collectively revealed the potential involvement of ROR, NER, and AKT signaling pathways in IHC-induced toxicity in *C. elegans* (Tables 1–2).

Table 1
Selected list of RNAi candidates that caused exacerbated or rescued reproductive toxicity to IHC exposure compared to EV-fed *C. elegans* (cut-off value > 2-fold). Relative unit indicates the difference of toxicity compare to empty vector. The full list is shown in Table S2. The statistical difference between the control group and treated group was analyzed using the two-tailed t-test (p value: $p^{*} < 0.05$, $p^{**} < 0.01$, and $p^{***} < 0.001$). Two independent experiments were performed per condition with 5 worms per RNAi.

Sequence	Genes	Human ortholog	Relative unit (Empty vector = 1)	P value
List of RNAi leading to rescued toxicity				
E02H9.8	nhr-121 (Nuclear Hormone Receptor family)	RORC	3.23	0.03
C07E3.5	ceh-57 (C. Elegans Homeobox)	TFIP11	2.90	0.07
Y53C10A.3	hsf-2 (Heat Shock Factor)		2.56	0.08
R53.3	egl-43 (EGg Laying defective)	MECOM	2.50	0.10
F34D10.5	lin-48 (abnormal cell LINeage)	OVOL2	2.41	0.05
C01H6.5	nhr-23 (Nuclear Hormone Receptor family)	RORA	2.40	0.22
ZK1025.9	nhr-113 (Nuclear Hormone Receptor family)	TRPT1	2.34	0.02
T09E11.2	nhr-217 (Nuclear Hormone Receptor family)	TRPT1	2.13	0.08
R04A9.5	ceh-93 (*C. elegans* Homeobox)	HHEX	2.11	0.14
F54A5.1	hmbx-1 (HMBOX (mammalian HoMeoBOX gene) homolog)	HMBOX1	2.11	0.04
C27A12.5	ceh-2 (C. Elegans Homeobox)	EMX1	2.09	0.02
F27E5.2	pax-3 (PAX (Paired box) transcription factor)		2.07	0.15
H01A20.1	nhr-3 (Nuclear Hormone Receptor family)		2.06	0.01
K12H6.1	nhr-119 (Nuclear Hormone Receptor family)	NR5A1	2.02	0.01
T23G7.1	dpl-1 (vertebrate transcription factor DP-Like)	TFDP2	2.02	0.02
List of RNAi leading to exacerbated toxicity				
C14C6.4	nhr-155 (Nuclear Hormone Receptor family)		0.487	0.76
T24A6.8	nhr-221 (Nuclear Hormone Receptor family)	RXRG	0.484	0.03
T16H12.4	T16H12.4 (General transcription factor)	GTF2H2	0.466	0.01
Y111B2D.e	Y111B2D.e		0.411	0.01

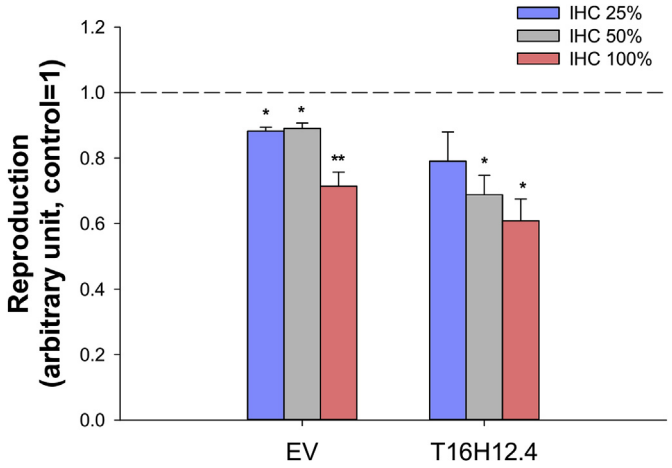

Fig. 1. Effects of IHC oil on the reproduction of T16H12.4 RNAi-fed *C. elegans*. The result was expressed compared to control (=1) as represented by the dashed line. Significance indicates the differences between unexposed control and treated group of either EV or T16H12.4 RNAi fed worms. Reproduction assays were performed by counting offsprings from 24 worms in average. The results were presented as the mean value compared to control (n = 3, mean ± SEM; two-tailed *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).

