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Polyethylene glycol used as a dispersant potentiates the toxicity of insecticides in mammalian cells rather than insects

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ABSTRACT

Insecticides are used in household products with various dispersants such as polyethylene glycol (PEG) and polyoxyethylene lauryl ether (PLE) to improve solubility. Although certain effects are expected, the combination effects of insecticides and dispersants remain elusive. Here, five different classes of insecticides (i.e., dinotefuran, fipronil, hydramethylnon, indoxacarb, and etofenprox) were dispersed in water, PEG, and PLE, and their lung inflammation potential was evaluated by bronchoalveolar lavage fluid analysis 24 h after intratracheal instillation into the lungs of rats. All chemicals dispersed in water caused no inflammation. However, among the five chemicals dispersed in PEG and PLE, only hydramethylnon showed significant neutrophilic inflammation and hydramethylnon in PEG showed 4-fold higher inflammogenic potential than that in PLE. The *in vitro* cytotoxic potential of hydramethylnon in PEG was 10–17 fold (in A549) or 12–14 fold (in dTHP-1) higher than that of hydramethylnon in PLE, and greater than 370 fold (in A549) or 65–169 fold (in dTHP-1) higher than that in water. PEG toxicity increased due to the micellar formulation of hydramethylnon in PEG, increasing cellular uptake by simple diffusion. Therefore, the observed potentiation effect highlights that the combination effect of formulation of hydrophobic compounds with dispersants should be carefully evaluated.

1. Introduction

Insecticides control insects in the agricultural, industrial, and domestic sectors to improve crop yields and quality of life. They are available as sprays, baits, dust, and gels, and their household usage has rapidly increased (Stejskal et al. 2021). Although insecticides are designed to benefit humans by protecting against various insect-spread diseases in crops, animals, and humans, their overuse in multiple fields can harm many species and damage ecosystems (Rezende-Teixeira et al. 2022; Yadav 2010). Furthermore, improper knowledge, poor storage, and excessive use of household chemicals contribute to indoor exposure and cause health risks (Sarwar 2016). Indoor air pollution from various sources including pesticides and volatile organic compounds is one of the greatest threats to public health, and upper-bound risks are much greater than the health risks associated with most other environmental problems (Wallace 1991). Furthermore, the tragic incidents related to humidifier disinfectants in South Korea warn regarding the safety of household chemicals, particularly inhalation exposure routes (Kim et al. 2014). The use of insecticides has exponentially increased owing to the coronavirus disease 2019 (COVID-19) pandemic (Choi et al. 2024) and the recent endemic outbreaks of bed bugs (Cimicidae), which have caused public concerns (Hasnaoui et al. 2023) not only about the effects of insects and pathogenic agents on human health, but also about resistance to insecticides in target organisms (Lewis et al. 2023).

Abbreviations: PEG, polyethylene glycol; PLE, polyoxyethylene lauryl ether; BALF, bronchoalveolar lavage fluid; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)–2H-tetrazolium; LDH, lactate dehydrogenase; MIP-1β, macrophage inflammatory protein-beta; IL, interleukin; TNF-α, tumor necrosis factor; SD, standard deviation; PBS, phosphate-buffered saline; DW, distilled water; FBS, fetal bovine serum; dTHP-1, differentiated THP-1; DMEM, Dulbecco's Modified Eagle's Medium; RPMI-1640, Roswell Park Memorial Institute 1640; EC, effective concentration.

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Among the various classes of synthetic insecticides, neonicotinoids, oxadiazines, phenylpyrazoles, pyrethroids, and amidinohydrazones are widely used because they are effective in controlling insects and are less toxic to humans (Hollingshaus 1987; Sparks and Bryant 2022). Neonicotinoids, including acetamiprid, dinotefuran, and imidacloprid, kill insects by inhibiting the nicotinic acetylcholine receptor (Casida 2018). Oxadiazines, such as indoxacarb, are broad-spectrum insecticides that block voltage-gated sodium channels (von Stein et al. 2013). Phenylpyrazoles, such as fipronil and ethiprole, block γ-aminobutyric acid-gated chloride channels (Bloomquist 2001). Pyrethroids, such as permethrin and cyfluthrin, are excitotoxic to axons by disrupting voltage-gated sodium channels (Richardson et al. 2019). Amidinohydrazones, such as hydramethylnon, are a metabolic inhibitor (Hollingshaus 1987). Furthermore, these chemicals are mixed in various household products, and many are used for spraying (Pomés and Schal 2020), as the mixture of multiple insecticides with different classes has advantages in controlling insects resistant to certain pesticides (Sparks and Nauen 2015).

Recent investigations on human exposure scenarios to biocides using a biocidal or household chemical product have shown that it contains multiple biocides in various combinations (Kim et al. 2021). According to European Union and South Korea chemical database investigations, 286 types of biocides have been used in combination with biocidal products, and 68 types have been used in household products, such as cleaning products, laundry products, and air fresheners (Kim et al. 2021). Etofenprox, hydramethylnon, and fipronil are among the most prevalent products of inhalation exposure in South Korea (Kim et al. 2021). However, this information can be underestimated because labeling and disclosure of the ingredients of these products are limited. Although humans are exposed to multiple chemicals and dispersants in combination with household products, their combined toxic effects remain largely unclear and require careful investigation.

Dispersants such as polyethylene glycol (PEG) and polyoxyethylene lauryl ether (PLE) have been used as nonionic surfactants in biocidal and household products to improve solubility, efficacy, storage, handling, and safety (Kato-Namba et al. 2023; Lang et al. 2024). Although these dispersants are considered inert and have no direct effect on the intrinsic properties of insecticides, their combined effects remain largely unclear, and many of the data generated by the manufacturers are kept confidential. However, even generally regarded as an inert dispersant like PEG can cause adverse effects in various organs such as the brain, lungs, and kidneys (Leth and Gregersen 2005). On the other hand, PEG has antagonistic effects on the toxicity of chlorpyrifos to common carp (*Cyprinus carpio*) (Hatami et al. 2019). Thus, the toxicity of PEG and its use in combination with insecticides should be carefully investigated. Furthermore, the toxic effects of PLE alone and in combination with other insecticides remain unexplored. Therefore, this study aims to investigate the comparative toxic effects of 5 different classes of insecticides w/wo dispersion with PEG or PLE using an *in vivo* acute lung inflammation model and *in vitro* cytotoxicity model. The test insecticides were dinotefuran (neonicotinoid), fipronil (phenylpyrazole), hydramethylnon (trifluoromethyl aminohydrazone), indoxacarb (oxadiazine), and etofenprox (pyrethroid).

2. Materials and methods

2.1. Selection of a panel of insecticides and dispersants

Five different classes of insecticides used in an inhalable spray product were selected: dinotefuran (neonicotinoid), fipronil (phenylpyrazole), hydramethylnon (trifluoromethyl amino hydrazone), indoxacarb (oxadiazine), and etofenprox (pyrethroid). Distilled water (DW), PEG 400, and PLE were selected as representative dispersants used in household products. Information regarding the test materials is presented in Table 1. All insecticides and dispersants were purchased from Sigma-Aldrich (St. Louis, MO, USA). The selected insecticides were dispersed in different dispersants, including water, 10 % PEG, and 0.625 mg/mL PLE. Dispersants applied in this study (i.e., PEG and PLE) were tested at a reasonable dose or concentration because the use of dispersants at extremely high levels is highly unlikely in the products. To prepare water dispersion, all insecticides were mixed in water at 80°C for fipronil, hydramethylnon, indoxacarb, and etofenprox or at 30 – 37 °C for dinotefuran. Then, the mixture was sonicated for 20 min at a controlled temperature using a water bath sonicator (Saehan Sonic, Seoul, Korea). The temperature applied for dispersion did not affect the structure of chemicals because all are stable under 140°C (Table 1). To prepare PEG dispersion, insecticides in PEG 400 were prepared by dissolving insecticides in 100 % PEG at 2 mg/mL, and solutions were sonicated using a bath sonicator (Saehan Sonic) for 15 min at room temperature. Further, DW was added at 1:9 (insecticide:DW; *v/v*) to make up the final concentration of 10 % PEG and sonicated for 15 min. To prepare PLE dispersion, the 1.25 mg/mL PLE in DW was prepared, and insecticides were dispersed in the prepared PLE at 5 mg/mL and sonicated for 15 min using a bath sonicator (Saehan Sonic). Subsequently, insecticides in PLE were diluted with DW at 1:1 (*v/v*) to make up the final concentration of 2.5 mg/mL insecticides and PLE (0.625 mg/mL). Further sonication for 15 min was applied to provide better dispersion and was used for experiments immediately after dispersion (e.g., within 1 min of preparation). The hydrodynamic size and dispersion stability of the final working solution for *in vivo* and *in vitro* studies were evaluated with the dynamic light scattering method using the ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK).

Table 1
The information on the test insecticides and dispersants used in this study.

