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Cytotoxicity of lambda-cyhalothrin on the macrophage cell line RAW 264.7

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Abstract

The wide use and wide-spectrum toxicity of synthetic pyrethroids (SPs) insecticides make them an emerging ecotoxicological concern. Some previous studies showed that SPs possessed cytotoxicity in some immune cells such as human lymphocytes and rat bone marrow. However, the cytotoxicity of SPs to macrophages, which are crucial to innate immunity, has not been explored. In the present report, we investigated a new pyrethroid insecticide, lambda-cyhalothrin (LCT), which may increase the generation of reactive oxygen species (ROS) and DNA damage levels and cause cytotoxicity in RAW 264.7 cells in dose- and time-dependent manners. The results for the first time implicated increased endogenous ROS and DNA damage as co-mediators of LCT-induced cytotoxicity in macrophages. Our results also suggested that macrophages were involved in synthetic pyrethroid-induced adverse immune effects. Considering the ubiquitous environmental presence of SPs, this study provided new information relative to the potential long-term physiological and immunological effects associated with chronic exposure to SPs. Hence, the potential immunotoxicity of SPs should be considered in assessing the safety of these compounds in sensitive environmental compartments.

Key words: lambda-cyhalothrin; macrophage; reactive oxygen species; cytotoxicity
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Introduction

Synthetic pyrethroids (SPs) are analogues of naturally occurring pyrethrins extracted from the dried flowers of *Chrysanthemum cinerariaefolium*. Synthetic pyrethroids are distinguished by three general characteristics: extreme hydrophobicity, rich stereochemistry, and broad-spectrum high-level insecticidal activity. These halogenated and lipophilic compounds are a group of potent insecticides that are environmentally compatible by virtue of low mammalian and avian toxicities, low potential to contaminate ground water, and relatively low application rates. In particular, SPs are commonly used in households to eradicate pests and insects (Naumann, 1990), and therefore the exposure of humans to SPs may be extensive. With restrictions on the use of organophosphorus insecticides, the use of SPs is expected to further increase. SPs can enter the aquatic environment through the direct spraying of water bodies and forest-spaying; thus, they may have negative effects on aquatic organisms (Bradbury and Coats, 1989) and sediment dwellers (Weston et al., 2004, 2005) and can be adsorpted by algas as many heavy metals acted (Ahmet and Mustafa, 2008; Ahmet et al., 2007a, 2007b). In addition, several aspects of carcinogenicity (Litchfield et al., 1985), genotoxicity (Carbonell et al., 1989; Puig et al., 1989) and estrogenic activity have been studied (Go et al., 1999; Chen et al., 2002). Therefore, the wide-spectrum toxicity of SPs makes them an emerging ecotoxicological concern.

Lambda-cyhalothrin (LCT) is a new pyrethroid insecticide used to control mosquitoes, fleas, cockroaches, flies, and ants around house. Previous studies using laboratory animals and *in vitro* models indicated a high aquatic toxicity of LCT (Liu et al., 2005; Bao et al., 2007; Carlos et al., 2007). Several studies focused on vertebrate toxicity, including cytotoxicity (Celik et al., 2005), developmental toxicity to zebrafish embryos (Xu et al., 2008), endocrine disruption (Wang et al., 2007; Zhao et al., 2008) and genotoxicity (Liu et al., 2008; Celik et al., 2005). Moreover, some *in vitro* assays also indicated immunotoxicity in *in vitro* models, such as human lymphocytes (Naravaneni and Jamil, 2005) and rat bone marrow (Celik et al., 2005). The possible mechanism of cytotoxicity of LCT could be nitric oxide production, increase of ROS, or DNA single strand breaks, as previous reported (Righi and Palermo-Neto, 2005). The immune system consists of complex and highly specialized cells, tissues and organs, and of innate and inducible immune functions to protect organisms from invaders. Among them, macrophages are crucial to innate immunity and play a key role in inflammation, host defense and reactions against a spectrum of autologous and foreign invaders (Enane et al., 1993; Ustyugova et al.,

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2007). The functional aspects of macrophages have been proposed for use as one of the important biomarkers for immunotoxic chemicals (Descotes, 2006). A well-known mouse macrophage cell line RAW264.7 has been used as the *in vitro* model for the assays. The mouse macrophage cell line RAW264.7 plays a significant role in the innate immune system (Zhao et al., 2009). However, information with regard to the cytotoxic effects of SPs in macrophages is still lack.

