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Synergistic Effects Induced by a Low Dose of Diesel Particulate Extract and Ultraviolet-A in *Caenorhabditis elegans*: DNA Damage-Triggered Germ Cell Apoptosis

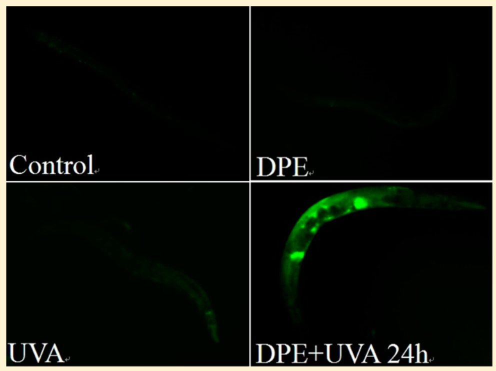
Xiaoying Guo,[†,‡] Po Bian,[*,†] Junting Liang,[†] Yichen Wang,[†] Luzhi Li,[†] Jun Wang,[†] Hang Yuan,[†] Shaopeng Chen,[†] An Xu,[†] and Lijun Wu[*,†,§]

[†]Key Laboratory of Ion Beam Bioengineering, Hefei Institutes of Physical Science, Chinese Academy of Sciences, P.O. Box 1138, Hefei, Anhui 230031, P.R. China

[‡]Institute of Agricultural Engineering, Anhui Academy of Agricultural Science, Hefei, Anhui 230031, P.R. China

[§]School of Nuclear Science and Technology, University of Science and Technology of China, Hefei, Anhui 230026, P.R. China

Supporting Information



ABSTRACT: Diesel exhaust has been classified as a potential carcinogen and is associated with various health effects. A previous study showed that the doses for manifesting the mutagenetic effects of diesel exhaust could be reduced when coexposed with ultraviolet-A (UVA) in a cellular system. However, the mechanisms underlying synergistic effects remain to be clarified, especially in an *in vivo* system. In the present study, using *Caenorhabditis elegans* (*C. elegans*) as an *in vivo* system we studied the synergistic effects of diesel particulate extract (DPE) plus UVA, and the underlying mechanisms were dissected genetically using related mutants. Our results demonstrated that though coexposure of wild type worms at young adult stage to low doses of DPE (20 μg/mL) plus UVA (0.2, 0.5, and 1.0 J/cm^2) did not affect worm development (mitotic germ cells and brood size), it resulted in a significant induction of germ cell death. Using the strain of *hus-1::gfp*, distinct foci of HUS-1::GFP was observed in proliferating germ cells, indicating the DNA damage after worms were treated with DPE plus UVA. Moreover, the induction of germ cell death by DPE plus UVA was alleviated in single-gene loss-of-function mutations of core apoptotic, checkpoint HUS-1, CEP-1/p53, and MAPK dependent signaling pathways. Using a reactive oxygen species (ROS) probe, it was found that the production of ROS in worms coexposed to DPE plus UVA increased in a time-dependent manner. In addition, employing a singlet oxygen ($^1O_2$) trapping probe, 2,2,6,6-tetramethyl-4-piperidone, coupled with electron spin resonance analysis, we demonstrated the increased $^1O_2$ production in worms coexposed to DPE plus UVA. These results indicated that UVA could enhance the apoptotic induction of DPE at low doses through a DNA damage-triggered pathway and that the production of ROS, especially $^1O_2$, played a pivotal role in initiating the synergistic process.

INTRODUCTION

Diesel exhaust, the dominant pollutant in ambient air, has been classified as a "potential" or "probable" human carcinogen by the International Agency for Research in Cancer.[1] Studies have found that diesel exhaust particles (DEPs) are associated with various health effects such as inflammation of the respiratory tract, lung cancer and cardiovascular diseases,[2−5] and their extracts, diesel particulate extract (DPE), are thought to be mainly responsible for these malignant effects.[3,6,7] DPE is a complex mixture composed of hundreds of organic chemical compounds including polycyclic aromatic hydrocarbons (PAHs), quinines, ketones, heterocyclic compounds, aldehydes, and other unidentified constituents,[8,9] many of which are promutagens that require subsequent activation by biotic and abiotic factors to show their mutagenic or carcinogenic effects.[10−15] For instance, the organic DEP extract and oxidized phospholipids synergistically affected the expression profile of several genes involved in pathways relevant to vascular inflammatory processes.[13] DEPs and bacterial lipopolysaccharides were reported to synergistically induce the generation of free radicals and neutrophilic inflammation in the lungs of rats.[14] The methtylation of T helper genes and IgE production were changed when mice were exposed to DEPs in combination with an allergen.[15] Our previous study also showed that in the human−hamster hybrid system, the cytotoxicity and genotoxicity of DPE at a low dose (20 μg/mL) could be activated by environmental physical factor ultraviolet A (UVA) radiation (0.5 J/cm^2).[16]

Ultraviolet (UV) radiation (UV-A, 320−400 nm; UV-B, 280−320 nm; UV-C, <290 nm) is the carcinogenic component of sunlight, and 95% of UV reaching the surface of earth is

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UVA.[12] Relative to the high carcinogenicity of UVB, UVA is usually considered to be less carcinogenic due to the weak absorption of UVA by DNA molecules.[17] However, recent evidence showed that UVA also caused various forms of DNA damage, such as cyclobutane pyrimidine dimers, single strand breaks, and DNA−protein cross-links and 8-oxoguanine in mammalian cells.[18,19] Furthermore, it was reported that UVA-induced DNA damage can be enhanced in the presence of either endogenous or exogenous photosensitizers, such as the diuretic agent hydrochlorothiazide and lomefloxacin.[18−20] Although the exposure to either diesel exhaust or UVA radiation alone or in combination with other agents has been identified as an essential risk factor for various benign or malignant human diseases,[20−22] the synergistic effects of diesel exhaust and UVA remain to be clarified, especially in an *in vivo* system.

Caenorhabditis elegans (*C. elegans*), a free-living nematode, is a simple multicellular eukaryote. Because of its short life cycle, small size of body, transparent body, and easy of cultivation in a laboratory, *C. elegans* has been adopted as an excellent model *in vivo* for toxicological tests and environmental evaluation.[23] Importantly, *C. elegans* shares cellular and molecular structures and signaling pathways with higher organisms; thus, biological information learned from *C. elegans* may be directly applicable to more complex organisms.[23] Moreover, genetically deficient strains of *C. elegans* are easily available, which facilitates further genetic dissection for the molecular mechanisms underlying the related biological events. Within *C. elegans*, the germ line is an intrinsic part of oogenesis, which establishes an unbroken chain between generations.[24] Abnormal germ line development, such as the induction of germ line apoptosis, would not only harm the organism but also disturb the species balance from generation to generation.[25−27] Normally, germ cell apoptosis occurs physiologically under normal conditions.[24] However, upon environmental stresses germ cell apoptosis was also induced sensitively through the signaling pathways that are distinct genetically from physiological apoptosis. It was reported that genotoxic insults (such as ionizing radiation, UV radiation, mutagens, oxidative stresses, heat, and salt etc.) induced germ line apoptosis likewise employed core apoptotic components but was dependent on the DNA damage checkpoint HUS-1 and regulator CEP-1.[28−31] In the present study, with the level of germ cell apoptosis as a main checking end point, our results showed that the coexposure of L4-stage or young adult worms to DPE plus UVA at low doses significantly enhanced the induction of germ cell apoptosis. The induction of germ cell apoptosis by DPE plus UVA might be triggered by DNA damage and involve ERK, JNK, and p38/MAPK signaling pathways.

■ MATERIALS AND METHODS

Worm Strains and Growth. Wild type *C. elegans* strain Bristol N2 was used for general experiments. In addition, the mutant strains *ced-3(n717)* and *ced-4(n1162)* were used for determining the nature of germ cell death. Strains with single-gene mutations of DNA damage-induced germ cell death machinery, *cep-1(w40)*, *cep-1(lg12501)*, and *hus-1(op241)*, were employed for investigating the signaling pathways involved in the induction of germ cell death by DPE and/or UVA. A worm line transgenic for *hus-1::gfp*, WS1433: *hus-1(op241)* I; *unc-119(ed3)*III; *opIs34*, was used for detecting the DNA damage in germ cells. Moreover, the strains deficient in the extracellular signaling-regulated protein kinases (ERK) signaling cascade, *lin-45(ku51)*, *mek-2 (n1989)*, and *mpk-1 (ku1)*; Jun N-terminal kinases (JNK) signaling cascade, *mek-1 (ks54)*, *jnk-1 (gk7)*, and *mkk-4 (ju91)*; and p38 MAPK signaling cascade, *nsy-1 (ag3)*, *sek-1 (ag1)*, and *pmk-1 (km25)*, were also adopted.