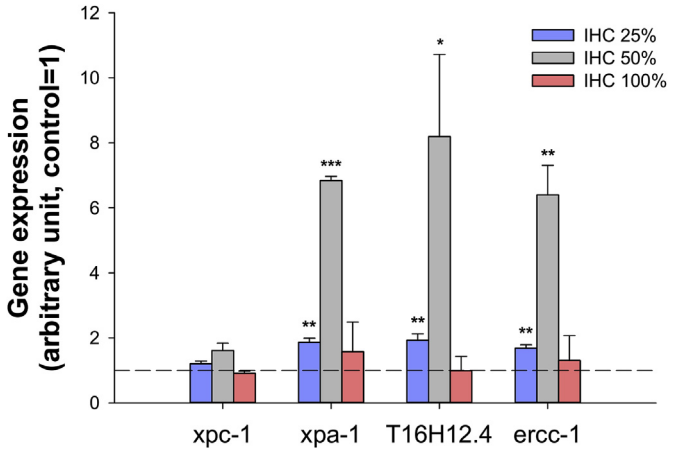

Fig. 2. Effects of IHC on the expression of NER pathway genes in *C. elegans*. Gene expression was measured on *C. elegans* exposed to 25, 50, and 100% of IHC oil (v/v). The result was expressed compared to control (=1) as represented by the dashed line. Significance indicates the differences between unexposed control and treated group of wild-type N2. The results were presented as the mean value compared to control (n = 3, mean ± SEM; two-tailed *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).

3.2. Nucleotide excision repair system is involved in IHC-induced toxicity

Since both KEGG and REACTOME analyses revealed the NER pathway, as a major involved pathway in response to IHC oil exposure, for the rest of the study, we focused on this pathway. IHC oil contains various PAHs, which would cause bulk DNA adducts leading, in turn, to evoke the NER pathway. NER is one of the DNA repair systems, which involves multiple processes that recognize and eliminate a wide range of damage causing DNA lesions, such as UV and chemical-induced damage (Scharer, 2013). Once PAHs in IHC oil enter the body, they can be converted to diol-epoxide by the phase I reaction, making bulky DNA adducts, which affect DNA replication. Thus, the NER pathway is an important defense mechanism for repairing DNA adducts due to IHC oil exposure. If NER is inefficient, DNA mutations will accumulate and possibly induce carcinogenesis (Stavric and Klassen, 1994). The importance of the NER pathway in IHC oil toxicity was also revealed by its occurrence in both the KEGG and REACTOME pathway analyses. To more thoroughly investigate the response of the NER system due to IHC oil exposure, the expression of a panel of genes in the NER pathway was examined. Each step of the pathway, such as recognition of distorted DNA (xpc-1: ortholog of human XPC), binding to a damaged strand (xpa-1: ortholog of human XPA), core subunit of transcription factor involved (T16H12.4: ortholog of human GTF2H2), and formation of single-strand breaks in DNA on the 5′ side of the damage (ercc-1: ortholog of human ERCC1), was successively investigated.

Expression of the NER pathway genes was investigated in *C. elegans* exposed to 25, 50, and 100% (v/v) of IHC oil (Fig. 2). An inverted u-shaped relationship was observed between IHC oil concentration and gene expression, except for the *xpc-1* gene. In response to 25% and 50% (v/v) IHC oil exposure, increased NER genes expression was observed. On the other hand, the highest exposure concentration, at 100% IHC oil, may destroy the worms' defense mechanisms leading to a reduced response.

Bulk DNA damage leading to adduct formation by PAHs is well characterized as the initiation stage of chemical carcinogenesis using benzo[*a*]pyrene as a model chemical (Stavric and Klassen, 1994). Involvement of the NER pathway by IHC oil was first revealed by RNAi screening (Tables 1 and 2) and later confirmed by individual gene expression analysis (Fig. 2), indicating that IHC oil causes severe DNA damage in *C. elegans*. Although our results cannot provide direct relationship between DNA repair and reproduction failure, PAHs, strong genotoxicants, may cause damage to germ cells, which eventually compromise reproductive function. Indeed, genotoxicity in germ cell of *C. elegans* is previously reported to induce reproductive toxicity (Du et al., 2015). In addition, the genotoxic potential of IHC oil was previously reported in an environmental monitoring study at the HSOS site using XPA-deficient cells (Ji et al., 2011). In that study, XPA-deficient cells showed more sensitive response than wild-type cells, when exposed to samples collected from oil contaminated sites. That study supported our result on activation of NER pathway by IHC oil exposure, as XPA plays an important role in appropriate excision of damaged DNA in the NER pathway.