In this study, we used LCT as a model SPs, and mouse macrophage cell line RAW 264.7 as an *in vitro* model, to evaluate the possible effects of LCT on innate immune functions through cell viability, intracellular ROS, and the comet assay. We expect that this study will be of great value for improving our understanding of SP-involved toxicity, especially potential immunotoxicity.

1 Materials and methods

1.1 Chemicals

Lambda-cyhalothrin (LCT, (*RS*)-α-cyano-3-phenoxybenzyl (1*R*)-*cis*-3-(*Z*)-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate, purity of 98%) was obtained from Danyang Agrochemicals (Jiangsu, China). A stock solution of 2000 mg/L of LCT was prepared using ethanol and stored at 4°C in darkness. Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (Logan, USA). Other chemicals or solvents used in this study were of cell culture, HPLC, or analytical grade.

1.2 Cell culture and treatment of RAW 264.7

The RAW 264.7 macrophage cell line was obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China; the original source is from ATCC (Manassas, USA)). The cells were cultured in DMEM medium with 10% of FBS supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Before treatment, the culture medium was replaced with the experimental medium (DMEM containing 2% FBS) for 1 day to reduce the effect of the serum. The culture media was refreshed every 2–3 days, and subculture was performed at a ratio of 1:3 with routine trypsinization every 5–6 days.

Based on the results of pre-tests, the cells were treated with experimental medium (with test compounds) at concentrations of 10^{-9}–10^{-5} mol/L for 3 days for the dose-dependent cell viability assay, 10^{-7} mol/L for 1, 2, and 3 days for the time-dependent cell viability assay, 10^{-7}, 5×10^{-7}, and 10^{-6} mol/L for 6 hr for the ROS generation assay and 1 day for the comet assay. Ethanol (0.1%, *V/V*) was used as the negative control.

1.3 Cell viability of RAW 264.7

Cell viability is one of the important endpoints for the assessment of SPs cytotoxicity. The cell viability was measured by a quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Following our previous study (Liu et al., 2008), cells were seeded in 96-well plates at an initial concentration of 4000 cells per well. After 1 day, the medium was removed from the wells, and MTT solution was added and then incubated at 37°C for 4 hr. After removing the medium from the wells, 150 μL of dimethyl sulfoxide (DMSO) was added to each well. After 10 min of shaking, the absorbance was measured at a wavelength of 490 nm with a Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories, USA). Results were expressed as the ratio of each exposure group to the vehicle control (0.1% ethanol, one-fold).

1.4 Detection of reactive oxygen species

Following our previous study (Liu et al., 2008), intracellular reactive oxygen species (ROS) generation was measured using a fluorescence probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma, USA), which can cross cell membranes and is hydrolysed to the nonfluorescent form, DCFH, by intracellular esterases. DCFH can be rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of reactive oxygen species such as H_2O_2 or low-molecular weight peroxyl radicals. In brief, RAW 264.7 cells were treated with vehicle or LCT at concentrations of 10^{-7}, 5×10^{-7}, and 10^{-6} mol/L for 6 hr, and then washed three times with ice-cold PBS. The cells were incubated with 10 μmol/L DCFH-DA (prepared in DMSO at 100 mmol/L) for 30 min at 37°C. At the end of incubation, the fluorescence intensity was measured at 485 nm excitation and 535 nm emission using a fluorescence spectrophotometer (Infinite M200, Tecan, Switzerland). The relative levels of ROS were expressed as fluorescence intensity ratio of the LCT-treated groups to the negative control.

1.5 Comet assay

The comet assay is a popular technique due to its simple procedure and high sensitivity for the detection of DNA damage at the level of the individual eukaryotic cell. The protocol used for the comet assay with cells from peripheral blood followed the guideline of Singh et al. (1988). Observations were made at a final magnification of 400 using an epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with filters suitable for ethidium bromide. A number of 50 randomly selected cells per sample (25 from each slide) were measured to evaluate tail length. Comet assay analyses were made by using a computerized image analysis system (Comet Assay II, Perceptive Instruments, UK).