Maintenance and genetic manipulation of *C. elegans* were carried out according to the standard procedures as described by Brenner.[32] All strains were grown at 20 °C on nematode growth medium (NGM) and fed with the bacterium *Escherichia coli* OP50. To obtain synchronized cultures, gravid hermaphrodites were lysed in an alkaline hypochlorite solution.

DPE Preparation. In the present study, DPE (standard reference material 1975) was provided by the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). SRM 1975 is a dichloromethane extract of the diesel particulate matter SRM 2975, which was generated by a forklift truck using an industrial diesel-powered engine and collected under specifically designed heavy-duty conditions (NIST 2000).

Exposure of Worms to DPE Plus UVA. The procedures for worm handling and chemical exposure were conducted as described previously.[33] Briefly, DPE was diluted to final concentrations in K-medium (containing 52 mM NaCl and 32 mM KCl). For the measurement of apoptosis,[34] the mitotic germ cells,[35] the brood size,[35] the foci of *hus-1::gfp*,[36] and the production of ROS,[37,38] age-synchronized young hermaphrodites were transferred into 30 mm-diameter Petri dishes containing K-medium with OP50 as a food source and treated with either DPE (20−400 μg/mL) or UVA (0.2−5.0 J/cm^2) alone or in combination (DPE + UVA) for determined times at 20. For the measurement of body size, the life span, and the percentage of adult worms, the hatched L1-stage larvae were employed to investigate the possible developmental effects of DPE plus UVA.[39,40] In the DPE plus UVA groups, worms were pretreated with 20 μg/mL DPE for 1 h and then irradiated with a determined dose of UVA. For UVA radiation, three UV lamps (BLE-IT151, Spectronics Co., Westbury, New York, USA) with an emission wavelength peak at 365 nm were used. The dishes were placed on a table that was 15 cm away from the UV lamps. During UV exposure, the dose rate was simultaneously measured by a radiometer (Photoelectric Instrument Factory of Beijing Normal University, Beijing, China) with a 365 nm detector located the same distance as the culture plates from the UV source. The worms were then grown at 20 °C for further testing.

Germ Cell Death/Apoptosis Assay. Germ cell corpses were measured by acridine orange (AO, Sigma) staining using a modified procedure developed by Kelly et al.[34] Briefly, the treated worms were stained for 1 h in the dark at 20 °C by transferring worms into a Costar 24-well plate containing 500 μL of 25 μg/mL AO and OP50 in M9 buffer (3 g of KH_2PO_4, 6g of Na_2HPO_4, 5 g of NaCl, 1 mL of 1 M $MgSO_4$, and H_2O to 1 L) and then transferred to NGM and allowed to recover for 40 min on bacterial lawns also in the dark. AO staining positive cell corpses were assessed under an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan). The apoptotic cells appeared yellow or yellow-orange, representing increased DNA fragmentation, while intact cells were uniformly green in color.

Mitotic Germ Cell Assessment. The procedures used to assess mitotic germ cells were developed by Craig et al.[35] To clearly assess the mitotic germ cells, the dissected gonads were stained by 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) for 10 min in the dark, rinsed 3 times for 5 min in PBST (PBS and 0.1% Tween-20), mounted in mounting solution (90% glycerol, 20 mM Tris at pH 8.0, and 1 mg/mL *p*-phenylenediamine), and then covered with a coverslip. The mitotic germ cells within 20-cell distance from the distal tip cell were counted under an Olympus IX71 fluorescence microscope.

Brood Size Assay. The procedures for brood size assay were conducted as described by Craig et al.[35] Synchronized young adult hermaphrodites were treated with either DPE (20 μg/mL) or UVA (0.2, 0.5, and 1.0 J/cm^2) alone or in combination (DPE + UVA) for 24 h. Worms were then transferred individually onto a NGM plate containing a bacterial lawn 1 cm in diameter in the center of the dish. The adult worms were removed onto a fresh NGM plate daily or every other day, and the number of eggs and hatched F1 larvae were counted under a dissection microscope. The brood size was calculated by combining the number of embryos and hatched larvae.

Body Size and Life Cycle Assay. The growth of *C. elegans* was measured according to Traunspurger et al.[39] Worms were photographed under a stereomicroscope equipped with a CCD camera at the time point of 72 h after L1-stage larvae were treated with either DPE (20 μg/mL) or UVA (0.2, 0.5, and 1.0 J/cm^2) alone or in combination (DPE + UVA). The body size was determined by measuring the flat surface area of the worms using ImageJ software. The life cycle was assayed by counting the percentage of adult worms in each treatment.

Life Span Assay. The life span was tested as described previously.[40] L1-stage larvae were treated with either DPE (20 μg/mL) or UVA (0.2, 0.5, and 1.0 J/cm^2) alone or in combination (DPE + UVA) throughout their life. In the experiment, worms were cultured individually in 96-well plates using OP50 as food at 20 °C. When the hermaphrodites developed to the gravid stage, they were transferred to fresh plates every other day to avoid confusing them with their progenies. Worms were checked every day and would be scored as dead when they would not respond to tapping with a pick.

DNA Damage Measurement. DNA damage in the *C. elegans* germ line was assessed with the strain *hus-1::gfp* as described previously.[36] Synchronized young adult hermaphrodites were treated with either DPE (20 μg/mL) or UVA (0.5 J/cm^2) alone or in combination (DPE + UVA) for 24 h. Worms were then mounted onto microscope slides in 0.2 mM of Levamisole (Sigma), and foci were counted in a single Z stack under a laser confocal microscope (LSM710 ZEISS, Germany), where about 40 mitotic germ cells in *C. elegans* were observed. Each experiment scored at least 40 germlines.

Effects of ROS Quenchers on the Induction of Germ Cell Apoptosis by DPE Plus UVA. The procedures were conducted as previously described.[37] Age-synchronized young hermaphrodites were treated with 0.5% and 1.0% dimethyl sulfoxide (DMSO) or 10 μM and 100 μM sodium azide (NaN_3) with or without concurrent treatment with DPE (20 μg/mL) for 1 h and then irradiated with UVA (0.5 J/cm^2). Then germ cell apoptosis was counted as described above. The dose of DMSO and NaN_3 in the present study was nontoxic and nonmutagenic.

Measurement of ROS Production *in Situ* in *C. elegans*. The level of ROS in *C. elegans* was measured with 2,7-dichlorodihydrofluorescein diacetate (DCF-DA), which is a general molecular probe that is used as an indicator of global ROS flux in intact animals.[37,38] After treatment, the worms were transferred into the wells of a Costar 24-well microtiter plate (black, clear, and flat-bottom wells) containing DCF-DA (final concentration of 10 μM in PBS) and incubated for 30 min in the dark at 20 °C. The relative fluorescence for worms was individually determined and analyzed using an Olympus IX71 fluorescence microscope with a CCD camera and Image-Pro Plus, version 6.0.

Analysis of $^1O_2$ in *C. elegans* by Electron Spin Resonance (ESR) Spectra. To detect $^1O_2$, we used the trap probe 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMP; purity of 95%). The probe, which has been shown to be specific for $^1O_2$ detection, reacts with $^1O_2$ to yield a stable nitroxide radical 4-oxo-2,2,6,6-tetramethylpiperidine-*N*-oxyl (4-O-TEMPO), having a known three-line ESR spectrum.[41] Age-synchronized young adult hermaphrodites were treated with DPE (20 μg/mL) for 1 h at 20 °C, and then TEMP (Sigma; 0.05 M) or the stable radical 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO; 10^{-6} M; Sigma) was added 30 min before UVA radiation. The treated worms were collected immediately and transferred into 25 μL capillaries after radiation. To eliminate the interference of $^1O_2$ generation in the culture medium, the remaining medium in capillaries was removed with filter paper.[42] Samples in 25 μL capillaries inserted into 4 mm quartz tubes were used for ESR analysis. ESR spectra were recorded at room temperature on a EMX-10/12 ESR spectrometer (Bruker, German). The measurements were repeated at least three times for each sample. We set the microwave source of the ESR at 9.0 GHz and the power at 3.0 mW. Modulation frequency and modulation amplitude were 100 kHz and 0.1 mT, respectively. The time constant was 0.3 s, and scan time was 120 s. The relative signal intensity of 4-O-TEMPO is represented by dividing the ratio of the 4-O-TEMPO signal intensity of the treated group by that of the control group.