Chemicals	Class	CAS No	Formula	Molecular weight (g/mol)	Solubility in water	Temperature (°C) stability
Dinotefuran	Neonicotinoid	165252–70-0	$C_7H_{14}N_4O_3$	202.21	39.83 g/L (EPA 2004)	185 (Salazar and jara 2020)
Fipronil	Phenylpyrazole	120068–37-3	$C_{12}H_4Cl_2F_6N_4OS$	437.1	1.9 mg/L	140 (Moyo et al. 2022)
Hydramethylnon	Trifluoromethyl aminohydrazone	67485–29-4	$C_{25}H_{24}F_6N_4$	494.5	6 μg/L	185 – 190
Indoxacarb	Oxadiazine	144171–61-9	$C_{22}H_{17}ClF_3N_3O_7$	527.8	0.20 mg/L	191 (Yang et al. 2021)
Etofenprox	Pyrethroid	80844–07-1	$C_{25}H_{28}NO_3$	376.5	<1 μg/L (Hwang et al. 2015)	200 (EFSA 2008)
PEG 400[a]	–	25322–68-3	$(C_2H_4O)_nH_2O$	380 – 420	100 %	180 (Royer and Gelin 2015)
PLE	–	9002–92-0	$C_{58}H_{118}O_{24}$	1199.5	100 %	100[a]

Unless otherwise specified, all data were collected from the PubChem website (https://pubchem.ncbi.nlm.nih.gov).
[a] Data were collected from the manufacturer (Sigma-Aldrich).

2.2. Intratracheal instillation of insecticides with different dispersants

The *in vivo* acute lung inflammation model using the rat intratracheal instillation method was applied to evaluate insecticide's acute toxic potentials and their mode of action in relation to the different dispersants. The deposition rates of test chemicals immediately after the intratracheal instillation in this study are likely to be more than 75 %, estimated from our previous publications with poorly soluble nanomaterials (Lee et al. 2020; Lee et al. 2024). Briefly, 6-week-old pathogen-free male Sprague Dawley rats were purchased from Central Laboratory Animal (Seoul, Korea) and acclimatized for a week before experimentation. Animals were maintained at 23 ± 1°C in an environment with 40–60 % humidity. All animal procedures were approved by the Institutional Animal Care and Use Committee of Dong-A University (DIACUC-2022–22-16). Intratracheal instillation was performed as previously described (Kim et al. 2020). Briefly, the rats were anesthetized using isoflurane in a rodent anesthesia system (VetEquip, Pleasanton, CA, USA) and the trachea was intubated using a 16-gage polycarbonate catheter (BD Biosciences, San Jose, CA, USA). Then, the solutions were instilled into the lungs of the rats using a 1 mL syringe. The instillation volume was 500 μL for all treatment groups. As the maximum feasible dose of insecticides in water was about 2 mg/mL and the instillation volume was 500 μL, insecticides in water were treated to rats at 0.5 and 1 mg/rat. In contrast, all insecticides in PEG had feasible doses higher than 1 mg/rat. Therefore, all insecticides were administered at a dose of 1 mg/rat for the first instance. Lower doses were tested when the insecticides induced lung inflammation. Finally, the PLE formulation was tested only when insecticides in PEG were toxic. Although the maximum feasible dose of insecticides in PEG and PLE was higher than 1.5 mg/rat, the higher dose was not tested because higher doses caused death due to neurotoxicity.

2.3. Evaluation of acute lung inflammation potential of insecticides

The lung inflammation patterns and comparative inflammation potential of insecticides with different dispersants were evaluated by bronchoalveolar lavage fluid (BALF) analysis at 24 h post-instillation. At 24 h post-instillation, the rats were anesthetized with isoflurane and euthanized by dissection of the inferior vena cava. A 14-gauge stainless catheter was inserted into the trachea and tightly tied using an elastic suture string. The lungs were lavaged four times with ice-cold phosphate-buffered saline (PBS). BALF samples were centrifuged at 250 × g for 5 min, and the supernatant of the first lavage was used for biochemical and cytokine analyses. Cell pellets from all lavages were resuspended in 1 mL PBS containing 10 % fetal bovine serum (FBS), and the total number of cells was counted using a NucleoCounter (Chemometec, Allerod, Denmark). A total of 4×10^4 cells were attached to a clean glass slide (Marienfeld, Lauda-Königshofen, Germany) at 15 × *g* for 5 min using Cytospin (Hanil, Seoul, Korea) and fixed in methanol for 5 min. Fixed cells were stained using Diff–Quik (Thermo Fisher Scientific, Waltham, MA, USA), and 300 cells per slide were counted using a light microscope (Nikon, Tokyo, Japan).

2.4. Measurement of the biochemical parameters and pro-inflammatory cytokines in BALF

Lactate dehydrogenase (LDH) and total protein levels were measured in the supernatant of the first lavage fluid to evaluate cytotoxicity and vascular permeability, respectively (Lee et al. 2016). LDH levels were measured using an LDH assay kit (Roche Diagnostics, Mannheim, Germany) and expressed as fold-changes versus a vehicle control group. Total protein levels were quantified using a bicinchoninic acid assay kit (Thermo Fisher Scientific) according to the instruction manual. When insecticides significantly increased inflammation, the levels of pro-inflammatory cytokines related to the acute inflammation, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, were measured using the commercial enzyme-linked immunosorbent assay (ELISA) kits. All the kits were purchased from R&D Systems (Minneapolis, MN, USA).

2.5. Evaluation of the effects of dispersants on the toxicity of hydramethylnon in vitro

The potentiating effect of dispersants on the toxicity of hydramethylnon, as shown in the animal experiment, was further confirmed using *in vitro* studies, including A549 lung epithelial cells and differentiated THP-1 (dTHP-1) macrophage-like cells. A549 cells (European Collection of Animal Cell Cultures, Salisbury, UK) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5 % FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 U/mL streptomycin. The cells were seeded in a 96-well plate at a density of 1×10^5 cells/mL. After 24 h of incubation, cells were washed with pre-warmed PBS three times and treated with different concentrations of hydramethylnon in DMEM. The working solution of hydramethylnon for A549 cells was prepared by serial dilution of the stock solution, which was prepared by dispersing hydramethylnon in water, 10 % PEG, or 0.625 mg/mL PLE at a 5-fold higher than the treatment concentrations. In this experiment, we tested hydramethylnon in different dispersants because, among the five insecticides, only hydramethylnon caused significant inflammation in rats. After 24 h of incubation with hydramethylnon, cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)–2H-tetrazolium (MTS) assay kit (Promega, Madison, WI, USA) and an LDH assay kit (Roche Diagnostics), according to the manufacturer's instructions. On the other hand, THP-1 cells (American Type Culture Collection; Rockville, MD, USA) were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) containing 10 % FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 U/mL streptomycin. Cells were seeded to a 96-well plate at 5×10^5 cells/mL in RPMI medium containing 10 ng/mL of phorbol myristate acetate (PMA; Sigma-Aldrich) to differentiate into macrophages. After 48 h of incubation, cells were washed with pre-warmed PBS three times and treated with different concentrations of hydramethylnon in RPMI-1640. The preparation of working solutions was performed with the same protocols applied in A549 cells. The viability of the dTHP-1 cells was measured after 24 h of treatment using an MTS assay kit (Promega) and an LDH assay kit (Roche Diagnostics). The effective concentration 20 % (EC_{20}) levels of cytotoxicity were selected as a toxicity endpoint, and the values were adapted only when Pearson's correlation coefficients in the dose–response curve fit were higher than 0.8. The linear regression plots and R^2 values are presented in Supporting Information (Figures S1 and S2 and Table S1). In addition, the levels of pro-inflammatory cytokines, including IL-1β, TNF-α, and macrophage inflammatory protein (MIP)-1β, were measured using Duoset ELISA kits (R&D Systems) according to the instruction manual.

2.6. Measurement of cellular uptake of hydramethylnon with different dispersants in vitro

Changes in the cellular delivery of active ingredients by different dispersants may be a possible mechanism of the combination effect. Thus, the cellular uptake levels of hydramethylnon with different dispersants were evaluated in A549 and dTHP-1 cells. Briefly, A549 cells at 1×10^5 cells/mL and dTHP-1 cells at 5×10^5 cells/mL were seeded in a 6-well plate. Then, hydramethylnon at 2 μg/mL was dispersed in DMEM (A549 cells) or RPMI-1640 (dTHP-1 cells) according to the same protocol applied in the cytotoxicity assays. This treatment dose was confirmed to be non-lethal for both cell lines. At 24 h after incubation, the cells were washed three times with pre-warmed PBS and lysed with a cell lysis buffer (RIPA buffer, Sigma-Aldrich). The solutions were then centrifuged at 250 × *g* for 5 min, and supernatants were used for quantification with a Xevo TQ-S micro liquid chromatography LC-MS/MS (Waters, Milford, MA, USA). The LC-MS/MS experiment was

performed from the contract research with the Center for Industrialization of Agricultural and Livestock Microorganisms (Jeollabuk-do, Korea).

