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Effects of fullerenol nanoparticles on acetamiprid induced cytoxicity and genotoxicity in cultured human lung fibroblasts

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ABSTRACT

The aim of this study was to investigate the effects of water soluble fullerene (fullerenol) nanoparticles on the in vitro genotoxicity induced by the insecticide acetamiprid. Healthy human lung cells (IMR-90) were treated with fullerenol $C_{60}(OH)_n$ (n: 18–22) alone and in combination with acetamiprid for 24 h. The micronucleus test, comet assay and γ-H2AX foci formation assays were used as genotoxicity endpoints. Cytotoxicity was evaluated using the clonogenic assay. The maximum tested concentration of fullerenol (1.600 μg/ml) induced 77% survival where as the lowest concentration (25 μg/ml) was not cytotoxic where as acetamiprid was cytotoxic. Fullerenol did not induce genotoxicity at tested concentrations (50–1600 μg/L). On the other hand, acetamiprid (>50 μM) significantly induced formation of micronuclei, and double and single stranded DNA breaks in IMR-90 cells. For simultaneous exposure studies, two non-cytotoxic concentrations (50 and 200 μg/ml) of fullerenol and three cytotoxic concentrations of acetamiprid (100, 200 and 400 μM) were selected. As a result, we observed that co-exposure with fullerenol significantly reduced the cytotoxicity and genotoxicity of acetamiprid in IMR-90 cells. Our results indicated the protective effect of water soluble fullerene particles on herbicide induced genotoxicity.

1. Introduction

Over the past decade, nanomaterials have received considerable attention due to their promising potentials for a wide range of industrial, biomedical, environmental and electronic applications [1–2]. As a result of the increasing diversity of nanoproducts, assessment of their potential impacts on human and environmental health has become crucial [3–5]. Among various types of nanomaterials, fullerenes attract a good deal of attention based on their electronic, mechanical, optical and chemical characteristics. A fullerene is any molecule composed entirely of carbon, in the form of sphere, ellipsoid or tube [6]. The best known of fullerenes is the "buckyball" a closed spherical molecule consists of sixty carbon atoms, so called C60 [7].

Pristine C60 fullerenes are nearly insoluble in water and forms aggregates in aqueous environments. However, chemical modifications of C60 fullerenes, into water soluble polyhydroxylated derivatives, make them available for biological systems [8]. Thus, hydroxylated fullerenes, also known as fullerenols, have emerged as an important class of fullerene-derived compounds with promising applications in bio-medical areas [9–10]. Results of previous studies generally demonstrated that fullerenols are not cytotoxic or genotoxic in biological systems [11–12]. However, there are also several in vitro studies that have shown fullerenol-induced cytotoxicity in various cell lines [13–14].

Pesticides are one of the most common pollutant groups in the world. They are known to be reactive compounds and can cause genotoxic effects on DNA or cytotoxic effects on cell [15–17]. Neonicotinoids are a relatively new class of pesticides, which are used as insecticides. *Acetamiprid* is a member of the neonicotinoid group of insecticides commonly used against a wide range of insect pests [18]. Although it acts as a selective agonists for the nicotinic acetylcholine receptors in insects, and thus is considered as safe for mammals, results of in vitro studies demonstrated that acetamiprid is cytotoxic and genotoxic on mammalian cells [15,17]. The proposed mechanisms underlying the cyto-genotoxicity of acetamiprid is mainly due to generation of reactive oxygen species (ROS) resulting in oxidative damage [19,20].

Polyhydroxylated fullerenes such as $C_{60}(OH)_{24}$, are being extensively studied due to their promising potential as antioxidants [10,21–23]. Protective effects of fullerenols against induced toxicity of different chemicals such as doxoribucin, cisplatin, taxol, levadopa, nitric oxide as well as radiation, have been demonstrated [12,21–25]. In the present study, we aimed to evaluate the modulating effects of water-soluble fullerene nanoparticles on the acetamiprid-induced cytotoxicity and genotoxicity in human cells in vitro. The human lung fibroblast cell line IMR-90 was selected

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for our experiments. Suitability of this cell line for cytotoxicity and genotoxicity experiments has been previously demonstrated [26,27]. The clonogenic assay and nuclear division index values were used to assess cytotoxicity [17,28,29]. The micronucleus, alkaline comet and γH2AX foci formation assays were used as genotoxicity endpoints [17,30–32].

2. Materials and methods

2.1. Chemicals

Technical grade (95%) acetamiprid (Shenzen Co. Ltd., China) was used for the experiments. Dulbecco's Modified Eagle Medium (MDMEM), fetal calf serum, phosphate buffered saline (PBS), cytochalasin-B and Gemsa stain were purchased from Sigma–Aldrich (St. Louis, MO). Anti phospho γH2AX primary and Alexafluor 488 labeled secondary antibodies were purchased from Thermo Scientific and Invitrogen, respectively. All other chemicals used in comet assay experiments such as low melting point agarose, normal melting point agarose, EDTA and Triton x-100 were also purchased from Sigma–Aldrich (St. Louis, MO). Polyhydroxy-C60 ($C_{60}(OH)_n$, fullerenol hereafter, $n \sim 18$–22) was purchased from BuckyUSA. An average of 20 OH groups per fullerenol molecule was assumed for all experiments. Hydrogen peroxide (H_2O_2) was used as positive control at a single concentration of 100 μM. Acetamiprid is soluble in water (4.25 g/L at 25 °C). Thus, prior to experiments, acetamiprid was dissolved in sterile distilled water in order to eliminate possible toxic effects organic solvents and sterile distilled water was used as solvent control at a maximum concentration of 0.5% (v/v).