Data Analysis. All experiments were performed at least three independent times. Values were expressed as the means ± standard error. Significant differences at the $P < 0.05$ level were tested using ANOVA followed by Tukey's multiple comparison test. For comparisons between different strains, statistical analysis was performed with 2-factor ANOVA with Dunnett's *t* tests.

■ RESULTS

Induction of Germ Cell Death in *C. elegans* Treated with DPE or UVA. DPE or UVA has been reported to exhibit significant genotoxicity and cytotoxicity in several cell models.[6,10] In this study, the genotoxicity of DPE or UVA was assessed with germ cell death as an end point. As shown in Figure 1, following treatment with DPE ranging from 20 to 50 μg/mL, germ cell death exhibited a basal level compared to that of the control populations (in all cases, $P > 0.05$), whereas the higher doses of DPE led to significant increases in the level of germ cell death in a dose-dependent manner (in all cases, $P < 0.05$). Similarly, exposure to UVA at low doses made no

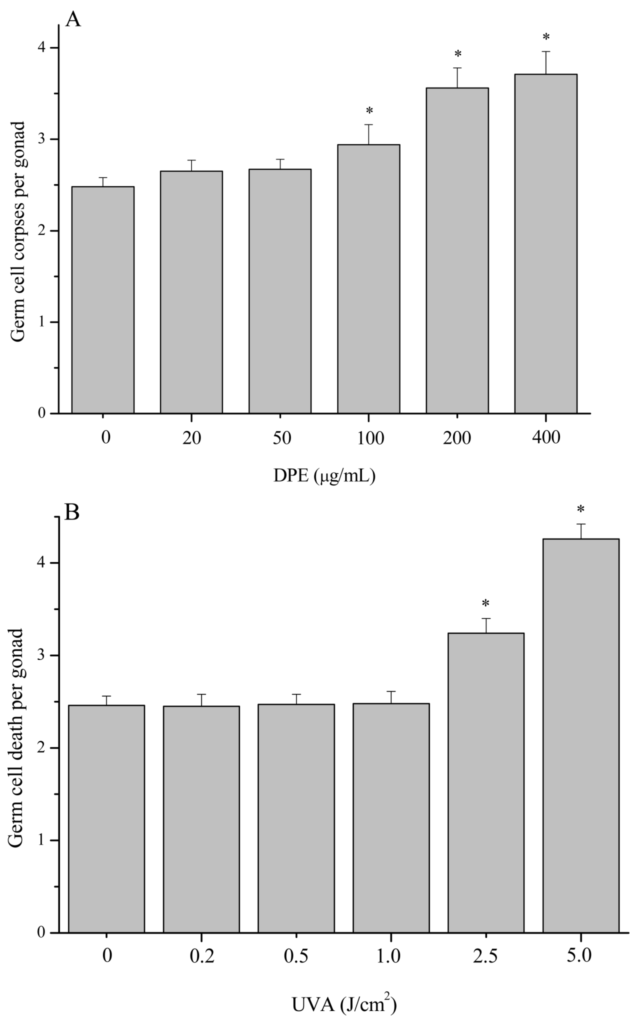

Figure 1. Effects of DPE or UVA on germ cell death in *C. elegans*. Synchronized young adult hermaphrodites were exposed to the indicated doses of DPE (A) or UVA (B), and germ cell corpses were scored 24 h after exposure. Data were pooled from at least three independent experiments. All values are presented as the means ± SE; $n \geq 40$, and * represents $P < 0.05$.

difference in germ cell death (in all cases, $P > 0.05$), and significant increases were observed when the exposure doses exceeded 2.5 J/cm^2 (in both cases, $P < 0.05$). The results agreed with our previous reports that the treatments with DPE at 20 μg/mL or UVA less than 1.0 J/cm^2 caused little toxic and mutagenic effects in the cell culture system.[16]

Synergistic Effects of Low-Dose Exposure to DPE Plus UVA on Germ Cell Death. To further clarify whether there are synergistic effects on the induction of germ cell death by low-doses of DPE plus UVA, worms at young adulthood were exposed to low doses of DPE plus UVA and then checked for the induction of germ cell death 24 h after cotreatment. As shown in Figure 2A, the application of 20 μg/mL DPE + 0.5 J/cm^2 UVA and 20 μg/mL DPE + 1.0 J/cm^2 UVA both led to a significantly enhanced induction of germ cell death (in both cases, $P < 0.05$).

Moreover, to avoid the interference of germ cell proliferation by DPE plus UVA on germ cell death, the numbers of mitotic germ cells were examined in the distal germ line.[35] As shown in Figure 2B, there was no significant changes in the number of mitotic germ cells in the groups of 20 μg/mL DPE + 0.2 J/cm^2 UVA, 20 μg/mL DPE + 0.5 J/cm^2 UVA, and 20 μg/mL DPE + 1.0 J/cm^2 UVA compared with that in the untreated worms (in all cases, $P > 0.05$). The results suggested that the enhanced germ cell death induced by DPE plus UVA was not due to the reduction of mitotic germ cell proliferation.

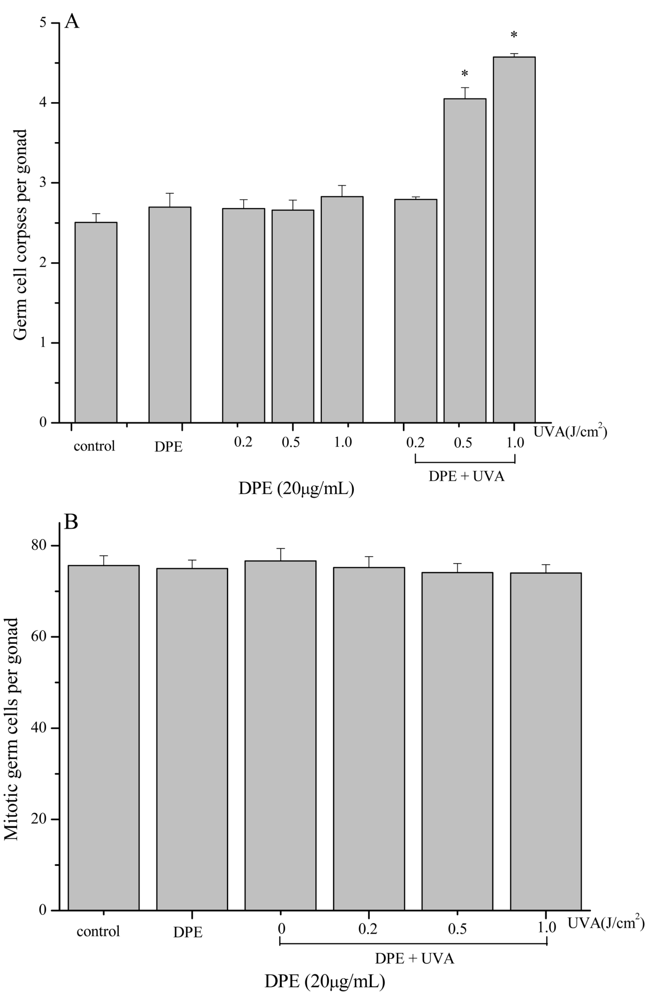

Figure 2. Synergistic germ cell death and cell cycle arrest induced by DPE plus UVA. Synchronized young adult hermaphrodites were treated with DPE and/or UVA, and germ cell death (A) and mitotic germ cells (B) were scored 24 h after exposure. Data are pooled from three independent experiments. All values are presented as the means ± SE; $n \geq 20$, and * represents $P < 0.05$.

