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Cytotoxicity of fullerenols on *Tetrahymena pyriformis*

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Abstract With the increasing use of fullerenes and their derivatives in a variety of fields, the toxicity and effects of fullerenes on humans and the environment have received considerable attention. In this study, the cytotoxicity of fullerene derivative, $C_{60}(OH)x$, on *Tetrahymena pyriformis* was investigated. Cell growth inhibition was evaluated by counting with an optical microscope, and the generation time was calculated. It was indicated that the fullerenols caused a dose-dependent growth inhibition of the cells. The morphologic change in the damaged macronucleus of cells was observed using a fluorescent microscope. Superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and glutathione reductase (GR) levels were also measured for *Tetrahymena pyriformis*, using conventional methods. The results showed that fullerenols could reduce GSH-PX and GR activities. But no noticeable difference in SOD activity was observed between the treated groups and the control group. This indicated that the antiproliferative effect of fullerenols might be mediated by the reduction in the activities of GSH-PX and GR of cells and the destruction of the macronucleus.

Key words Fullerenol, Cytotoxity, *Tetrahymena pyriformis*

CLC numbers Q689, X174

1 Introduction

Nanotechnology has vast potential uses, such as in drug delivery devices and personal care products, in the fields of biology and medicine. Fullerene is one type of man-made nanoparticle. They have unique optical, electrical, chemical, and physical properties. Although scientists from several different research fields believe that fullerene might have wide applications, it is prudent to take into account the possible toxicity of nanoproducts before their widespread use. Nanomaterials including fullerenes can affect wildlife if they accidentally enter the environment. It is likely that they will eventually be found in the environment at measurable concentrations. Therefore, their toxicity, both *in vitro* and *in vivo*, is an important characteristic for defining and constraining their applications. There are a number of articles that report that the phototoxicity of fullerene molecules is a feature that is useful for therapeutics [1,2]. Other studies have focused on minimizing the toxicity of fullerenes to enable their use in drug delivery applications [3]. The study of fullerenes is also important for understanding the eventual fate and environment implications of fullerenes used in certain products [4]. Our attention was drawn to this issue because of the recent interest in the toxicity and the environment problem effected by fullerenes. Fullerene aggregates were found to induce oxidative stress in the brains of fish in water systems [5]. Because of their sensitivity to environmental alterations, protozoa have been proposed as biological indicators of water pollution [6].

Moreover, the ease with which protozoa can be handled in the laboratory is an essential requirement

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that makes these unicellular eukaryotes a suitable alternative for aquatic toxicity evaluation of pollutants. To our knowledge, the toxicological database of the effects of fullerens on protozoans is rather sparse. In this study, *Tetrahymena pyriformis* was used as the model organism to assess the cytotoxicity of fullerens, for the first time. The tests carried out in this study include morphological and physiological state of the cells exposed to different concentrations of fullerens.

2 Materials and methods

2.1 Fullerenols $C_{60}(OH)_x$

The polyhydroxylated derivative of fullerene, fullerenols $C_{60}(OH)_x$ (x = 18–22), used in this study was synthesized according to a previously reported procedure[7]. The fullerenols were dissolved in distilled water and a stock solution of concentration of 2.27 mg·mL^{-1} was prepared. Sterile solutions for cell experiments were obtained by filtering these solutions through 0.22-μm pore membranes before adding to the culture.

2.2 Cell cultures and exposure to fullerenes

Tetrahymena pyriformis were obtained from the Institute of Hydrobiology, the Chinese Academy of Sciences. The cells were grown at exponential phase in Proteose Peptone Yeast Extract Medium (PPY), 2% proteose peptone and 0.5% yeast extract at pH 7.0–7.5, at 24±2 °C.The density of *Tetrahymena pyriformis* cultures was adjusted in fresh PPY in order to obtain at least 10^4 cells per mL. Fullerenols were added to the cells at 0.06, 0.10, 0.15, 0.20, and 0.25 mg·mL^{-1}, and the cells that were not exposed to fullerenols were used as control. Untreated and treated *Tetrahymena pyriformis* were incubated in 4 mL eppendorf tubes in a total volume of 2 mL. Samples from six independent assays were taken for the determination of growth inhibition of the cells after 24 h and 48 h of incubation.

2.3 Determination of generation time

Aliquots of 100 μL were immediately taken (T_0) from the control and the exposed cultures and subsequently at 24 h and 48 h. The samples were appropriately diluted in distilled water and fixed with neutral-buffered formalin (NBF) containing 10% (*V/V*) formalin in phosphate-buffered saline (PBS) (pH 7.4) at a final concentration of 2%–5% for 1 h. The cell number was determined by counting every cell present in each of two 30-μL subsamples using an inverted optical microscope (Fenghuang, Chongqing) at 100× magnification. The *Tetrahymena pyriformis* were characterized by their generation time (g) required for doubling the population. Generation time was calculated using the following formulae. [8]

Number of generations n is given by

$$n = \frac{\log N_1 - \log N_0}{\log 2} \quad (1)$$

and generation time g is given by

$$g = \frac{\text{time of growth}}{n} \quad (2)$$

where N_1 is the number of cells at 24 h, N_0 is the number of cells at T_0, and "time of growth" = 24 h.

2.4 Fluorescence microscopy observation

Tetrahymena pyriformis were incubated in the solution of fullerenes with concentration of 0.25 mg mL^{-1} for 24 h. Then, the cells were washed thrice with PBS. After washing, cells were stained with acridine orange (AO) and examined using a Zeiss Axioskop2 plus fluorescent microscope, and the macronuclei of the cells appeared in green at 488 nm.

2.5 Determination of enzyme and lipid peroxidation

Experimental samples were divided into two groups: untreated control group and fullerenols groups (F). In the case of the (F) groups, fullerenols were added to cell cultures with the final concentration of 0.06 and 0.25 mg·mL^{-1}, respectively. After exposure for 24 h, the cells were washed three times with PBS and sonicated for 6–8 min in an ice-cold water bath. The homogenate was centrifuged for 10 min at 3000 r min^{-1} at 4°C and the supernatants were used to assay the activities of glutathione peroxidase (GSH-PX), glutathione reductase (GR), and superoxide dismutase (SOD). The assay was conducted according to the methods described in the Detection Kits (Nanjing Jiancheng Bioengineering Institute). The results were