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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

expressed as U·mg^{-1}protein for GSH-PX, GR, and SOD.

2.6 Statistical analysis

All the experiments were repeated three times or more, and the results were expressed as mean and standard deviation (SD) values. Student's *t*-test was used to analyze the differences between the groups.

3 Results

3.1 Determination of generation time

Under the culture conditions used in this study, the initial cell density was 1.5×10^4 and the normal generation time of *Tetrahymena pyriformis* was about 7.2 h. The experiments showed that addition of fullereonls affected gradually generation time (Table 1) in dose-dependent manner. The fullerenols of lower concentration (0.06, 0.10, and 0.15 mg·mL^{-1}) did not have a significant effect ($p < 0.005$) on generation time *of Tetrahymena pyriformis*. However, generation time was increased at higher concentrations (0.20 and 0.25 mg·mL^{-1}) of fullerenols, indicating that fullerenols of higher concentration inhibited the growth of *Tetrahymena pyriformis*.

3.2 Alterations in the macronucleus in fullerenols-treated cells

Fig. 1 shows the fluorescent images of *Tetrahymena pyriformis* after being stained with acridine orange (AO). The morphology of a normal cell (Fig. 1(a)) was complete, and the macronucleus with complete shape, showing green fluorescence (indicated by an arrow) was located in the middle of the cell. However, after 24 h of incubation in a solution of fullerenols with final concentration of 0.25 mg·mL^{-1}, the macronucleus was destroyed or differently chipped (Fig. 1(b)).

Table 1 Effect of fullerenols on generation time of *Tetrahymena pyriformis* after 24 h of growth

Fullerenol concentrations / mg·mL^{-1}	Generation time / h
0	7.23 ± 0.03
0.06	7.30 ± 0.44
0.10	7.51 ± 0.61
0.15	7.59 ± 0.77
0.20	8.97 ± 1.23
0.25	9.97 ± 1.54

3.3 Effect of fullerenols on enzymes reactivity

As is shown in Fig. 2-I, the GSH-PX levels of the fullerenols groups were significantly lower than those of the control group ($p < 0.01$). The GR levels (Fig. 2-II) of the control group were evidently higher than those of the fullerenols group with concentration of 0.25 mg·mL^{-1} and slightly higher than those of the fullerenols group with concentration of 0.06 mg·mL^{-1}. According to Fig. 2-III, the SOD activities did not show significant difference between the control group and the treated groups.

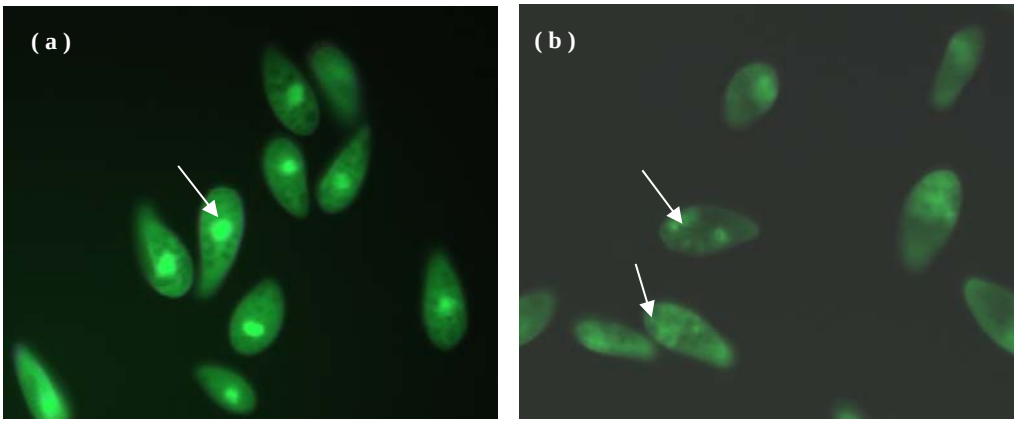
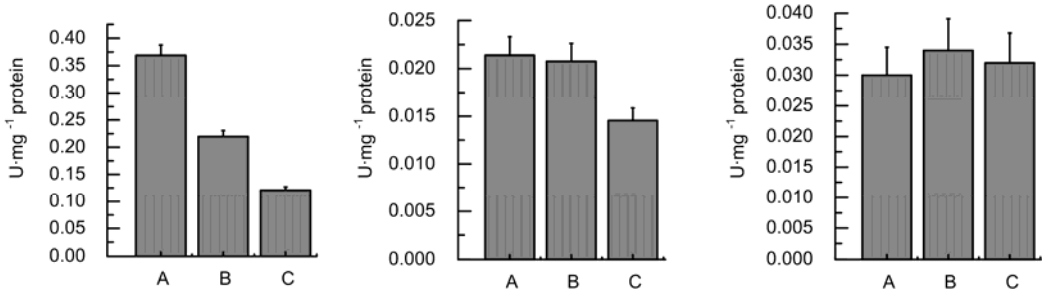


Fig. 1 Fluorescence images of *Tetrahymena pyriformis* (in green due to the macronuclei stained with acridine orange (AO), indicated by an arrow) (10 × 10). (a) Control. (b) Cells after 24 h of incubation with fullerenols at final concentration of 0.25 mg·mL^{-1}.