Time Course of Germ Cell Death Induced by Coexposure to DPE Plus UVA. In *C. elegans*, the spatial and temporal organization of the germ line allows one to investigate damage effects in various meiotic progressions through a reverse time course analysis.[24] As shown in Figure 3A, compared to the control or single-treated populations, the worms coexposed to 20 μg/mL DPE + 0.2 J/cm^2 UVA exhibited slight increases in germ cell death at the time points of 6 and 12 h (in both cases, $P < 0.05$) and recovered to the basal level at the time points of 24 and 36 h (in both cases, $P > 0.05$). However, for the groups of 20 μg/mL DPE + 0.5 J/cm^2 UVA and 20 μg/mL DPE + 1.0 J/cm^2 UVA, the worms both exhibited significant increases in germ cell death at all of the tested time points (in all cases, $P < 0.05$), and the largest induction of germ cell death occurred at the time point of 24 h.

Germ Cell Death Induced by Coexposure to DPE Plus UVA Was Apoptotic Death. To further clarify the nature of germ cell death induced after coexposure to DPE plus UVA, *C. elegans* strains with single-gene mutations of the *ced-3(n717)* and *ced-4(n1162)* genes were employed. CED-3 and CED-4 are two critical components of the core apoptotic pathway within *C. elegans*.[43] As shown in Figure 4, the synergistic induction of germ cell death was significantly inhibited in both *ced-3(n717)* and *ced-4(n1162)* mutant strains (in both cases, $P > 0.05$), suggesting that the germ cell death induced by coexposure to DPE plus UVA might be apoptotic death in nature.

Coexposure of Worms to DPE Plus UVA Had Little Effect on Worm Development. Environmental stresses could modify the developmental processes when the larvae were exposed to toxicants either in embryonic development or early developmental stages.[39] In *C. elegans*, germ cell apoptosis commences in early adulthood and increases over time.[24] To exclude the changes of background value, we investigated the developmental effects by DPE plus UVA at different stages. As shown in Figure 2B and Figure 5A, worms coexposed to DPE plus UVA at the L4 stage had little effect on the index of mitotic germ cells and brood size. In addition, the body size and the life span of worms exposed to DPE plus UVA at the L1 stage were not changed obviously as well (Figure 5B and C). However, there was a slight decrease in the percentage of adult worms compared to that in the single treatment of DPE or UVA, or to the control (in all cases, $P > 0.05$) when worms were coexposed to DPE plus UVA at the L1 stage (Figure 5D). The results indicated that the enhanced levels of germ cell apoptosis after coexposure to DPE plus UVA at the late stage did not result from the modification of the developmental procedure.

Synergistic Induction of Germ Cell Apoptosis by DPE Plus UVA through DNA Damage Machinery. The classic DNA damage-induced germ cell death machinery has been reported to be involved in the induction of apoptosis in addition to physiological germ cell apoptosis in *C. elegans*.[35,44] To clarify whether *C. elegans* employed this death machinery for the induction of germ cell apoptosis after coexposure to DPE plus UVA, worm strains with single-gene loss-of-function mutations of this death machinery, *cep-1(w40)*, *cep-1(lg12501)*, and *hus-1(op241)*, were used. As shown in Figure 6A, in the worms with null mutations of the *hus-1* and *cep-1* genes, the

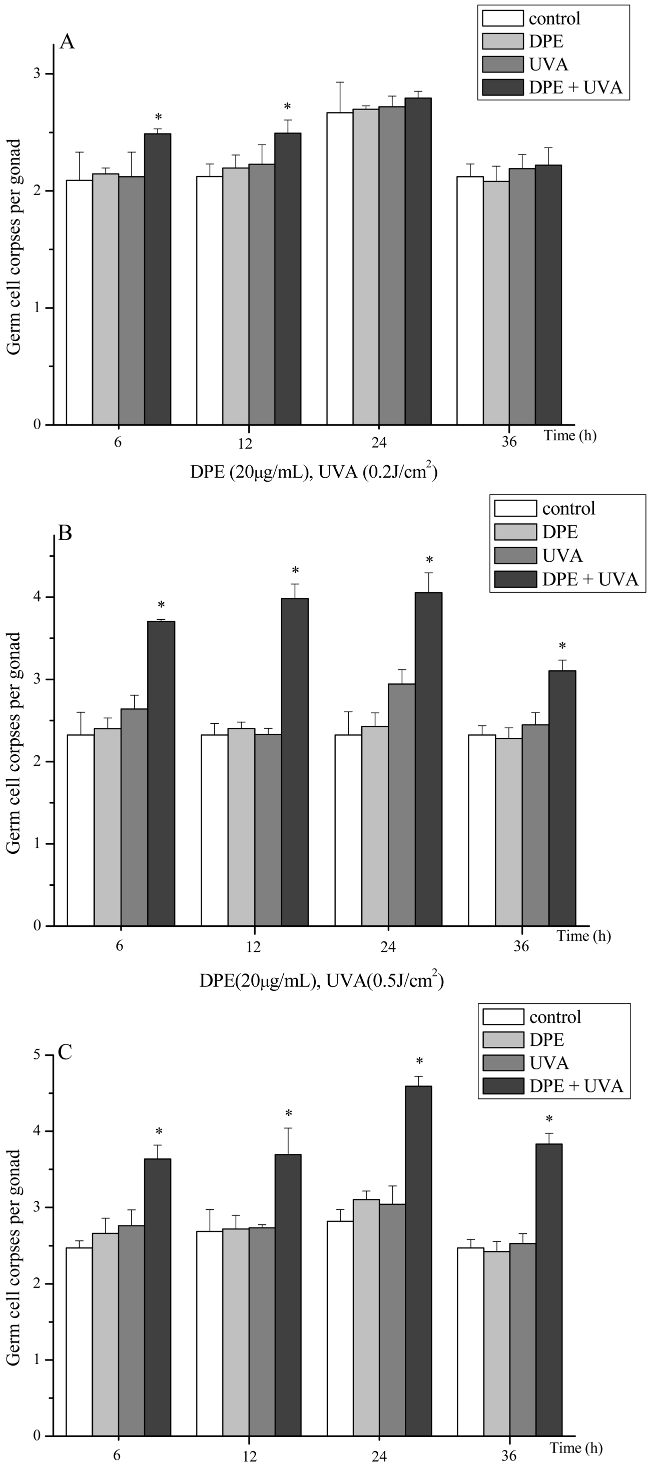

Figure 3. Time course of germ cell death in *C. elegans* induced by 20 μg/mL DPE + 0.2 J/cm^2 UVA (A), 20 μg/mL DPE + 0.5 J/cm^2 UVA (B), and 20 μg/mL DPE + 1.0 J/cm^2 UVA (C). Synchronized young adult hermaphrodites were exposed to DPE, UVA, or DPE + UVA, and germ cell corpses were scored at time points of 6, 12, 24, and 36 h, respectively. Data are pooled from three independent experiments. All values are presented as the means ± SE; $n \geq 20$, and * represents $P < 0.05$.

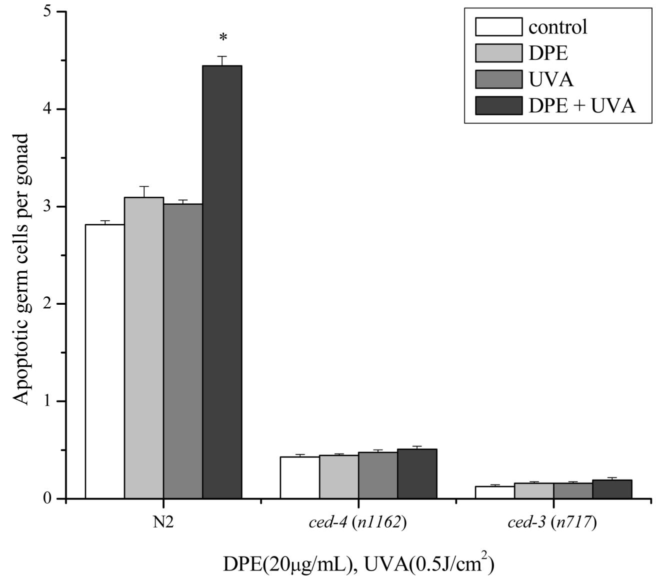

Figure 4. Germ cell death induced by DPE plus UVA was apoptotic cell death. The muations of the *ced-3* and *ced-4* genes significantly inhibited the induction of germ cell death by exposure to DPE plus UVA. Data were pooled from at least three independent experiments. All values are presented as the means ± SE; $n \geq 40$, and * represents $P < 0.05$.

induction of germ cell death was significantly inhibited after coexposure to DPE (20 μg/mL) plus UVA (0.5 J/cm^2) (in all cases, $P > 0.05$), while the wild type and the strain with partial loss-of-function of the *cep-1* gene showed a significant induction of germ cell apoptosis.