1.6 Statistical analysis

All experiments performed were repeated at least three times. The statistical analysis of the data was conducted with the statistical program package SPSS 11.0. All data are expressed as mean ± standard deviation (SD). Value comparison between groups was performed by one way ANOVA followed by Dunnett's multiple-comparison test, and $p < 0.05$ was considered statistically significant.

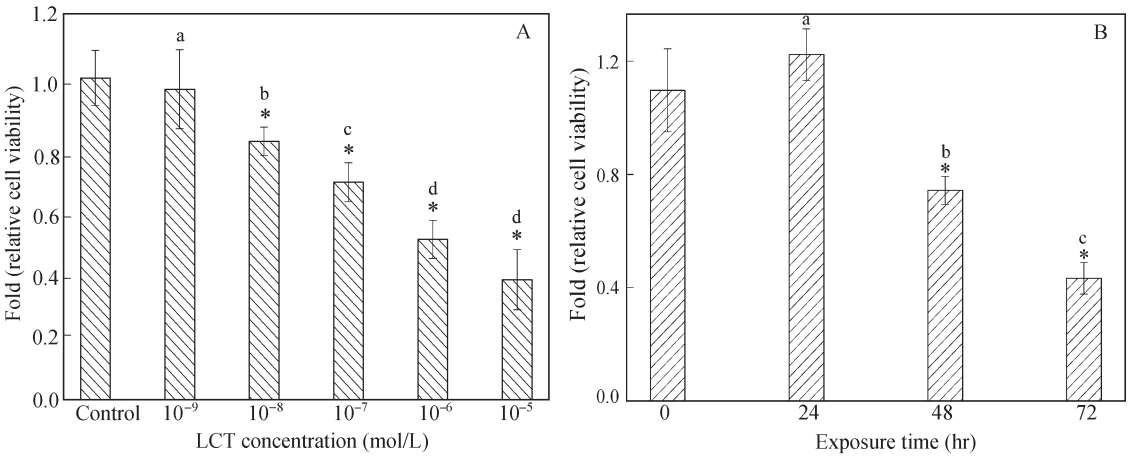

Fig. 1 Effect of lambda-cyhalothrin on RAW 264.7 cell viability. (A) RAW 264.7 cells were incubated with different concentrations of lambda-cyhalothrin for 72 hr, followed by the MTT assay. Asterisk above adjacent bars indicate a significant difference ($p < 0.05$, $n = 5$) between the treatment and control; (B) RAW 264.7 cells were incubated in lambda-cyhalothrin at a concentration of 10^{-7} mol/L for 0, 24, 48, and 72 hr, followed by the MTT assay. Asterisk above adjacent bars indicate a significant difference ($p < 0.05$, $n = 5$) between the treatment and control. Different letters above asterisk indicate a significant difference ($p < 0.05$, $n = 5$) among each concentration.

2 Results and discussion

The cytotoxicity assay is a type of *in vitro* assay used to examine the toxic effects of environmental chemicals by analyzing cell growth. Our results indicated the dose-response and time-response of cell growth inhibition after incubation with LCT. The relative viability of RAW 264.7 cells was decreased with increasing concentrations of LCT. A significant difference was observed in the range of 10^{-8}–10^{-5} mol/L (Fig. 1A). The cytotoxic effect was also strongly dependent upon time at the concentration of 10^{-7} mol/L (Fig. 1B). The marked difference occurred 48 and 72 hr after exposure to LCT. All of the results together suggested a clear cytotoxicity of LCT on the macrophage cell line. We proposed that the adverse effect on the macrophage line may be one of the possible mechanisms of immunotoxicity of SPs, especially following long exposure at low concentrations. Although the cytotoxicty of SPs carried out *in vitro* and *in vivo* models including LCT are controversial, our results were in agreement with that reported by previous research. The SPs can induce cytotoxicity and genotoxicity in *in vivo* and *in vitro* models. The cytotoxic and genotoxic effect of LCT has been observed in bone marrow cells of female albino rats at the concentration of 0.8 mg/kg bw (body weight) or above, which was similar to the exposure level in our present study (Ayla et al., 2003). Lambda-cyhalothrin has been reported to have an obvious negative effect on human lymphocytes in an *in vitro* model (Naravaneni and Jamil, 2005). This cytotoxicity phenomenon also occurred in other SPs. For instance, *cis*-bifenthrin caused apoptosis in the human amnion epithelial (FL) and human hepatocellular liver carcinoma (Hep G2) cell line mediated by oxidative damage (Liu et al., 2008, 2009). Cypermethrin induced DNA damage in organs and tissues of the mouse (Patel et al., 2006). While, in our best knowledge, the cytotoxic potential of LCT on macrophage was firstly reported by our study.