Table 2
Pathway analysis from RNAi screening results using KEGG mapper and REACTOME databases. The gene list shown in Table 1 was used and pathways involved in IHC oil exposure were identified.

Software	RNAi candidates leading to exacerbated toxicity		RNAi candidates leading to rescued toxicity	
	Biological pathways	Entry genes	Biological pathways	Entry genes
KEGG			Nucleotide excision repair	T16H12.4
			Basal transcription factor	T16H12.4
REACTOME	PIP3 activates AKT signaling	egl-43	RNA Polymerase II Transcription	T16H12.4
			Nucleotide excision repair	T16H12.4

3.3. Alkylated naphthalene is a major contributor to NER toxicity by IHC oil

In the next step, we therefore, tried to identify which components in IHC oil contribute more to the identified toxicity in this study, specifically to activation in the NER system. In a previously conducted toxicity study using the luminescence inhibition of a marine bacterium (*Aliivibrio fischeri*) and growth inhibition of the microalga, *Raphidocelis subcapitata*, researchers reported that alkylated PAHs contributed significantly to oil toxicity (Kang et al., 2016a). In a previously conducted genotoxicity monitoring study using DT40 cells, which are deficient in nucleotide excision repair (XPA) pathways, they also found that weathered oil, which generally undergoes chemical substitutions, such as alkylation, has greater cytotoxic potential than fresh oil (Ji et al., 2011).

Based on these previous reports, we hypothesized that C3-naphthalene might be one of the major contributors to NER system activation observed by whole IHC oil exposure. As mentioned above, C3- and C4-naphthalenes and other alkylated PAHs are expected to be the most predominate aromatic hydrocarbons, contributing to the toxicity of IHC oil (Kang et al., 2014). Due to solubility limit of high molecular weight PAHs, C3-naphthalene was suggested as one of main component to exhibit toxicities. Therefore, here we used C-3 naphtalene fraction, as an alkylated naphthalene enriched fraction of whole crude oil and 1,4,5-TMN as a surrogate of C3-naphtalene. Effects on NER were studied by measuring the reproductive toxicity of T16H12.4-fed RNAi (Fig. 3A) and examining the expression of genes involved in the NER pathway (Fig. 3B). Reproductive toxicity was more important in T16H12.4-fed RNAi than in EV (Fig. 3A). 1,4,5-TMN exposure caused a clear dose-response relationship with regard to gene expression (Fig. 3B). As we found that exposure of 1,4,5-TMN led increased expression of NER pathway genes, we further investigated whether the C3-naphtalene is a main contributor to the toxicity of IHC oil by comparing the response of C3-naphtalene fraction with that from whole IHC oil exposure (Figs. 1 and 2).

The same approach was conducted using the sub-fraction of IHC oil, which contains C3-naphthalene (C3-naphthalene-enriched fraction) (Fig. 4). As with IHC oil and 1,4,5-TMN results, the number of offsprings decreased following to exposure of C3-naphtalene enriched fraction, in T16H12.4 RNAi-fed worms (Fig. 4A). Exposure to the C3-naphthalene-enriched fraction caused an inverted u-shaped concentration-response relationship for *xpc-1* and *xpa-1* gene expression (Fig. 4B), which is similar to whole IHC oil exposure but with higher intensity (Fig. 2B). Though we did not investigate other fractions, this result insinuates that chemicals in the C3-naphthalene-enriched fraction may be an important contributor of by IHC oil. On the other hand, exposure concentration-dependent decrease was observed in T16H12.4 and *ercc-1* gene expression (Fig. 4B). In the environment, we are exposed to various chemicals that are usually in mixtures and identification of sources or major contributors is important for the proper management of chemical mixtures. Our results show a tiered approach, using sub-fraction from the whole-fraction mixture is efficient for the identification of the major contributing players in the toxicity of chemical mixtures. Moreover, our results also suggest that efficiency of the tiered approach can be maximized when using mechanism-based endpoints rather than apical ones. The difference between whole IHC oil and C3-naphthalene is not significant when using apical endpoints, such as reproduction (Figs. 1, and 4A). However, clear differences were observed when using mechanism-based endpoints, such as NER (Figs. 2, and 4B).