2.7. Evaluation of the cellular delivery mechanism of hydramethylnon

The mechanism of cellular delivery of hydramethylnon dispersed in PEG was evaluated by treating cells with cytochalasin-D, a chemical used to block cellular phagocytosis (Cho et al. 2013). Briefly, dTHP-1 cells at 5×10^5 cells/mL were prepared and washed three times with pre-warmed PBS. Then, 2 μg/mL of PEG-formulated hydramethylnon in RPMI-1640 w/wo 0.25 μM of cytochalasin-D (Sigma-Aldrich) were treated for 24 h, and the cytotoxicity and cellular uptake levels were evaluated according to methods described above.

2.8. Statistical analysis

The data are presented as mean ± standard deviation (SD). GraphPad Prism software (ver. 9.2.0; La Jolla, CA, USA) was used for graphs and statistical analysis. One-way ANOVA with post-hoc Tukey analysis was used to compare between groups. Dispersion stability between time 0 and 1 h post-dispersion was analyzed with a non-parametric Mann-Whitney *U* test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Hydrodynamic size and dispersion stability of five insecticides with different dispersants

Among the five insecticides, fipronil was excluded from water dispersion because of its poor solubility in water. In water dispersion, the hydrodynamic sizes of four insecticides ranged 344 – 1337 nm (Fig. 1A). The efficacy of water dispersion was in the order of dinotefuran ≈ etofenprox ≈ hydramethylnon > indoxacarb. On the other hand, although PEG showed slightly better dispersion than PLE, both dispersants produced an improved dispersion than water (Fig. 1B and C). The hydrodynamic sizes of all tested chemicals in PEG and PLE ranged 12 – 2378 nm and 51 – 2673 nm, respectively. The dispersion efficacy of test chemicals dispersed in PEG was in the order of dinotefuran > hydramethylnon > etofenprox ≈ indoxacarb > fipronil (Fig. 1B). While that of in PLE was dinotefuran > hydramethylnon ≈ indoxacarb ≈ etofenprox > fipronil (Fig. 1C). In addition, the dispersion stability of tested chemicals was maintained for 1 h (Fig. 1A – C). Thus the intratracheal instillation was performed with inhalable particle size with a stable dispersion condition because the instillation was performed immediately after dispersion. Likewise, *in vitro* studies with hydramethylnon in RPMI-1640 and DMEM medium were performed in a consistent dispersion status, although hydramethylnon in DW showed a slightly

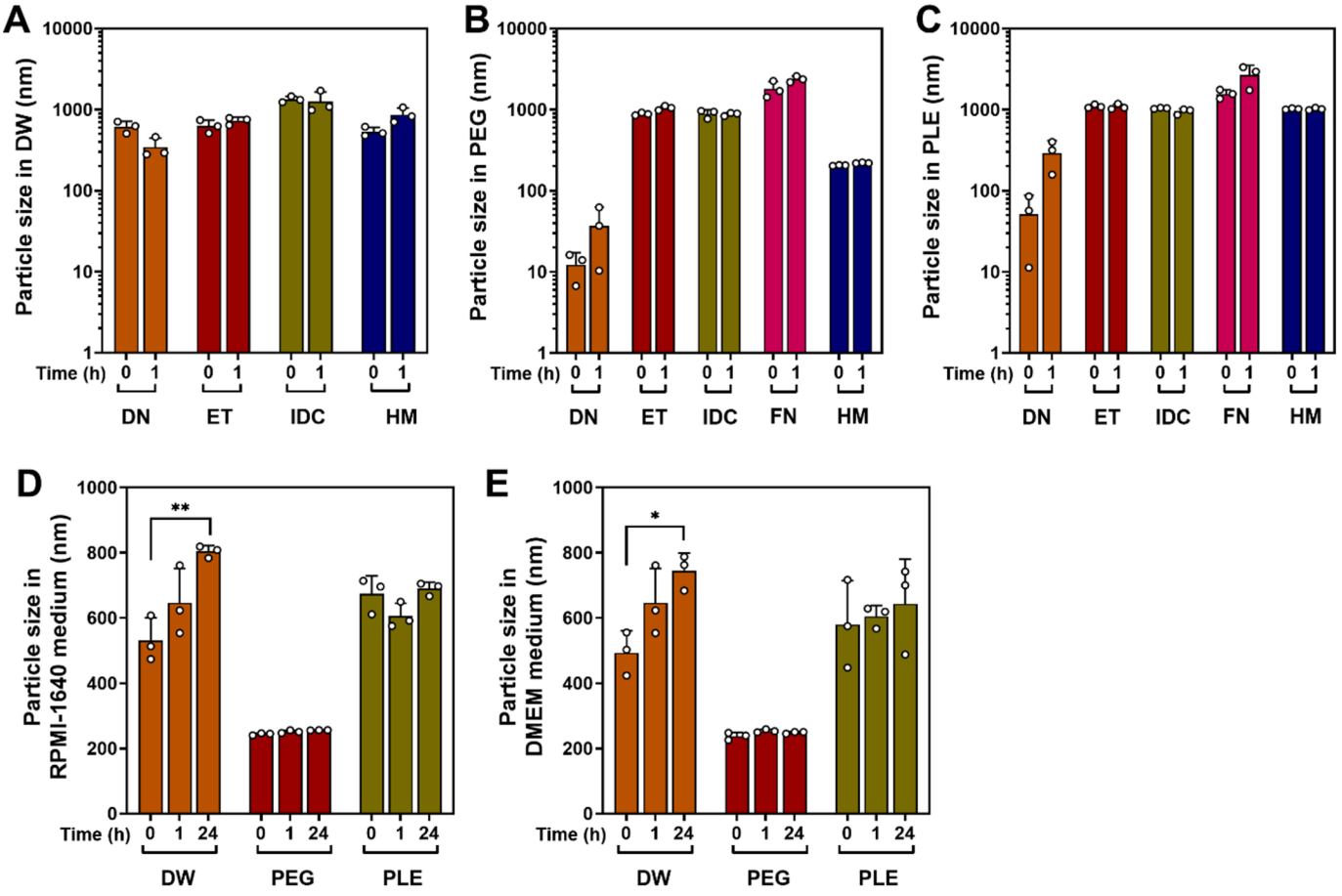

Fig. 1. Hydrodynamic size and dispersion stability of five insecticides with different dispersants. Dinotefuran (DN), etofenprox (ET), indoxacarb (IDC), fipronil (FN), and hydramethylnon (HM) were tested for dispersion in (A) distilled water (DW), (B) polyethylene glycol-400 (PEG), (C) polyoxyethylene lauryl ether (PLE). Note that FN was excluded from DW dispersion because of its poor solubility in water. Tested insecticides in PEG showed slightly better dispersion than PLE, although both dispersants produced an improved dispersion than DW. The dispersion stability of tested chemicals was maintained for 1 h, which implies that the intratracheal instillation was performed with inhalable particle size with a stable dispersion condition because the instillation was performed immediately after dispersion. The hydrodynamic sizes of hydramethylnon for *in vitro* studies were tested in (D) RPMI-1640 and (E) DMEM medium. All tested chemicals in both media showed a consistent dispersion status, although hydramethylnon in DW was slightly agglomerated over time. Data are mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to between groups.

increased particle size over time (Fig. 1D and E).

3.2. Comparative lung inflammation potential of five insecticides dispersed in water

Fipronil was excluded from the water dispersion study due to its insoluble nature under any conditions. At 24 h post-instillation, all tested insecticides did not recruit any inflammatory cells in the alveoli (Fig. 2). In addition, LDH and total protein levels did not show significant changes in any insecticide group compared with the vehicle control group (Fig. 2). Therefore, the four tested insecticides in water dispersion did not induce pulmonary inflammation at the feasible tested doses.

3.3. Lung inflammation potential of five insecticides dispersed in 10 % PEG

All insecticides were further tested by dispersing them in 10 % PEG to determine their toxic potential in the presence of dispersants. Among five insecticides, only hydramethylnon showed a significant neutrophilic inflammation (approximately 30 % neutrophilia in BALF) (Fig. 3). Consistent with the increased levels of neutrophils, LDH and total protein levels were also significantly increased compared to those in the vehicle control group (Fig. 3). Conversely, the other four insecticides showed no significant changes in BALF analysis (Fig. 3). These results indicated that the PEG formulation of hydramethylnon resulted in higher lung inflammation than the other insecticides or vehicle control.

3.4. Comparative lung inflammation potential of hydramethylnon in different dispersants

As only hydramethylnon showed toxic potential among the five insecticides, the comparative inflammatory potential of hydramethylnon in different dispersants (such as water, PEG, and PLE) was further investigated to evaluate the dose–response and dispersant-specific effects. Among the three dispersants, PEG induced the highest neutrophilic inflammation in a dose-dependent manner (Fig. 4). The PLE dispersion showed a slightly lower inflammation potential than PEG; however, it also showed significant neutrophilia in BALF in a dose-dependent manner (Fig. 4). The LDH and total protein levels also showed similar patterns to the cytological analysis of BALF (Fig. 4). Meanwhile, the groups treated with hydramethylnon dispersed in water showed no significant inflammation. The levels of pro-inflammatory cytokines, including IL-1β, IL-4, IL-6, and TNF-α in BALF, showed no significant changes in any treatment groups compared to the vehicle control group (data not shown). The calculated values of effective dose 20 % (ED_{20}) from the linear regression of the percentage of neutrophils were 0.37 mg/rat and 1.48 mg/rat for PEG and PLE dispersion, respectively. Thus, the inflammatory potential of hydramethylnon in PEG was 4-fold higher than that of the PLE dispersion.