Oxidative damage and DNA damage are two important factors in the induction of cytotoxicity of SPs. To evaluate the relationship between cytotoxicity and oxidative damage in the macrophage cell line RAW 264.7 by LCT, the ROS were detected using a fluorescence probe, DCFH-DA. Our results illustrated that ROS were enhanced in a dose-dependent manner, and ROS generation was increased approximately 1.6-fold after exposure to 10^{-6} mol/L of LCT (Fig. 2). Our present study is in good agreement with other reports (Bachowski et al., 1998; Liu et al., 2008) about some pesticides inducing ROS generation in other cell line models. Previous studies showed that ROS generation caused by some pesticides is one of the important factors in cytotoxicity. Our recent investigation exhibited that *cis*-bifenthrin can induce human FL cell apoptosis that is mediated by a dose-dependent accumulation of intracellular ROS. The molecular mechanism of induction of ROS generation by LCT is unknown. Some previous studies about oxidative damage caused by organochlorine pesticides such as acetofenate, dieldrin and DDT indicated that ROS generation plays an important role in cytotoxicity (Zhao et al., 2009; Zhao and Liu, 2009); Bachowski et al., 1998; Pérez-Maldonado et al., 2005). Because LCT contains chlorine atoms, we proposed that LCT may share a similar mechanism of oxidative damage with organochlorine pesticides.

Many studies have confirmed that ROS generation may cause DNA damage (Liu et al., 2008). In this study, we chose the comet assay for the investigation of DNA damage induced by LCT in the macrophage cell line RAW 264.7. The results showed that LCT induces DNA damage, which was reflected by an increase in tail length and a decrease in integrity of the nucleolus. Regarding tail length (Fig. 3A), a significant dose-dependent induction was observed after incubation with 10^{-7}, 5×10^{-7}, and 10^{-6} mol/L of LCT. At the same time, the integrity of the nucleolus was decreased in cells after exposure to similar concentrations of LCT, exhibiting obvious DNA damage with dose-dependence (Fig. 3B). It is well-known that there is a close relationship between ROS generation and DNA damage (Chung et al., 2000). Our former results indicated that the generation of ROS was an important

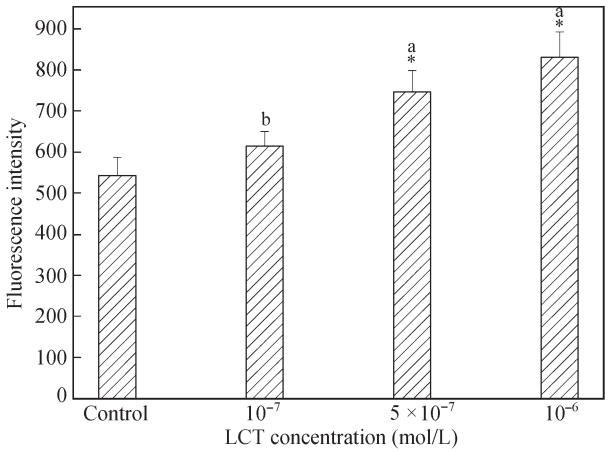

Fig. 2 Effect of lambda-cyhalothrin on intracellular reactive oxygen species (ROS) generation. RAW 264.7 cells exposed to different concentrations of lambda-cyhalothrin for 6 hr, followed by ROS determination. Asterisk above adjacent bars indicate a significant difference ($p < 0.05$, $n = 5$) between treatment and the control. Different letters above asterisk indicate a significant difference ($p < 0.05$, $n = 5$) among each treatment time.