The differences in concentrations (IHC oil, C-3 naphthalene enriched fraction, 1,4,5-TMN) need to be considered in the interpretation of the toxicity results. Worms are exposed to mixture chemicals (i.e. C-3 naphtalene fraction and whole crude oil) via passive dosing method whereas, 1,4,5-TMN is spiked to the exposure media, therefore their actual exposure concentrations cannot be comparable. We focused on the fact that the same mechanistic pathway of toxicity was observed for mixtures and a single surrogate chemical, 1,4,5-TMN. Though it is not possible to directly compare exposure concentrations of three scenarios, overall, potentially very different doses for 1,4,5-TMN, C3-naphtalene enriched fractions, and complete IHC oil exposure may explain the differences observed for gene expression levels.

3.4. Activation of the NER system by IHC oil exposure is conserved in the zebrafish model

We confirmed activation of the NER system by IHC oil using a whole mixture and its enriched fraction and C3-naphthalene in *C. elegans* (Figs. 1–4). The NER system is known to be conserved throughout the animal kingdom (Wirth et al., 2016). We next investigated whether the toxicity mechanism identified in the *C. elegans* model is conserved in other model organisms, such as zebrafish.

The NER pathway genes identified in *C. elegans* are conserved to those in zebrafish, such as *xpc, xpa, gtf2h2,* and *ercc1*, and were investigated in zebrafish following exposure to IHC oil. As with

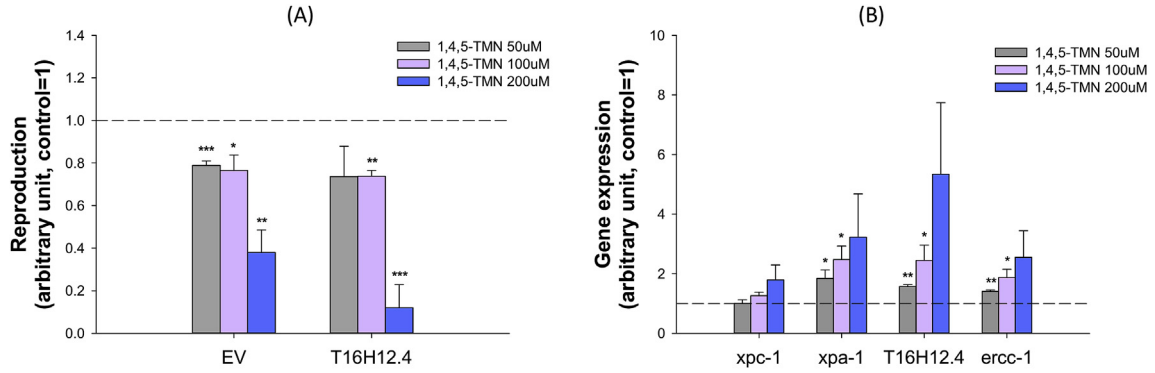

Fig. 3. Effects of 1,4,5-TMN on the NER pathway in *C. elegans*. (A) Effects of 1,4,5-TMN on reproduction of T16H12.4 RNAi-fed *C. elegans*. Significance indicates the differences between unexposed control and treated group of either EV or T16H12.4 RNAi fed worms. (B) Effects of 1,4,5-TMN on the expression of NER genes in *C. elegans*. Statistical difference was compared to unexposed the gene expression level of wild-type N2. *C. elegans* exposed to 50, 100, and 200 μM of 1,4,5-TMN. The Reproduction assays were performed by counting offsprings from 24 worms in average. The result was expressed compared to control (=1) as represented by the dashed line. The results were presented as the mean value compared to control (n = 3, mean ± SEM; two-tailed *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).