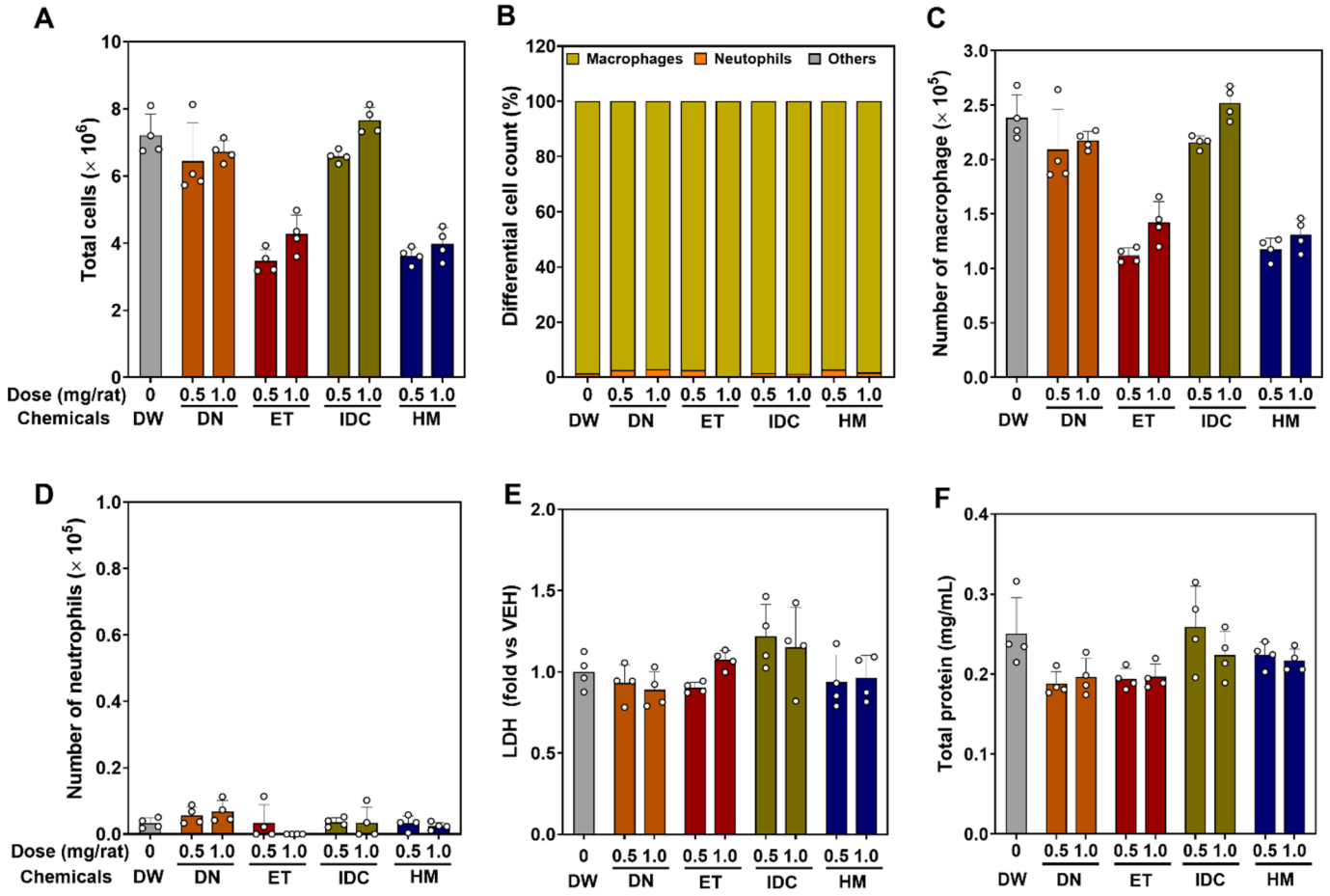

Fig. 2. Lung inflammation potential of four insecticides dispersed in water. Four insecticides dispersed in water were instilled into the lungs of rats at 0.5 and 1 mg/rat in rats. Lung inflammation was evaluated by the bronchoalveolar lavage fluid analysis at 24 h post-instillation. (A) The number of total cells in BALF. (B) Differential cell count expressed as percentages (%). The number values of (C) alveolar macrophages and (D) neutrophils in BALF suggest that all tested chemicals are not inflammogenic. The levels of (E) lactate dehydrogenase (LDH) and (F) total protein also showed that these materials in water dispersion did not cause changes in the cytotoxicity and vascular permeability compared to the vehicle control group (DW). DW, distilled water; DN, dinotefuran; ET, etofenprox; IDC, indoxacarb; HM, hydramethylnon. Data are mean ± SD (n = 4 per group).

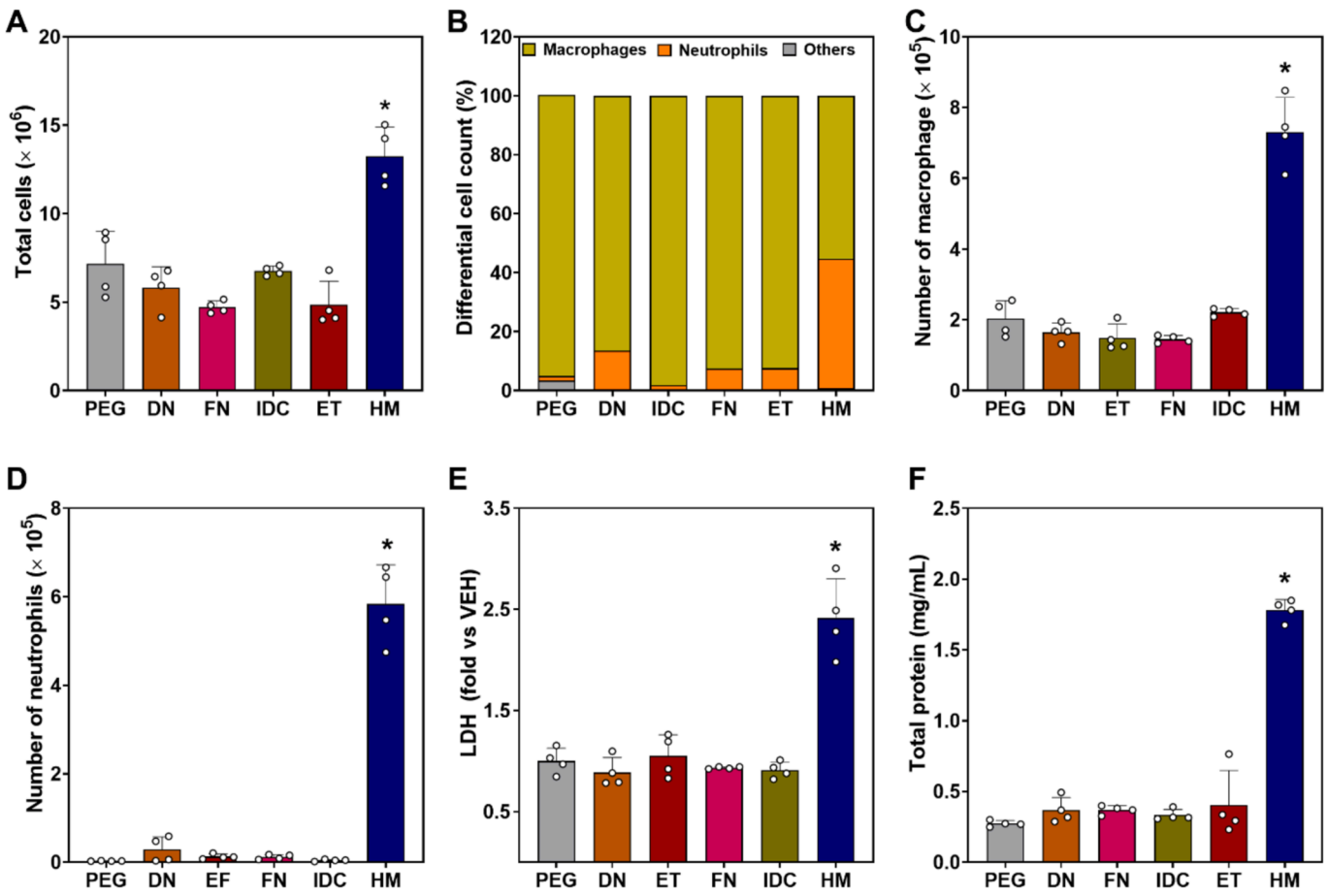

Fig. 3. Lung inflammation potential of 5 insecticides dispersed in 10 % PEG. Five insecticides dispersed in PEG were instilled into the lungs of rats at 1 mg/rat. Lung inflammation was evaluated by the bronchoalveolar lavage fluid analysis at 24 h post-instillation. (A) The number of total cells in BALF. (B) Differential cell count expressed as percentages (%). The number values of (C) alveolar macrophages and (D) neutrophils in BALF suggest that only hydramethylnon (HM) in PEG dispersion produces a significant neutrophilic inflammation compared to the vehicle control group. The levels of (E) lactate dehydrogenase (LDH) and (F) total protein also showed that HM in PEG dispersion significantly increased cytotoxicity and vascular permeability compared to the vehicle control group (PEG). PEG, polyethylene glycol 400; DN, dinotefuran; ET, etofenprox; FN, fipronil; IDC, indoxacarb. *$p < 0.05$ compared to the vehicle control group (PEG). Data are mean ± SD ($n = 4$ per group).