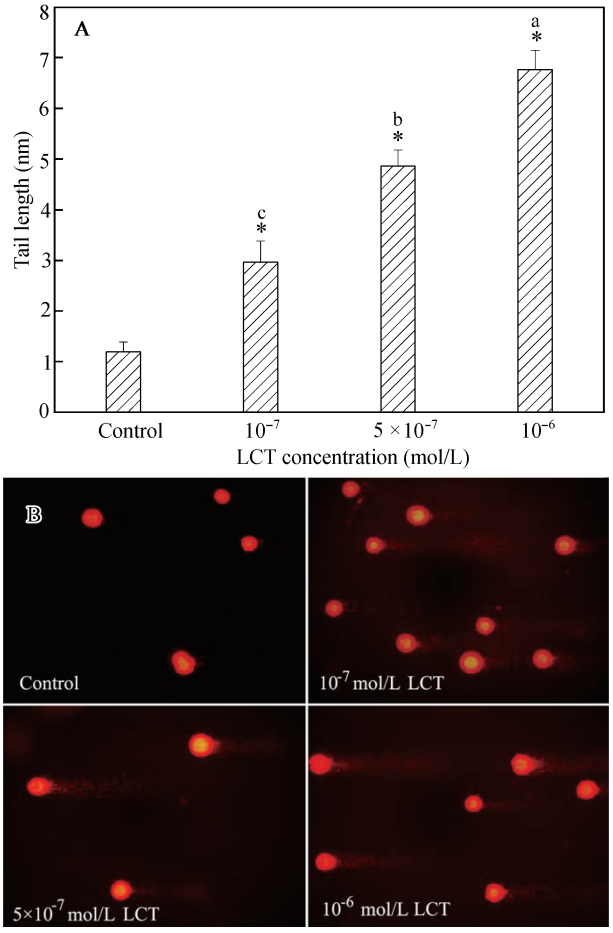

Fig. 3 Effect of lambda-cyhalothrin on DNA damage in RAW264.7 by the comet assay. (A) tail length data of RAW 264.7 cells. RAW 264.7 cells exposed to lambda-cyhalothrin at concentrations of 10^{-7}, 5×10^{-7}, and 10^{-6} mol/L for 24 hr, followed by the comet assay. Asterisk above adjacent bars indicate a significant difference ($p < 0.05$, $n = 5$) between treatment and the control. Different letters above asterisk indicate a significant difference ($p < 0.05$, $n = 5$) among each concentration. (B) typical comet assay pictures.

apoptotic signal in FL cells and macrophage when they were treated with *cis*-bifenthrin and acetofenate, respectively (Liu et al., 2008; Zhao et al., 2009). Reactive oxygen species including $O_2\cdot$, $\cdot OH$ and H_2O_2 enhance oxidative process and produce lipid peroxidative damage to cell membranes. The $\cdot OH$ has been proposed as an initiator of lactiperoxidase (LPO) through an iron-catalysed Fenton reaction (Halliwell et al., 1985) and the macrophage cells may be susceptible to oxidative damage. Our present research further demonstrated that there was a strong dependence of ROS-induced DNA damage on LCT. It is interesting to note that the cytotoxicities to macrophage cells, ROS generation and DNA damage by LCT were reasonably consistent. As a result of the linkage, the ROS increase and DNA damage may be the major factors influencing LCT-induced macrophage cytotoxicity.

3 Conclusions

In summary, we explored, for the first time, the role of LCT in inducing cytotoxicity in macrophage cell line RAW 264.7. Our results suggested that LCT possesses potential immunotoxicity mediated by toxicity to macrophages. The phenomena observed indicated that the cytotoxicity in macrophages could be contributed by the high level of intracellular ROS and DNA damage. Given the widespread use of SPs, it would be imperative to carry out comprehensive research and epidemic investigation to understand the potential immunotoxicity to non-target organisms of SPs.

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