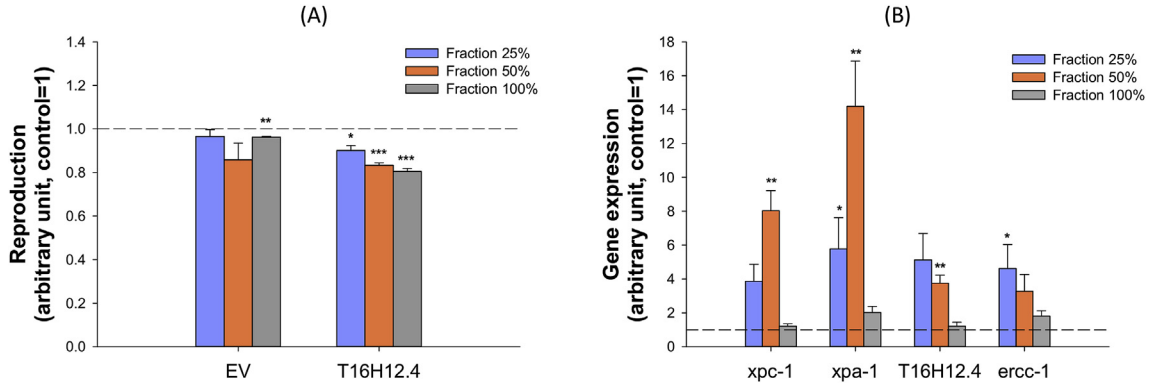

Fig. 4. Effects of the C3-naphthalene-enriched fraction on the NER pathway in *C. elegans*. (A) Effects of C3-naphthalene-enriched fraction on reproduction of T16H12.4 RNAi-fed *C. elegans*. Significance indicates the differences between unexposed control and treated group of either EV or T16H12.4 RNAi fed worms. (B) Effects of C3-naphthalene-enriched fraction on the expression of NER genes in *C. elegans*. Statistical difference was compared to unexposed the gene expression level of wild-type N2. *C. elegans* exposed to 25, 50, and 100% of the C3-naphthalene-enriched fraction. Reproduction assays were performed by counting offsprings from 24 worms in average. The result was expressed compared to control (=1) as represented by the dashed line. The results were presented as the mean value compared to control (n = 3, mean ± SEM; two-tailed *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).

C. elegans, the response of zebrafish to whole IHC oil and C3-naphthalene-enriched fraction was also investigated (Fig. 5). IHC oil exposure activated NER systems in zebrafish and as with *C. elegans*, the highest increase in expression was observed with 50% IHC oil while decreased expression was observed with 100% IHC oil exposure (Fig. 5A). A similar trend was also observed with the C3-naphthalene-enriched fraction, with a much stronger response when compared to whole IHC oil exposure (Fig. 5B). A clear exposure dose-response was observed with 1,4,5-TMN exposure (Fig. 5C). This result suggests that 1,4,5-TMN is also an important chemical in NER activation by IHC oil in the zebrafish model.

In this study we used 1,4,5-TMN, as a surrogate of C3-naphtalene and C-3 naphtalene fraction, as an alkylated naphthalene enriched fraction of whole IHC oil, to identify major contributor of toxicity. As far as comparison of responses of zebrafish (Fig. 5), as well as, of *C. elegans* (Figs. 1–4), across different exposure scenario, the response to whole IHC and C3-naphtalene fractions is comparable, as they are both mixture of chemicals and are expressed as % unit.

3.5. NER pathway identified in C. elegans and zebrafish is translated to humans

Finally, we investigated whether the activated NER pathway caused by IHC oil exposure that was identified in model organisms is also related to toxicity in humans. For this, the HSOS cohort was used. In HSOS Cohort, the population living near and far from the accident site, represents high and low exposure to oils, respectively. Clear differences were observed between groups that were exposed to either low or high amounts of the spilled oils when studying the four human NER genes (i.e. *xpc, xpa, ercc1*, and *gtf2h2*) in blood samples from the cohort. Increased expression in the high exposure group was at least three-fold more than the low exposure group (Fig. 6). The result suggests that the mechanism identified in the model organisms can be applied to humans. It is also important to note that samples were collected from the subjects seven years after the oil spill accident. In a previously conducted monitoring study, the genotoxic potential was found in samples that were collected two years after the HSOS (Ji et al., 2011). Increased oxidative stress biomarkers in the high exposure group were also reported in the HSOS cohort six years after the accident (Kim et al., 2017b). Big differences in NER gene expression between the high and low exposure groups suggests that if a population who was exposed to high levels of oils, DNA damage continues to induce NER activity years later, even though exposure to the oils has ceased. Though current results cannot provide clear explanation for this, our result reinforces the importance of long-term monitoring of health effects for populations exposed to environmental accidents, such as oil spills, even when the population was only exposed to the hazardous chemicals for short period of time.