3.5. Potentiation effect of dispersants on the cytotoxicity and cellular uptake of hydramethylnon in A549 cells

As the potentiation effect of hydramethylnon with PEG and PLE dispersions was observed *in vivo,* we further investigated these effects in A549 cells, a human alveolar type II cell-derived adenocarcinoma cell line. The cytotoxicity of hydramethylnon was highly dependent on the dispersion medium and the potential was in the order of PEG > PLE > water (Fig. 5). A comparative evaluation of the effects of the dispersants was performed with the EC_{20} values calculated by linear regression of the dose–response curves. It should be noted that the EC_{20} value was selected as a toxicity endpoint because cytotoxicity was a common endpoint, and EC_{20} was more accurate than EC_{50} with the data obtained in this study (Figures S1 and S2, see Supporting Information). Furthermore, EC_{10} was excluded in this study because effects with 10 % can be within the normal range. The EC_{20} values suggested that the cytotoxic potential of hydramethylnon in PEG was 10–17 fold higher than that in PLE and greater than 370 fold higher than that in water (Table 2). The cellular uptake levels in A549 cells of hydramethylnon were in the order of PEG > PLE > water, which implies that higher cellular uptake makes higher toxicity (Fig. 5C). The percentage of cellular uptake in A549 cells per nominal treated concentration was 48.2, 5.5, and 4.6 % for PEG, PLE, and water, respectively (Fig. 5D). The correlation plot between cellular uptake levels and EC_{20} values of the MTS assay further supports that the toxic potential of hydramethylnon is closely related to the efficacy of cellular delivery (Fig. 5E).

3.6. Potentiation effect of dispersants on the cytotoxicity and cellular uptake of hydramethylnon in dTHP-1 cells

As one of the primary target cells for exogenous aerosol particles is alveolar macrophage (Oberdörster et al. 1992), the effect of different dispersants on the toxicity of hydramethylnon was evaluated in dTHP-1 cells. The LDH values of hydramethylnon showed a pattern similar to that observed in A549 cells (Fig. 6). The concentrations that induced cytotoxicity in dTHP-1 cells were similar to those in A549 cells. The cytotoxic potential (i.e., EC_{20} values) of hydramethylnon in PEG to dTHP-1 cells was 12–14 fold higher than that of the PLE dispersion and 65–169-fold higher than that of the water dispersion (Table 2). The levels of IL-1β and MIP-1β in cell culture supernatants from dTHP-1 cells showed dose-dependent increases in all tested dispersants (Fig. 6). However, TNF-α levels increased only in the hydramethylnon in PLE (Fig. 6E), which implies that the mechanism of insecticide toxicity can differ depending on the dispersion medium. The cellular uptake levels of hydramethylnon in dTHP-1 cells were in the order of PEG > PLE > water (Fig. 6F). The percentage of cellular uptake in dTHP-1 cells per nominal treated concentration was 56, 10, and 5.5 % for PEG, PLE, and water, respectively (Fig. 6G). The correlation plot between cellular uptake

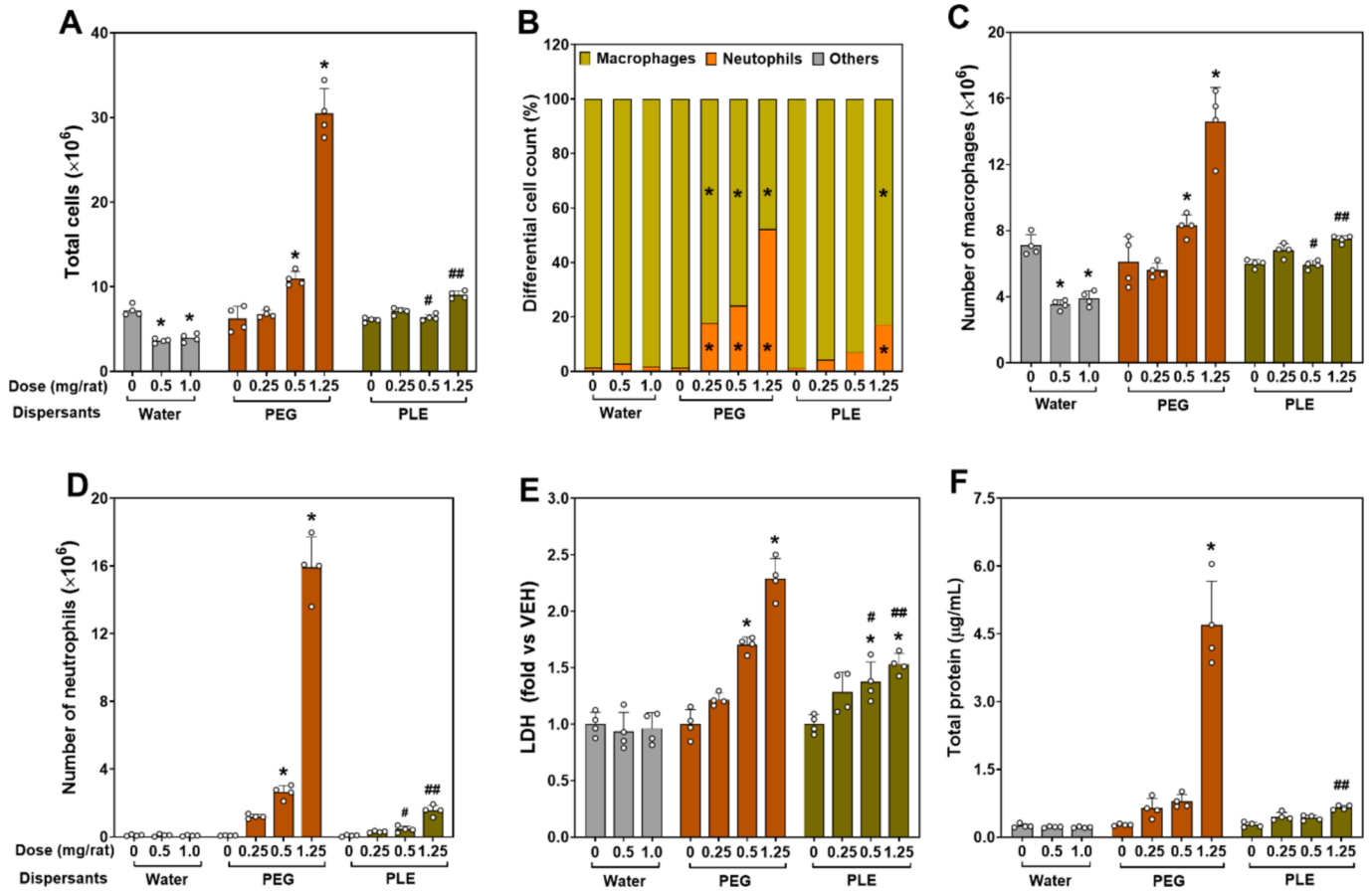

Fig. 4. Comparative evaluation of lung inflammation potential of hydramethylnon in different dispersants. Hydramethylnon samples dispersed in different dispersants, such as water, polyethylene glycol (PEG), and polyoxyethylene lauryl ether (PLE), were instilled into the lungs of rats. The treatment doses for water dispersion were 0, 0.5, and 1 mg/rat, while those for PEG and PLE were 0.25, 0.5, and 1.25 mg/rat. Lung inflammation potential was evaluated at 24 h post-instillation by the bronchoalveolar lavage fluid (BALF) analysis. (A) The number of total cells in BALF. (B) Differential cell count expressed as percentages (%). The number values of (C) alveolar macrophages and (D) neutrophils in BALF. The levels of (E) lactate dehydrogenase (LDH) and (F) total protein in BALF. Note that hydramethylnon dispersed in PEG and PLE induced significant lung inflammation, and the inflammation potential was in the order of PEG > PLE. $^{*}p < 0.05$ compared to the vehicle control group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ between PEG and PLE at the same dose level. Data are mean ± SD ($n = 4$ per group).

levels and EC_{20} values of the MTS assay further supports that the toxic potential of hydramethylnon is closely related to the efficacy of cellular delivery (Fig. 6H).