To further determine the role of DNA damage in the induction of germ cell apoptosis by DPE plus UVA, the worms transgenic for *hus-1::gfp* were employed. In the *C. elegans* germ line, HUS-1::GFP diffuses in proliferating germ nuclei, which relocalize and form distinct foci following DNA damage.[36] As shown in Figure 6B, distinct foci of HUS-1::GFP could be observed in a small number of mitotic germ cells at the time point of 24 h after worms were coexposed to DPE (20 μg/mL) plus UVA (0.5 J/cm^2) but nearly none in the single treatment of DPE or UVA, or in the control worms. These results indicated that the DNA-damage-induced germ cell death machinery played a pivotal role in the synergistic induction of germ cell apoptosis by DPE plus UVA.

MAPK Signaling Pathways Took Part in the Induction of Germ Cell Apoptosis by Coexposure to DPE Plus UVA. It has been shown that the P53 protein can functionally interact with the mitogen-activated protein kinases (MAPKs).[45] Once MAP kinases are activated, they function as effectors to phosphorylate and activate p53, leading to a p53-mediated cellular response, including apoptosis.[45] To explore the possible role of MAPK signaling pathways in the induction of germ cell apoptosis of DPE plus UVA, the strains with the loss-of-function of genes related to MAPK pathways were used. The MAPK signaling pathways mainly include ERK, JNK, and p38 MAPK cascades in *C. elegans*.[46] In *C. elegans*, LIN-45 (MAPKKK), MEK-2 (MAPKK), and MPK-1 (MAPK) are the components of the ERK signaling pathway.[47] As shown in Figure 7A, the worm strains with loss-of-function of the *lin-45(ku51)*, *mek-2 (n1989)*, and *mpk-1 (ku1)* genes exhibited a basal level of germ cell apoptosis after coexposure to DPE plus UVA compared to that of their respective controls (in all cases, $P > 0.05$). JKK-1 and MEK-1 are members of MAPK kinase (MAPKK), and JNK-1 is a member of the JNK homologue.[48] In our experiments, the loss-of-function of these genes

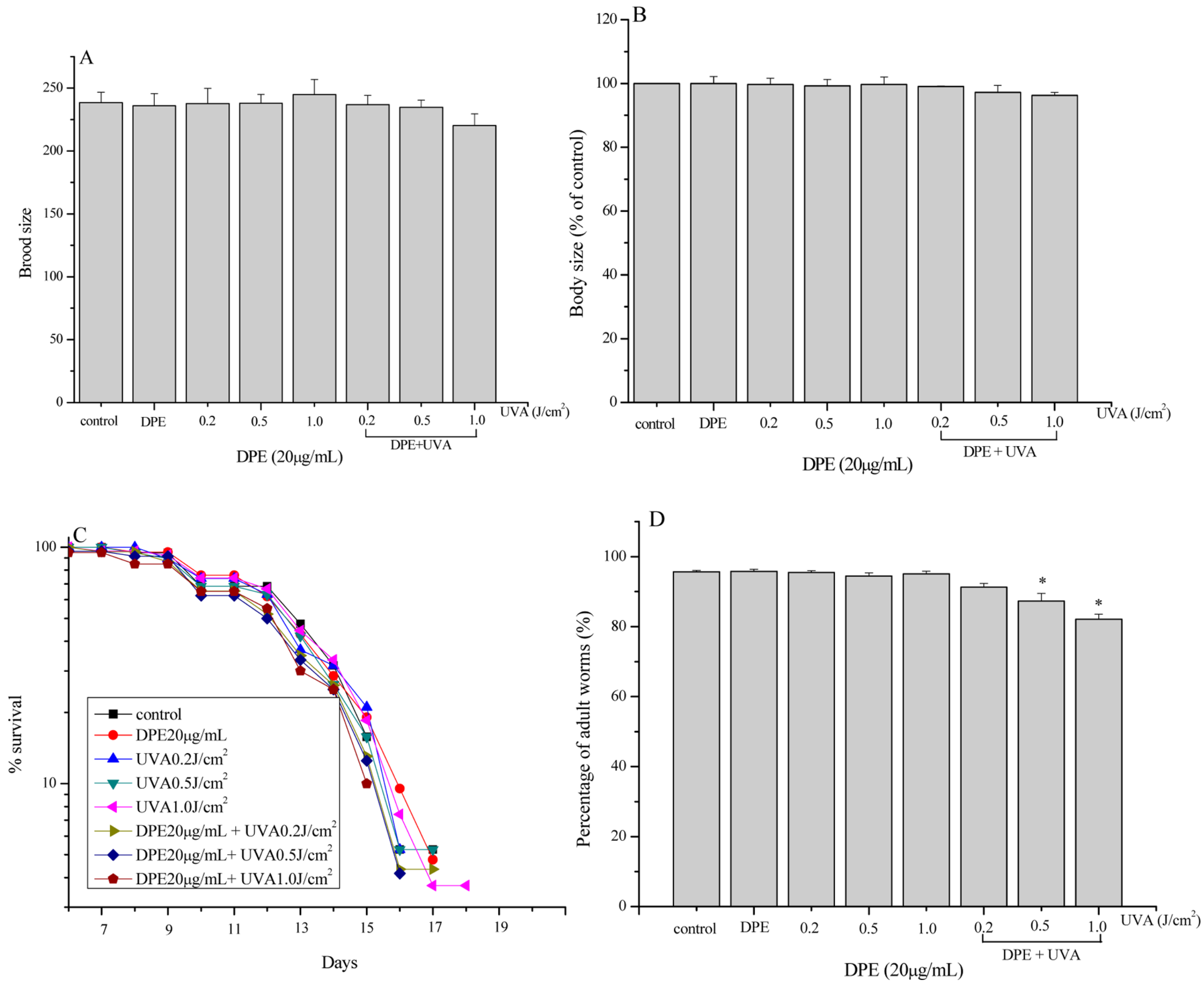

Figure 5. Effects of DPE plus UVA on the worms' development. (A) Age-synchronized young hermaphrodites were treated with either DPE (20 μg/mL) or UVA (0.2−1.0 J/cm^2) alone or in combination (DPE + UVA) for 24 h at 20 °C, then the brood size was counted. (B) The body sizes were determined by measuring the flat surface area of the worms using ImageJ software, and there was no difference among all treatments after L1-stage larvae were treated with DPE and/or UVA for 72 h. (C) Life span curves of worms and (D) the percentage of adult worms were scored after L1-stage larvae were treated with DPE and/or UVA for 72 h. Data were pooled from three independent experiments. All values are presented as the means ± SE; $n \geq 20$, and * represents $P < 0.05$.

significantly inhibited the induction of germ cell apoptosis by coexposure to DPE plus UVA (in all cases, $P > 0.05$), as shown in Figure 7B. In the p38 MAPK pathway of *C. elegans*, NSY-1 encodes a MAPK kinase kinase (MAPKKK), SEK-1 is a member of MAPKK, and PMK-1 is the p38 MAPK homologue.[49] In the present study, the strains with single-gene loss-of-function mutations of the *nsy-1 (ag3)*, *sek-1 (ag1)*, and *pmk-1 (km25)* genes were coexposed to DPE plus UVA, respectively, and no significant induction of germ cell apoptosis was observed in all of the mutant strains (in all cases, $P > 0.05$), as shown in Figure 7C. The results suggested that MAPK signal pathways, including ERK, JNK, and p38/MAPK, might play a pivotal role in the induction of germ cell apoptosis by coexposure to DPE plus UVA.