In this study, the mechanism of toxicity of IHC oil was identified in an unbiased way, using *C. elegans* RNAi screening, which was later extrapolated to a zebrafish model. Using two complementary

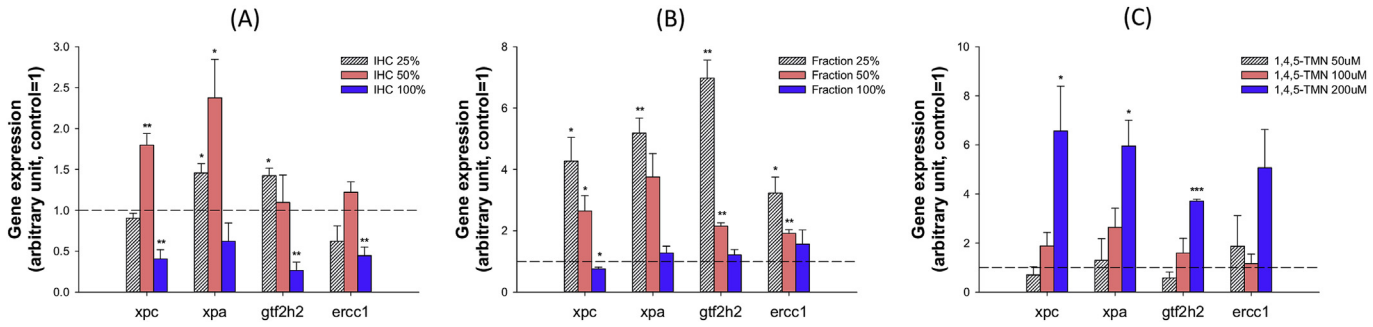

Fig. 5. Effects of IHC, its enriched fraction, and 1,4,5-TMN on the expression of nucleotide excision repair pathway genes in zebrafish. (A) Effects of IHC oil, (B) C3-naphtalene enriched fraction and (C) 1,4,5-TMN on zebrafish NER gene expression. Zebrafish were exposed to 25, 50, and 100% of IHC oil or the C3-naphthalene enriched fraction and to 50, 100 and 200 μM 1,4,5-TMN. Statistical difference was compared to unexposed the gene expression level of wild-type N2. The result was expressed compared to control (=1) as represented by the dashed line. The results were presented as the mean value compared to control (n = 3, mean ± SEM; two-tailed *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).

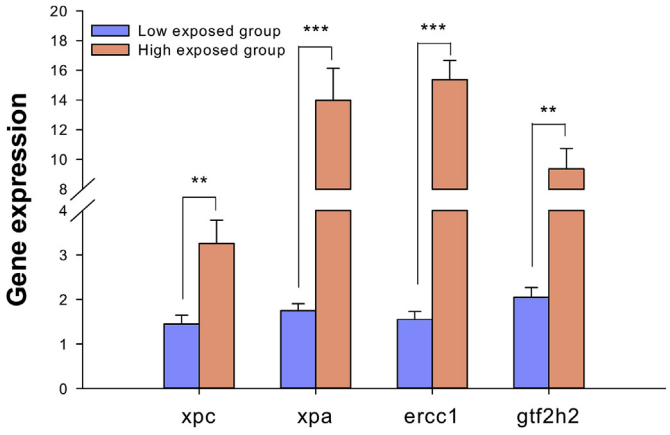

Fig. 6. Expression of NER pathway genes in the high and low exposed subjects from the Cohorts of the Hebei Spirit oil spill study. The results are showed as mean values compared by two-tailed *t*-test (n = 20, mean ± SEM, p value: $p^{*} < 0.05$, $p^{**} < 0.01$, and $p^{***} < 0.001$). Gene expression level was normalized using housekeeping gene *gapdh*.

model organisms has the potential to reveal a much more detailed view of the mechanism of toxicity than when used individually. Thus, simultaneous use of complementary models is a prudent way to diagnose chemical toxicity and plan for chemical management. Our study also shows that the mechanism of toxicity identified in model organisms can be translated to humans. Toxicological and epidemiological approaches can be complementarily used for identifying the toxicity of chemicals and their consequences to human health. As we used the same oils that were spilled in the accident and the population that experienced the accident, our study shows this complementary approach can be successfully implemented in real environmental settings.

4. Conclusion

In conclusion, model organism-based toxicological and cohort-based epidemiological studies collectively suggest that involvement of the NER pathway in the toxicity of IHC oil from the 2007 HSOS. Alkylated PAHs in the oil may have caused bulk DNA damage to the exposed population. As DNA damage is directly related to carcinogenesis, long-term health effects of the HSOS should be investigated in the exposed population.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.112997.

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