3.7. *Mechanism of potentiation effect by PEG dispersion: Not particle size but the efficacy of intracellular delivery*

Considering that THP-1 cells are much more sensitive than A549 cells to toxic particulate matter (Cho et al. 2013), the similar EC_{20} values imply that the toxic effect of hydramethylnon in PEG and PLE is not derived from phagocytosis, but rather from diffusion-like mechanisms. To further evaluate this hypothesis, the effects of particle size (i.e., hydrodynamic size) and intracellular delivery efficacy were evaluated by testing different percentages of PEG (i.e., 2–10 %). The EC_{20} value of LDH cytotoxicity for hydramethylnon in 10 % PEG was 2.5-fold higher than that in 2 % PEG (Fig. 7A). Similarly, the EC_{20} value of MTS cytotoxicity for hydramethylnon in 10 % PEG was 2.68 fold higher than that in 2 % PEG (Fig. 7B). Considering that the hydrodynamic size of hydramethylnon dispersed in different concentrations of PEG (i.e., 2–10 %) showed similar ranges, hydrodynamic size was not a key factor in the potentiation effect of hydramethylnon (Fig. 7C). Thus, we further tested the intracellular delivery efficacy of hydramethylnon using different dispersants. Hydramethylnon was treated to dTHP-1 cells for 24 h at 2 μg/mL (a non-lethal dose), and the levels of intracellular uptake of hydramethylnon were measured by LC-MS/MS. The cellular levels of hydramethylnon showed that the PEG dispersion had 5-fold higher levels than the water dispersion (Fig. 7D). Thus, the higher cytotoxicity of hydramethylnon dispersed in PEG compared to other dispersants was due to its greatly increased cellular delivery efficacy. Moreover, we investigated the mechanism of intracellular delivery of the PEG formulation by treatment with cytochalasin D, an inhibitor of phagocytosis. Co-treatment with cytochalasin D and hydramethylnon dispersed in PEG showed similar or even higher levels of cytotoxicity than hydramethylnon dispersed in PEG (Fig. 7E). Furthermore, the level of intracellular delivery of hydramethylnon in PEG with cytochalasin D was higher than that in PEG without cytochalasin D (Fig. 7F). These results imply that the dispersion medium can alter the cellular delivery efficacy, subcellular localization, and mode of action for hydrophobic insecticides, such as hydramethylnon.

4. Discussion

Climate change impacts insect populations and worsens insect-associated pests (Outhwaite et al. 2022; Porter et al. 1991). In the meantime, the use of insecticides has exponentially increased due to the COVID-19 pandemic outbreaks, and their usage has been extended after the endemic situation (Choi et al. 2024). The exposure of humans to insecticides is not limited to occupational settings but is much broader in the general population due to the household use of insecticide products (Class and Kintrup 1991; Loroño-Pino et al. 2014). However,

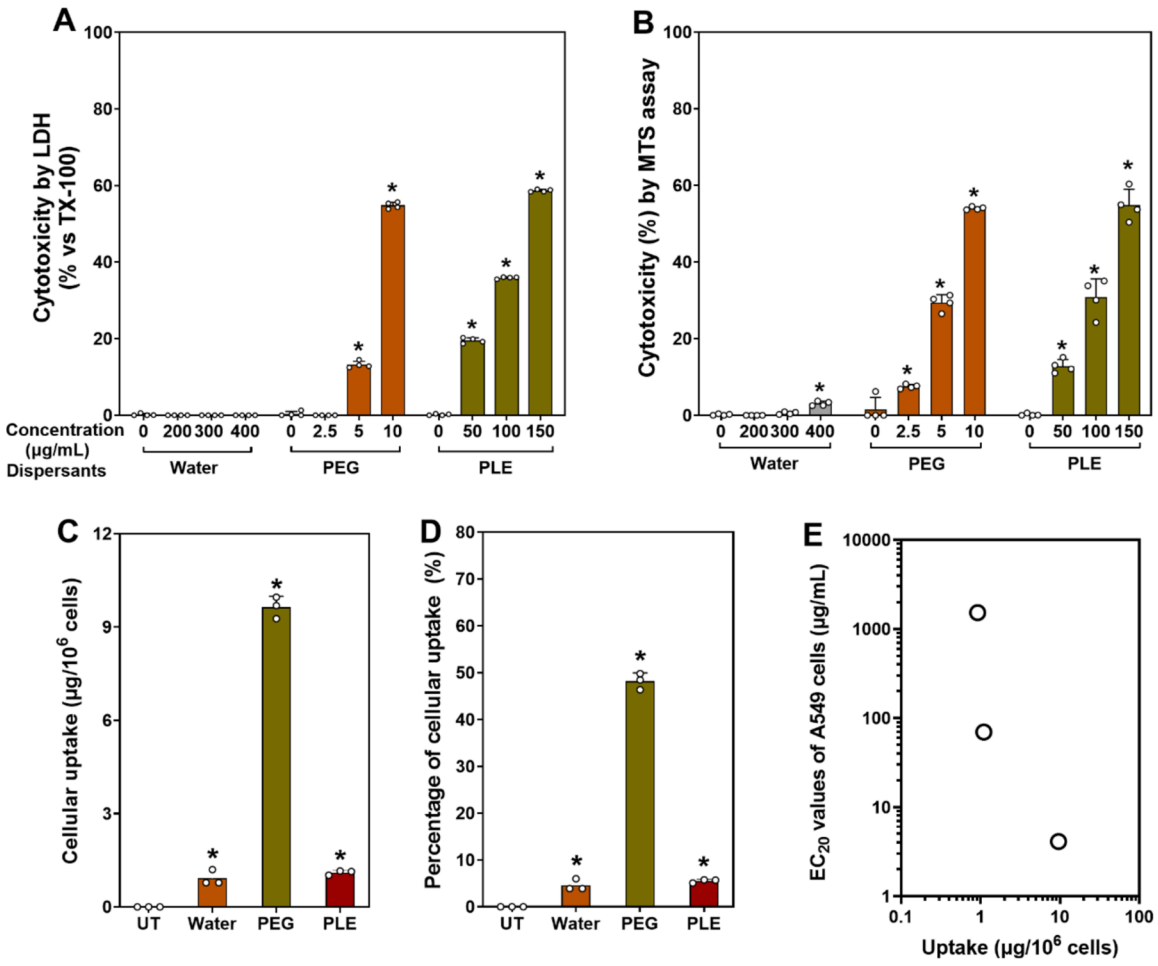

Fig. 5. The comparative evaluation of the cytotoxic potential and cellular uptake levels of hydramethylnon in A549 cells. Hydramethylnon was dispersed in water, polyethylene glycol (PEG), and polyoxyethylene lauryl ether (PLE) and treated to A549 cells for 24 h. The cytotoxicity was evaluated by levels (A) of lactate dehydrogenase (LDH) and (B) 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)–2H-tetrazolium (MTS). The effective concentration 20 % (EC_{20}) values from the linear correlation analysis are presented in Table 2. The cellular uptake levels of hydramethylnon expressed as (C) mass value per cell and (D) percentage compared to treatment concentration. At 24 h post-treatment of hydramethylnon in A549 cells at 2 μg/mL, the levels of hydramethylnon in cells were quantified using the LC-MS/MS. (E) The correlation plot between cellular uptake levels of hydramethylnon in A549 cells and EC_{20} values from three different dispersants. UT, untreated control group. Data are mean ± SD ($n = 4$ per group). *$p < 0.05$ compared to the vehicle control group or UT.

Table 2
EC_{20} values for the cytotoxicity of hydramethylnon on A549 and dTHP-1 cells.

Dispersant	LDH (A549)	MTS (A549)	LDH (dTHP-1)	MTS (dTHP-1)
PEG	5.48 μg/mL	4.12 μg/mL	5.33 μg/mL	2.28 μg/mL
PLE	53.95 μg/mL	69.49 μg/mL	76.69 μg/mL	26.69 μg/mL
– fold vs PEG[a]	10	17	14	12
Water	ND	1523.58 μg/mL	901.73 μg/mL	147.64 μg/mL
– fold vs PEG[a]	ND	370	169	65

EC_{20} values were calculated from the simple linear regression test of Figs. 5 and 6.

[a] The cytotoxic potentials were calculated by the fold changes of EC_{20} values. ND, not determined. ND means that the EC_{20} values cannot be determined due to the non-toxic response.

information regarding the inhalation toxicity of insecticides or their modes of action is scarce. In this study, only hydramethylnon induced lung inflammation in rats among the five different types of insecticides. Furthermore, the inflammatory potential of hydramethylnon was potentiated when combined with PEG 400. We further concluded that the potentiation effect of hydramethylnon was due to increased cellular delivery by PEG via a simple diffusion mechanism rather than the particle size effect or endocytosis mechanisms.