2.2. Preparation and characterization of fullerenol

Fullerenol powder was dissolved in demineralized water at concentration of 20 mg/ml and then diluted with DMEM medium to set a treatment solution. Characterizations of fullerenol particles were performed using Dynamic Light Scattering (DLS) and zeta potential measurements. Fullerenol particles further characterized using transmission electron microscopy (TEM). Fullerenol at a concentration of 100 μg/ml placed onto 300 mesh formvar-coated copper grids and allowed to dry. Samples were examined under TEM (FEI/Tecnai Spirit G2) operating at 120 kV. Digital images were taken with the MORADA camera using Soft Image System.

2.3. Cell culture

The human lung fibroblast cell line IMR-90 obtained from ATCC was used for the experiments. Cells with passage number between 25 and 30 were used in this study. The IMR-90 cells were grown in DMEM medium supplemented with 15% fetal calf serum (FCS), penicillin (100 IU ml21) and streptomycin (100 lg ml21), 10 mM L-glutamine, 10 mM non essential amino acids and sodium pyruvate. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were grown in 75 cm^2 flasks and subcultured once a week.

2.4. Cytotoxicity

Cytotoxicity was determined using the clonogenic assay [29]. Approximately ten thousand cells were seeded into a 6 well tissue culture plate and allowed to grow for 48 h. The cultures were then treated for 24 h with serial concentrations of acetamiprid (50, 100, 200, 400, 800 and 1,600 μM) and fullerenol (50, 100, 200, 400, 800 and 1,600 μg/mL). Following the exposure period, the cells were collected by trypsinization and centrifugation and then counted using Cedex XS (Roche) cell counter. Then cells were re-seeded at colony forming density (1000 cells per well). Colonies were allowed to grow for 15 days, fixed with 100% methanol, stained with crystal violet, and counted. Four dishes were used for each treatment and experiments were repeated in triplicate.

2.5. Cytokinesis-block micronucleus test

To perform cytokinesis-block micronucleus test, the IMR-90 cells were seeded in 60 mm diameter sterile cell culture dishes at a density of 5×10^4 cells/dish and allowed 48 h to establish normal growth. Cells were then treated with selected concentrations of acetamiprid, fullerenol, alone and or in combination for 24 h. After treatment, cells were further cultured for 24 h with new medium containing cytochalasin-B. The cells were then harvested and resuspended in hypotonic solution (0.075 M KCl) for 2.5 min. Following fixation in ethanol:glacial acetic acid (3:1), cells were spread on precleaned glass slides and stained with 5% giemsa for 10 min. Dried slides were analyzed and under light microscope (40×) and the number of binucleated (BNC) cells with micronuclei (MNBNC) was recorded in 2000 cells per treatment group [17,30].

Cytotoxicity was further estimated by using the nuclear division index (NDI) as described elsewhere [17,28]. Briefly, the numbers of cells with one to four nuclei were determined in 1000 cells and NDI was calculated using the following formula: NDI = (1 × M1 + 2 × M2 + 3 × M3 + 4 × M4)/1000; where M1 through M4 represent the number of cells with one to four nuclei.

2.6. Immunofluorescence for γH2A.X foci formation

Gamma-H2A.X foci assay, used to detect double strand DNA breaks, was performed on 8 well chamber slides as previously described [17,32]. Briefly, the cells were treated with selected concentrations of acetamiprid and fullerenol for 24 h. Following fixation with 4% paraformaldehyde and permeabilization with 0.2% Triton X-100, blocking was performed with 1% bovine serum albumin (BSA) for 1 h. Cells were then incubated with anti-γH2A.X antibody at 4 °C overnight and then incubated with a AlexaFluor 488-conjugated second antibody for 1 h. Nuclei were counterstained with DAPI. The slides were mounted and viewed with a Nikon Fluorescence Microscope. Three independent experiments were performed and γH2A.X foci were counted in 100 cells per treatment concentration.

2.7. Alkaline comet assay

Alkaline comet assay was used to detect acetamiprid and fullerenol induced single strand DNA breaks. IMR-90 cells treated in 60 mm dishes were harvested and embedded in 0.8% low melting agarose on slides precoated with normal melting point agarose. Slides were then placed in lysis solution and incubated overnight at 4 °C. Electrophoresis as performed at 25 V and 300 mA for 20 min following denaturation in alkaline buffer. The slides were immersed in neutralization buffer for 10 min and than dehydrated in 70% ethanol. The slides were air dried and stained with ethidium bromide. Comets were analyzed by visual scoring as described elsewhere [33] and genetic damage index (GDI) values were calculated according to Pitarque et al. [34].

2.8. Statistical analyses

After assessing the normality of distribution of the data, both parametric and nonparametric tests were used in order to detect differences at the 0.05 level of significance. Cytotoxicity data was analyzed on the percentage of cells that survived compared to the control. ANOVA with LSD (least significant difference) post