Role of ROS, Especially $^1O_2$, in the Synergistic Induction of Germ Cell Apoptosis by DPE Plus UVA. ROS was reported to activate the mitogen-activated protein kinases, and played an important role in the induction of DNA damage.[48,50] To find out the role of ROS in the induction of germ cell apoptosis by DPE plus UVA, the ROS quenchers, NaN_3 and DMSO, were employed. As shown in Figure 8A, the induction of germ cell apoptosis by coexposure to DPE (20 μg/mL) + UVA (0.5 J/cm^2) was significantly inhibited in the presence of NaN_3 (in both cases, $P < 0.05$) but only partially inhibited in the presence of DMSO (in both cases, $P > 0.05$). In addition, the production of ROS in individual worm coexposure to DPE plus UVA increased in a time-dependent manner and reached the highest level at a time point of 24 h compared with that of the control or single-treated populations and decreased afterward (Figure 8B and C).

Since NaN_3 has been found to be an efficient quencher for singlet oxygen ($^1O_2$),[51,52] we further analyzed the $^1O_2$ production by the $^1O_2$ trapping probe, 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMP), coupled with electron spin resonance (ESR) spectroscopy.[16] As shown in Figure 8D and E, 4-O-TEMPO triplet spectra and the relative signal intensity

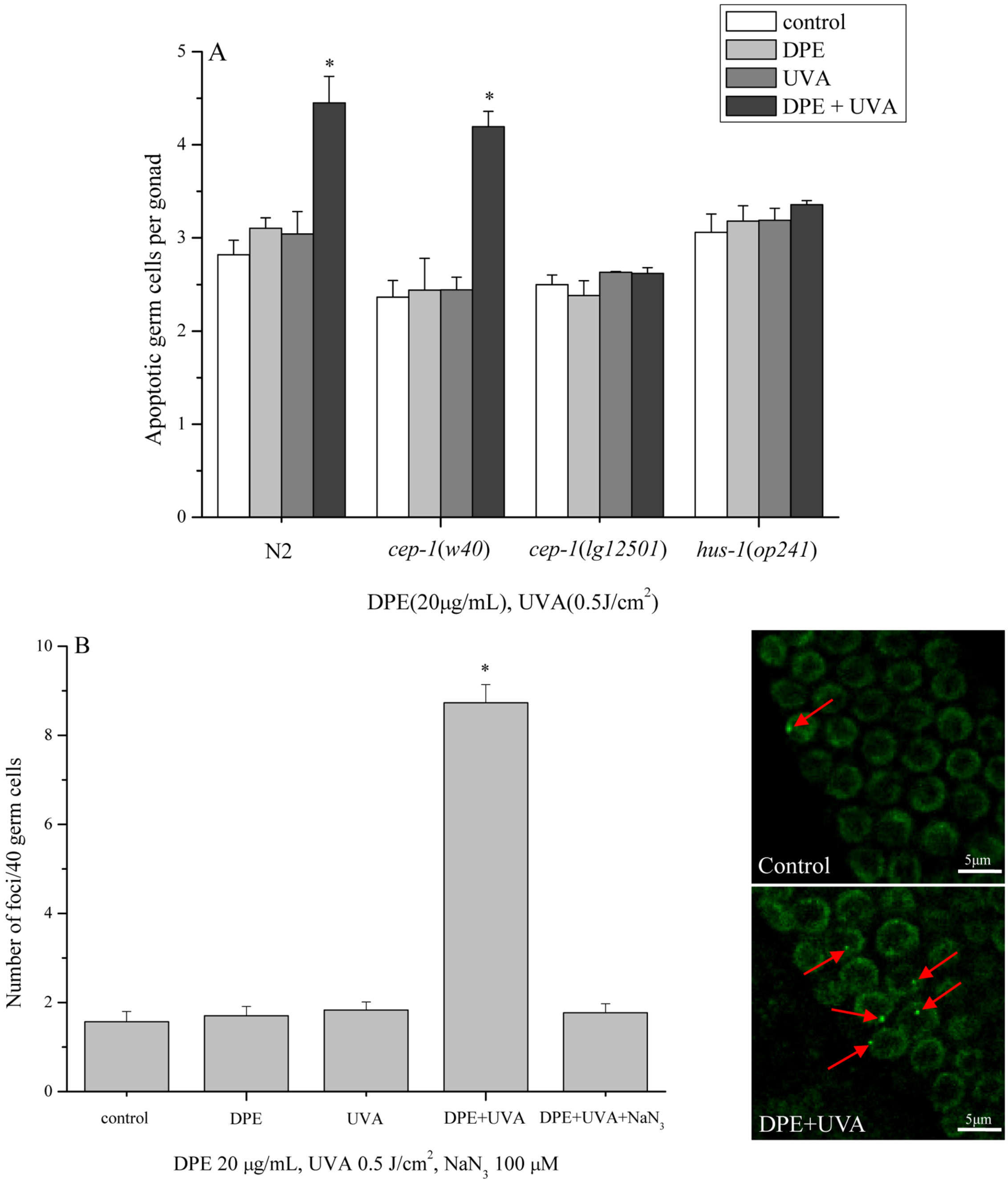

Figure 6. Role of DNA damage in the induction of germ cell apoptosis by exposure to DPE, UVA, or DPE + UVA. (A) There was significantly enhanced induction of germ cell apoptosis in the partial loss-of-function strain of *cep-1(w40)*, while the null mutation strains of *hus-1(op241)* and *cep-1(lg12501)* significantly inhibited the induction of germ cell apoptosis by DPE plus UVA. (B) Quantification of HUS-1::GFP foci in the mitotic germ cells after worms were treated with DPE plus UVA for 24 h. Foci were scored in 40 proliferating germ cells. Fluorescent microscopy of proliferating germ cells expressing HUS-1::GFP. HUS-1::GFP diffuses in control worms. Distinct foci of HUS-1::GFP could be observed in a small number of the mitotic germ cells in *C. elegans* coexposed to DPE plus UVA at the time point of 24 h. The scale bar represents 5 μm. These results suggested that the classic DNA damage-induced germ cell death machinery might be employed in germ cell apoptosis induced by DPE plus UVA. Data were pooled from at least three independent experiments. All values are presented as the means ± SE; $n \geq 40$, and * represents $P < 0.05$.

increased considerably in worms coexposed to DPE (20 μg/mL) plus UVA (0.5 J/cm^2) compared with those in the single treatment of DPE or UVA, or with the control worms, and NaN_3 (100 μM) significantly reduced this signal ($P < 0.05$). Taken together, the results indicated that the ROS, especially $^1O_2$, played a pivotal role in the induction of germ cell apoptosis within *C. elegans* by coexposure to DPE plus UVA.

■ DISCUSSION

Epidemiologic studies have shown that exposure to diesel exhaust is associated with various health effects, such as cancer induction.[2−5] However, the cytotoxicity and genotoxicity of DPE in *in vitro* or *in vivo* studies were normally discovered at relatively higher doses. It was reported that cell death and apoptosis in macrophages were only significantly enhanced following an exposure dose of DPEs higher than 100 μg/