In this study, among the five insecticides, only hydramethylnon caused inflammation in rats, whereas other insecticides, such as dinotefuran, etofenprox, fipronil, and indoxacarb, did not cause any inflammation up to 1 mg/rat. A dispersion of over 2.5 mg/rat caused death owing to neurological disorders (data not shown). To the best of our knowledge, information on the respiratory toxicity of these insecticides is scarce. Although the respiratory toxicity of dinotefuran, a neonicotinoid, has not been reported, an epidemiological study suggested that neonicotinoid inhalation reduced lung function in occupationally exposed farmers (Hernández et al. 2008). In addition, dinotefuran caused anxiety and depression in mice (Takada et al. 2020) and reproductive toxicity (Thompson et al. 2020). Etofenprox, a pyrethroid, induced liver toxicity (e.g., hepatocyte enlargement and fat metabolism dysfunction), thyroid toxicity (e.g., increased number of micro follicles and reduced levels of thyroxine), and adrenal gland toxicity (e.g., elevated adrenal weight and increased adrenal cortical thickness) in a 90-day inhalation toxicity study with the no observed adverse effect concentration (NOAEC) as 0.042 mg/L air (Authority 2009). Fipronil, a phenylpyrazole, induced lung inflammation in mice when intranasally exposed at 8 mg/kg/day for 7 days (Merkowsky et al. 2016). Inhalation exposure to fipronil in paddy farmers of Malaysia did

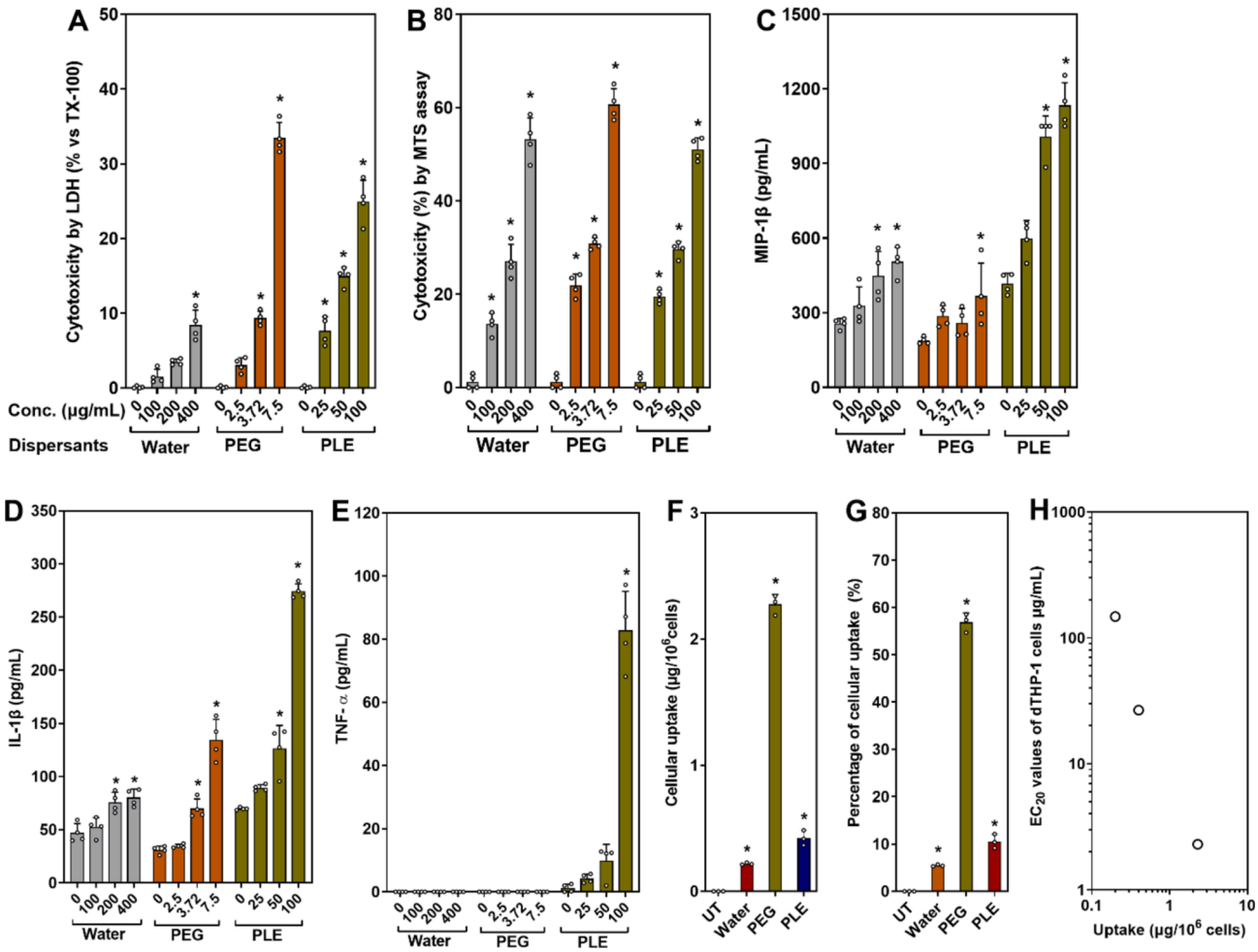

Fig. 6. Evaluation of cytotoxicity, pro-inflammatory cytokines, and cellular uptake levels of hydramethylnon in differentiated THP-1 (dTHP-1) cells. dTHP-1 cells were treated with hydramethylnon with different dispersants, such as water, polyethylene glycol (PEG), and polyoxyethylene lauryl ether (PLE). Cytotoxicity was evaluated by (A) levels of lactate dehydrogenase (LDH) and (B) mitochondrial activity analysis using MTS assay. The calculated EC_{20} values in LDH and MTS assay are presented in Table 2. The pro-inflammatory cytokine levels including (C) MIP-1β and (D) IL-1β showed similar patterns between different dispersants. However, the levels of (E) TNF-α treated with hydramethylnon were only increased when dispersed in PLE, which suggests that the dispersant can change the mechanism of toxicity. The cellular uptake levels of hydramethylnon expressed as (F) mass value per cell and (G) percentage compared to treatment concentration. At 24 h post-treatment of hydramethylnon in dTHP-1 cells at 2 μg/mL, the levels of hydramethylnon in cells were quantified using the LC-MS/MS. (H) The correlation plot between cellular uptake levels of hydramethylnon in dTHP-1 cells and EC_{20} values from three different dispersants. UT, untreated control group. Data are mean ± SD ($n = 4$ per group). *$p < 0.05$ compared to the vehicle control group (VEH) or UT.

not have significant health risks (Hamsan et al. 2017). Indoxacarb, an oxadiazine, increased spleen weight, increased hematopoiesis in the spleen tissue, and hematological changes in a 28-day inhalation toxicity study in rats (Sandeep et al. 2016). Lastly, hydramethylnon, a trifluoromethyl aminohydrazone, is classified as Toxicity Category IV for the inhalation route, which implies that it is practically non-toxic to humans (EPA 1998). Therefore, the literature review implies that the inhalation toxicity of insecticides has not been well reported so far, although occupational and consumer exposure to household products has been increasing. Thus, the comparative studies for their lung toxicity and combination effects should be carefully investigated because insecticides and dispersants are mixed in products (Kim et al. 2021). The doses or concentrations applied in this study were chosen based on the dose responses rather than real-world exposure scenarios to perform the comparative toxicity evaluation among 5 different classes of insecticides.

Among the five insecticides tested in this study, only hydramethylnon caused pulmonary toxicity; however, its inflammatory potential was highly dependent on the dispersion medium. Although hydramethylnon in water was not inflammogenic, its dispersion in PEG or PLE significantly induced neutrophilic inflammation, which implies a potentiation effect because neither dispersion medium caused inflammation at the tested concentrations. The *in vitro* cytotoxicity data in this study further suggested the potentiating effect of PEG and PLE on the toxicity of hydramethylnon in human type 2-like cells (A549 cells) and human macrophage-like cells (dTHP-1 cells). The difference in the toxic potential of hydramethylnon according to the dispersion medium may be due to differences in the cellular uptake mechanism of hydramethylnon. The measurement of cellular uptake of hydramethylnon and cytochalasin D treatment studies shown in this study suggests that PEG delivers insecticides to cells via simple diffusion as hydrophobic hydramethylnon simply across the cytoplasmic membrane by encapsulated with PEG micelles (Wang et al. 2012). The higher toxicity of hydramethylnon with increasing PEG concentration without a difference in hydrodynamic size shown in this study further supports the idea that the potentiation effect of PEG depends not on particle size but on micellar delivery.