I. GSH-PX level II. GR level III. SOD level

Fig. 2 Effect of fullerenols on enzymes reactivity after 24 h of incubation.

A: Control group**.** B: Fullerenols (0.06 mg·mL^{-1}) group. C: Fullerenols (0.25 mg·mL^{-1}) group.

4 Discussion

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. However, we were unable to obtain information in the literature on the biochemical parameters of *Tetrahymena pyriformis* that reflected the effects of fullerenes, although there are some studies concerning the effect of fullerenes on cells. Therefore, the data of this study should be compared with those of other studies on the effects of fullerenes on biochemical parameters of cells.

There are several articles concerning the influence of C_{60} and its derivatives on cell growth [1]. Most of them indicated that the cytotoxicity of C_{60} was photodependent. However, Bogdanovie *et al.* [9] reported that the fullerenols $C_{60}(OH)_{22}$ inhibited the growth of human breast cancer cell lines T47D, MCF-7, and MDA-MB-231 at a range of nanomolar concentrations and without photoinduction. This result is in accordance with the assumption that fullerenols is retained by the cells; thus, cell growth is impaired. Some studies reported the antiproliferative effects of fullerenol ($C_{60}(OH)_n$, $n = 18-20$) on normal vascular smooth muscle cells and tumor cell lines as well. The results can be explained by the interaction of $^1O_2$, (generated upon light excitation of fullerenols), with cells or by the direct interaction between the fullerene excited state and cells [10]. In the study of Lu *et al* [11], the inhibitory effect on cell growth was explained by the inhibition of signal transduction through tyrosine kinase pathway. Sayes *et al* [12,13] reported that some water-soluble fullerenes have cytotoxicity for different human cell lines under ambient conditions in water. Oxidative damage to the cell membranes was observed in the case where fullerene exposure led to cell's death. In this study, it was observed that high concentration of fullerenol led to a dose-dependent inhibition of growth of *Tetrahymena pyriformis*, whereas low concentration of fullerenol had no inhibitory effect (Table 1). The results showed that fullerenes might decrease the reactivity of some antioxidant enzymes. For the group with concentration of 0.25 mg·mL^{-1}, the activities of GSH-PX and GR were lower than the control group ($p < 0.01$). The activity of GSH-PX of the 0.06 mg·mL^{-1} group was lower than that of the control group ($p < 0.01$), whereas the GR level of this group was not significantly different from that of the control group. With regard to the SOD level, shown in Fig. 3-III, there was not much difference between the treated groups and the control group. It indicated that high concentration of fullerenols can reduce the cell's activities of GSH-PX and GR. This implied that fullerenols caused damage of *Tetrahymena pyriformis.* In our study, macronucleus was observed using fluorescent microscope and their damage (Fig. 1(b)) and dysfunction were observed to be primary characteristic of cell death. Hence, fullerenol can cause growth inhibition of *Tetrahymena pyriformis*.

5 Conclusions

In our study**,** high concentrations of fullerenol derivative, $C_{60}(OH)x$, caused a dose-dependent growth

inhibition of *Tetrahymena pyriformis*. The results suggest that the antiproliferative effect of fullerenols may be mediated by reducing the activities of GSH-PX and GR of cells and damaging the macronucleus. Because the eukaryotic unicellular protozoan is highly prevalent in the aqueous environment and is an important member of the food chain in fresh water, the results obtained in this study have provided significant information to evaluate the effect of fullerenol on the environment.

References

1 Yang X L, Fan C H, Zhu H S, Toxicol in Vitro, 2002, **16**: 41-46

2 Yang A, Cardona D L, Barile F A, Cell Biol Toxicol, 2002, **18**: 97-108

3 Gonzalez K A, Wilson L J, Wu W J *et al.* Bioorg Med Chem, 2002, **10**: 1991-1997.

4 Colvin V L, Nat Biotechnol, 2003, **21**: 1166-1170

5 Oberdrster E, Environmental Health Perspectives, 2004, **112**: 1058-1062

6 Nicolau A, Mota M, Lima N, FEMS Microbiology Ecology, 1999, **30**: 209-216

7 Li T B, Huang K X, Li X H, *et al*. J. Chem Commun**,** 1999**,** **4**: 30-32

8 Dias N, Mortara R A, Lima N. Toxicol in Vitro, 2003, **17**: 357-366

9 Bogdanović G, Kojić V, Đorđević A , *et al*. Toxicol in Vitro, 2004, **18**: 629-637

10 Lu L H, Lee Y T, Chen H W, *et al*. British Journal of Pharmacology, 1998, **123**: 1097-1102

11 Nakamura E, Tokuyama H, Yamago S, *et al*. Bulletin of the Chemical Society of Japan, 1996, **69**: 2143-2151.

12 Sayes C M, Fortner J D, Guo W, Nano Letters. 2004, **10**: 1881-1887

13 Sayes C M, Colvin V L. Biomaterials, 2005, **26**: 7587-7595