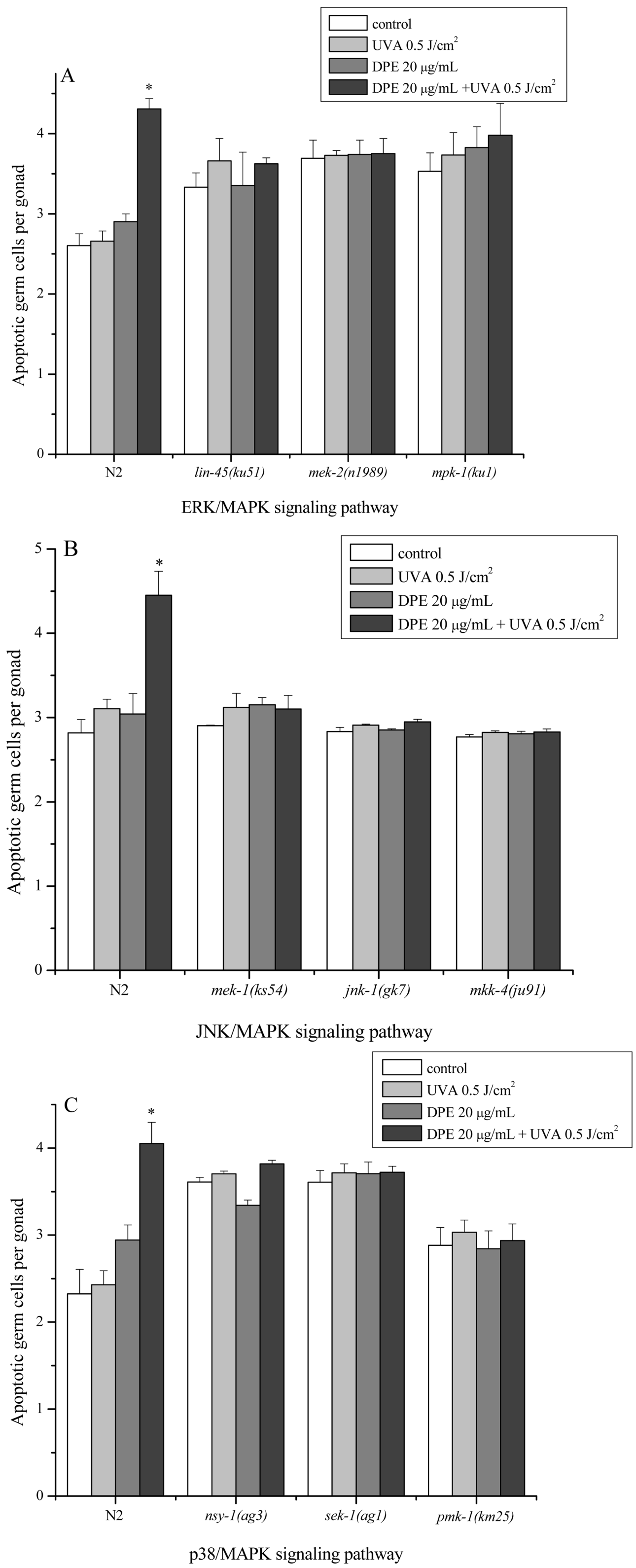

Figure 7. Induction of germ cell apoptosis by exposure to DPE, UVA, or DPE + UVA in worms deficient in ERK (A), JNK (B), and p38/MAPK (C) signaling pathways. Germ cell apoptosis was significantly inhibited in all of the mutant strains after exposure to DPE plus UVA, suggesting that the MAPK signaling pathways play a pivotal role in germ cell apoptosis induced by DPE plus UVA. Data were pooled from at least three independent experiments. All values are presented as the means ± SE; $n \geq 20$, and * represents $P < 0.05$.

mL.[53,54] The organic extract of DEPs at the dose of 140 μg/mL increased ROS production in human neutrophil granulocytes and rat alveolar macrophages *in vitro* assayed with DCFH-DA.[55] Consistent with these results, we found that a significant induction of germ cell death was only shown at a dose of DPE greater than 100 μg/mL. In our previous study, we found that lower concentrations of DPE could manifest its cytotoxicity (10 μg/mL) and genotoxcity (20 μg/mL) in an A_L cell culture system with 0.5 J/cm^2 of UVA radiation.[16] The question is whether the deleterious effects of diesel exhaust could be manifested by the environmental factor of UVA at low doses in an *in vivo* system, as well as the underlying mechanisms. With a *C. elegans* system, we further demonstrated that the cyto- and genotoxicity of low-dose exposure of DPE could be activated synergistically by UVA radiation (0.5 J/cm^2) in the context of the whole organism. It is notable that this dose of UVA radiation is much lower than those to show the genetic effects in single-exposure experiments (>24 J/cm^2).[56,57]

For the activation of DPE by UVA radiation in synergistic effects, one of the important ways is through photoactivation. After absorbing sufficient UVA light energy, xenobiotics in DPE can be elevated from ground state to an excited state. The excited molecules can not only react with biological molecules but also transfer their energy to molecular oxygen to create ROS.[58] It was reported that benzo[α]pyrene, a component of DPE, became highly toxic or carcinogenic in *in vitro* and *in vivo* experiments in the manner of photoactivation.[59,60] Moreover, some components of DPE can also be metabolically activated. Their metabolic products, such as diol epoxides and diones, are highly carcinogenic and can induce covalent DNA adducts and oxidative DNA lesions.[61] Metabolically activated xenobiotics in DPE also exerted stimulatory or toxic effects via the generation of ROS.[62,63] By employing a ROS probe (DCF-DA) and quenchers (NaN_3 and DMSO), the present study found that ROS levels in worms coexposed to DPE (20 μg/mL) plus UVA (0.5 J/cm^2) significantly increased in a time-dependent manner, and the induction of germ cell apoptosis in worms treated with DPE plus UVA was effectively restored to the basal level but not for 0.5% and 1.0% DMSO treatment groups (Figure 8A). NaN_3 has been reported to be an efficient $^1O_2$ quencher, and DMSO mainly eliminates the effect of the hydroxyl radical (HO·).[51,52,64] Price et al. showed that 1 mM NaN_3 efficiently quenched $^1O_2$ formation in Murine leukemia L1210 cells, while 1.0% DMSO had no effect.[64] To further elucidate the pivotal role of $^1O_2$, using a $^1O_2$ trapping probe, TEMP, coupled with ESR spectroscopy, we found increased $^1O_2$ production in worms coexposed to DPE plus UVA. These results indicated that the production of ROS, especially $^1O_2$, played a pivotal role in the induction of germ cell apoptosis by DPE plus UVA in *C. elegans*, which was consistent with the previous findings that $^1O_2$ was mainly responsible for UVA-activated toxicity of DPE in mammalian cells.[16]

In *C. elegans*, germ line apoptosis could be physiological and also stress-induced.[24,35,65] Unlike stress-induced apoptosis, physiological germ cell apoptosis is a highly controlled process, which commences in early adulthood and increases over time.[24] As physiological germ cell apoptosis that is usually scored as background value in the measurement of germ cell death could be affected with worm development,[24,35] it is quite important to assess the modification of developments by DPE plus UVA under different worm stages. By exposing worms at the L1 or young adult stage, it was found that worms coexposed to DPE plus UVA at young adult stage had little effect on the index of