In water dispersions, hydramethylnon hardly translocates to cells via simple diffusion mechanisms. In comparison with dTHP-1 and A549 cells, the difference in sensitivity for the cytotoxic potential of

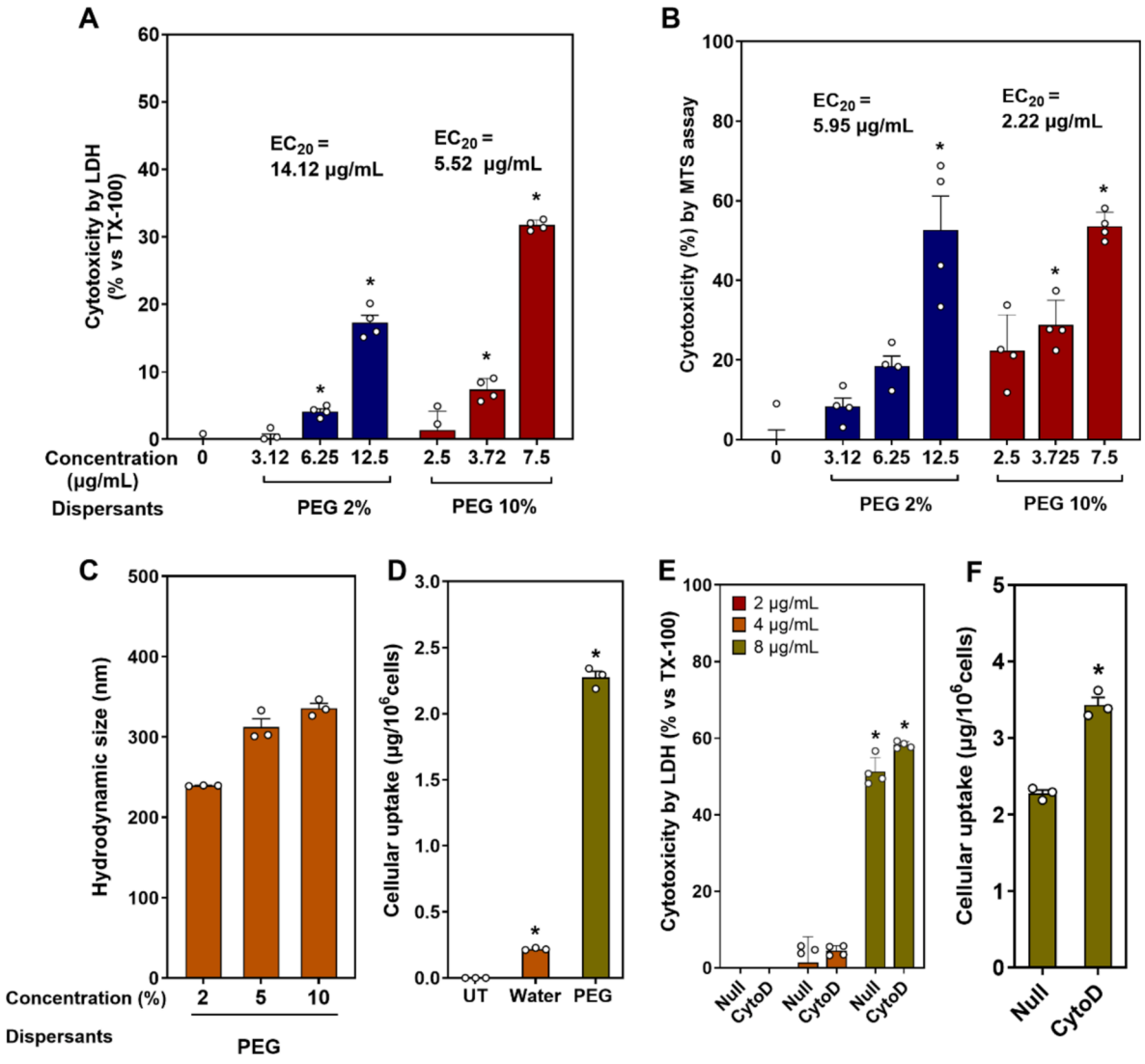

Fig. 7. The mechanism of potentiation effect of polyethylene glycol (PEG) on the toxicity of hydramethylnon in differentiated THP-1 (dTHP-1) cells. Hydramethylnon with different concentrations of PEG and PLE was treated to dTHP-1 cells, and the cytotoxicity was evaluated by (A) the lactate dehydrogenase (LDH) assay and (B) mitochondrial activity analysis using MTS assay. Note that hydramethylnon in 10 % PEG showed 2.5 – 2.6 fold higher cytotoxicity levels than in 2 % PEG. *$p < 0.05$ compared to the vehicle control group. (C) The hydrodynamic size of hydramethylnon dispersed in 2 % − 10 % PEG. (D) The cellular uptake levels of hydramethylnon at 24 h treatment to dTHP-1 cells at 2 µg/mL. Note that levels of hydramethylnon in 10 % PEG have 5-fold higher cellular uptake than water dispersant. *$p < 0.05$ compared to UT (E) The cytotoxicity of hydramethylnon measured by LDH assay showed that cytotoxicity of hydramethylnon in PEG dispersion was not affected by the cytochalasin D (cytoD) treatment, a blockage of phagocytosis activity. *$p < 0.05$ compared to the vehicle control group. (F) The levels of hydramethylnon in PEG dispersion w/wo cytoD show that the blockage of phagocytosis activity has no effect or even higher cellular uptake than that without cytoD. *$p < 0.05$ compared to Null (without cytoD). These results imply that the cellular uptake of hydramethylnon is mediated by simple diffusion rather than endocytosis mechanisms. UT, untreated control group. Data are mean ± SD ($n = 4$ per group).

hydramethylnon measured by MTS assay was most remarkable in the water dispersion (approximately 10-fold difference), whereas PEG and PLE showed approximately 1.8- and 2.6-fold differences, respectively. This result might be due to the cellular uptake mechanism of hydramethylnon in water dispersions, which is mainly mediated by endocytosis mechanisms, such as phagocytosis and pinocytosis (Wang et al. 2012), and dTHP-1 cells are specialized phagocytic cells, whereas A549 cells are not (Hwang et al. 2016). PLE is also a detergent, which can form micellar structures in aqueous solution with hydrophobic materials (Gao et al. 2002); however, its molecular weight (MW) is much higher than PEG 400 (PLE: 1199.5 g/mol; PEG 400: 380–420 g/mol). Based on the PEG studies, PEGs with low MW (e.g., 750 and 2,000 g/mol) show easier passive diffusion to cell membranes than higher MW PEGs (e.g., 5,000 g/mol) (Wang et al. 2020). Furthermore, the high MW micelles are more involved with caveolae-mediated endocytosis (Wang et al. 2020), which explains why the PLE dispersion is less toxic than PEG dispersion and why the difference between PEG and PLE in nonphagocytic cells (such as A549 cells: 17-fold difference) is higher than in specialized phagocytic cells (i.e., dTHP-1 cells: 12-fold difference).

The potentiating effect of PEG and PLE on the toxicity of insecticides shown in this study highlights the importance of surfactants in insecticide dispersants and their potential implications for human health, as insecticide-containing household products contain a mixture of insecticides and detergents (Kim et al. 2021). In other words, the delivery of hydrophobic insecticides to the human body is greatly enhanced by dispersing them in detergents because they can change the delivery mechanism to cells from endocytosis to simple diffusion. However, more importantly, this effect was only valid for cells with phospholipid bilayers and less effective for insects covered with chitin layers (Zhu et al. 2016). This result highlights that adding detergents increases the

sensitivity of non-target organisms, such as humans and farm animals. Furthermore, the target organisms covered with chitin layers are more sensitive to insecticides with water formulation than with PEG formulation because the sprayed hydrophobic insecticides with water formulation are quickly dried and easily absorbed. However, the insecticides with PEG formulation are likely to persist in their micellar structures after being sprayed on insects, which retards absorption from the integument. Therefore, the PEG formulation can reduce the lethal effect of the hydrophobic insecticides.

Although this study informs the differential role of dispersants on the cellular delivery of insecticides, further studies with a wider array of dispersants and solubilizers are needed on this issue for more robust conclusions and guidance for safer product development. In addition, evaluation of the effect of different types of PEGs with various molecular weights on the cellular delivery and toxicity of insecticides is also needed in future studies to propose more efficient products with safer in non-target organisms. Likewise, the effect of dispersants or solubilizers on the toxicity of aquatic organisms, such as plants, fish, and daphnids, is also needed for such assessments to ensure the broader ecological safety of these substances.

5. Conclusion

The results of this study suggest that adding detergents, such as PEG and PLE, changes the delivery mechanism of insecticides to rodents and human cells from endocytosis to simple diffusion, drastically increasing the cellular delivery load. Furthermore, this effect is more prominent in non-target organisms, such as humans and farm animals, than in insects, which are less permeable to micellar structures. Therefore, detergents should be considered for their comparative toxicity to target and non-target organisms, in addition to the dispersion efficacy of hydrophobic insecticides.

CRediT authorship contribution statement

Thillaichidambaram Muneeswaran: Writing – original draft, Methodology, Investigation, Formal analysis. **Tanya Bhatt:** Writing – original draft, Methodology, Investigation, Formal analysis. **Su Hwan Park:** Writing – review & editing, Conceptualization. **Muthuchamy Maruthupandy:** Writing – review & editing, Methodology, Conceptualization. **Minsik Kim:** Writing – review & editing, Methodology, Conceptualization. **Vartika Mathur:** Formal analysis, Writing – review & editing. **Jong-Ho Lee:** Writing – review & editing, Methodology, Conceptualization. **Min-Seok Kim:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Wan-Seob Cho:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109307.

Data availability

Data will be made available on request.

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