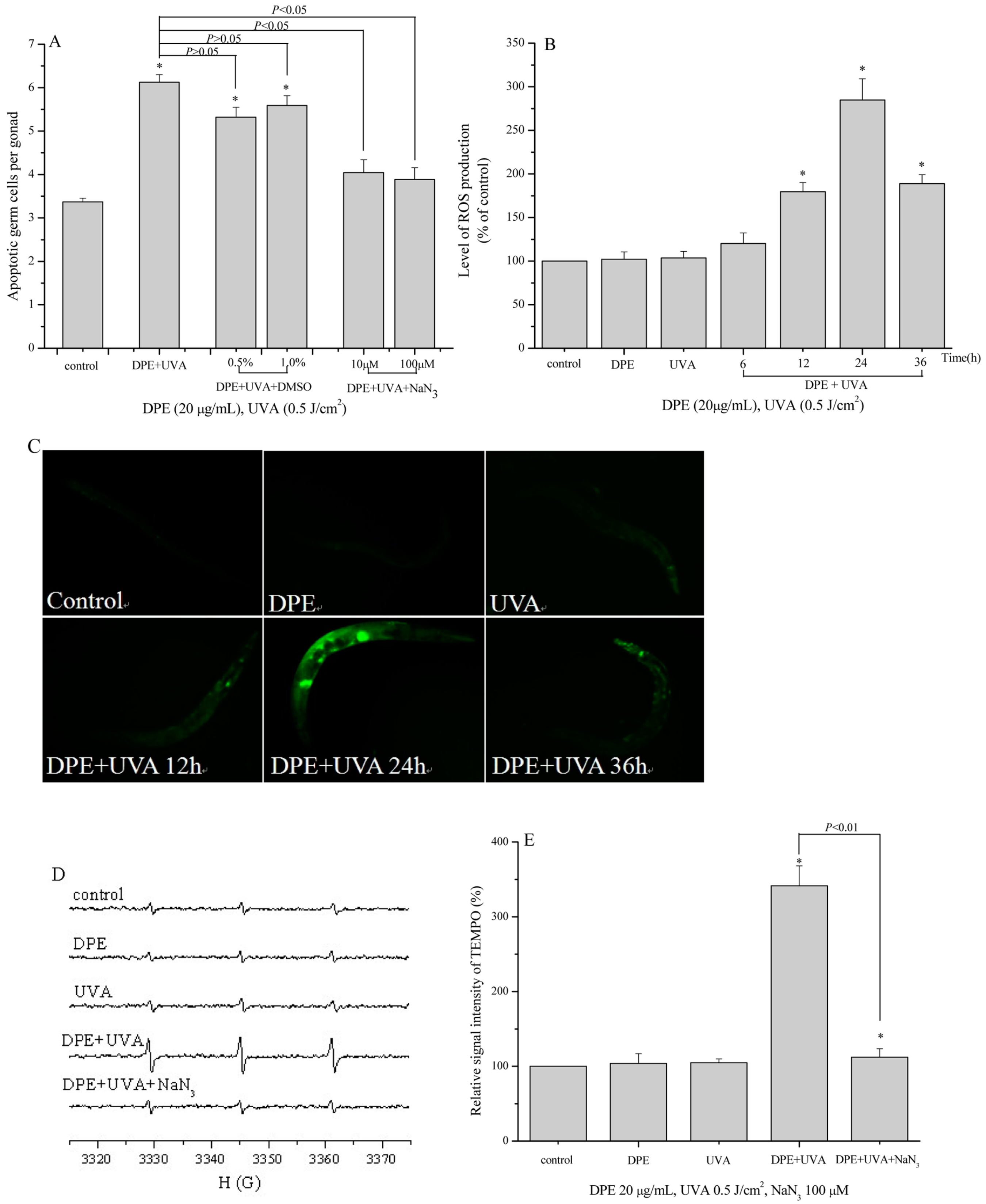

Figure 8. ROS, especially $^1O_2$, play a crucial role in germ cell apoptosis induced by DPE (20 μg/mL) plus UVA (0.5 J/cm^2) . (A) The induction of germ cell apoptosis by DPE plus UVA was effectively rescued by NaN_3, a specific $^1O_2$ scavenger. (B) The *in situ* expression of fluorescence was measured using DCF-DA (a molecular probe) in single whole worms. (C) The relative fluorescence was determined using Image-Pro Plus, version 6.0. (D) Three-line ESR spectra of the 4-O-TEMPO signal. (E) Relative signal intensity of 4-O-TEMPO. All these results suggested that ROS, especially $^1O_2$, play a pivotal role in the induction of germ cell apoptosis by DPE plus UVA. Data were pooled from at least three independent experiments. All values are presented as the means ± SE; $n \geq 40$, and * represents $P < 0.05$.

the mitotic germ cells and the brood size. In addition, there were no effects on the body size and the life span when worms were exposed at the L1 stage (Figure 5B and C). However, a slight decrease was found in the percentage of adult worms compared to the single treatment of DPE or UVA, or to the control (in all cases, $P > 0.05$) when worms were exposed at the L1 stage (Figure 5D). These findings were consistent with the results by Xing et al., showing that a significant decrease in locomotion was observed after L1-stage larvae were exposed to Pb and Hg at a concentration of 2.5 μM, while no obvious difference was observed in young adult worms exposed to 100 μM of the examined metals.[66] Therefore, germ cell apoptosis induced by UVA plus DPE in young adult worms in the present study was not interfered, or was less, by the changes of physiological germ cell apoptosis. To find out the nature of apoptosis induced by DPE plus UVA, we used mutant strains, such as DNA damage response checkpoint protein HUS-1 and the regulator CEP-1/p53. It has been reported that UV radiation-induced germ cell apoptosis in *C. elegans* was dependent on both the CEP-1/p53 and the checkpoint HUS-1.[67,68] Although there is no evidence yet for the role of CEP-1/p53 in the induction of germ cell apoptosis by DPE in *C. elegans*, p53-dependent cell apoptosis was reported in the J774A.1 macrophage cell line after exposure to DPE.[69] In the present study, the lack of induction of germ cell apoptosis by coexposure in *hus-1* and *cep-1* mutants suggested that DNA-damage-induced germ cell death machinery was involved in the synergistic induction of germ cell apoptosis by DPE plus UVA. The enhanced induction of germ cell apoptosis in the W40 strain might be due to the partial loss-of-function of *cep-1* and could not effectively and completely block damage signaling transduction.[70] Moreover, using the strain of *hus-1::gfp*, we found that distinct foci of HUS-1::GFP could be observed in a small number of mitotic germ cells after worms were coexposed to DPE (20 μg/mL) plus UVA (0.5 J/cm^2) (Figure 6B). HUS-1 is a part of the 9:1:1 complex, which encodes one of the checkpoint proteins that act as the DNA damage sensors in *C. elegans*. It was reported that HUS-1::GFP diffuses in proliferating germ nuclei and can be relocalized to distinct foci following DNA damage.[36] Hence, the foci of HUS-1::GFP in *C. elegans* germ cells indicated clearly that DNA-damage-induced germ cell death machinery played a pivotal role in the synergistic induction of germ cell apoptosis by DPE plus UVA. Furthermore, the decreased survival rates in the F1 progenies of young adult worms with DPE (20 μg/mL) plus UVA (0.2, 0.5, and 1.0 J/cm^2) also proposed the occurrence of DNA damage in the process (Figure S1, Supporting Information).

In addition to the oxidative damage to DNA molecules, increased oxidative stress (ROS) can also activate MAPK signaling cascades.[65,71] In this study, the MAPK signaling pathways including ERK, JNK, and p38 MAPK were shown to take part in the synergistic induction of germ cell apoptosis by DPE plus UVA. Each of them is essential for germ cell apoptosis induced by coexposure to DPE plus UVA, and blockage of any one can inhibit induction, suggesting an elaborate cooperation among three signal cascades in the synergistic induction of germ cell apoptosis. It has been shown that activation of MAPKs can phosphorylate and activate a number of signaling pathways, including p53.[45] Therefore, in light of the above results, we hypothesize that synergistic germ cell apoptosis induced by DPE plus UVA in *C. elegans* occur via DPE plus UVA-induced ROS generation that activates MAPK signaling pathways; subsequently, activation of p53 induces *ced-4* and *ced-3*, which finally leads to apoptosis. Moreover, it is not excluded that these signaling pathways were separately used by the DPE plus UVA-initiated events due to their distinct activation mechanisms. In addition, the blockage of DNA-damage-induced signaling pathway (HUS-1) could also inhibit the synergistic induction of germ cell apoptosis in the presence of MAPK signaling pathways, suggesting interplay between two types of signaling pathways. This might be another possible reason for the necessity of each signaling pathway for the induction of germ cell apoptosis by coexposure to DPE plus UVA.

In summary, our results suggested that UVA radiation synergistically enhanced the toxicity of DPE at low-dose exposures in the context of the animal *in vivo*. The synergistic induction of germ cell apoptosis by DPE plus UVA should mainly be triggered by DNA damage, and the DPE plus UVA generated ROS, especially $^1O_2$, might be one of the factors that lead to DNA damage. These data might have some significant implications for exactly assessing the health risk of diesel exhaust and for adopting protective measures for the population exposed to diesel exhaust.

■ ASSOCIATED CONTENT

Supporting Information

The effect of DPE plus UVA on the survival rate of F1 progenies, the germ cell death induced by NaN_3, and quantification of HUS-1::GFP foci in all mitotic germ cells in *C. elegans*. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*(P.B.) Phone: 86-551-65591602. Fax: 86-551-65595670. E-mail: ljw@ipp.ac.cn.
*(L.W.) Phone: 86-551-65591602. Fax: 86-551-65595670. E-mail: ljw@ipp.ac.cn.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AO, acridine orange; *C. elegans*, *Caenorhabditis elegans*; DDR, DNA damage response; DEPs, diesel exhaust particles; DMSO, dimethyl sulfoxide; DPE, diesel particulate extract; ERK, extracellular signaling-regulated protein kinase; ESR, electron spin resonance; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NaN_3, sodium azide; NGM, nematode growth medium; $^1O_2$, singlet oxygen; 4-O-TEMPO, 4-oxo-2,2,6,6-tetramethyl-piperidine-*N*-oxyl; PAHs, polycyclic aromatic hydrocarbons; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TEMP, 2,2,6,6-tetramethyl-4-piperidone; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; UVA, ultraviolet A

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