Effects of low level tributyltin chloride on DNA in *Euglena gracilis* Z and SMZ strains

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Tributyltin chloride (TBTCl) has been widely used as marine anti-fouling and caused serious toxicity to organisms. In this study, effects of low concentration TBTCl exposure to *Euglena gracilis* Z and SMZ, particularly, on cell growth and DNA strand were examined. The inhibition of cell growth for 3 days exposure to TBTCl was observed dose dependently from 1 to 4 μM in both the strains. Comet assay showed DNA strand breaks at 1 μM TBTCl exposure in both the strains. These results suggest that micro-molar TBTCl exposure inhibited cell growth and induced DNA breaks to *E. gracilis* Z and SMZ.

Key words: Tributyltin chloride (TBTCl), comet assay, DNA strand breaks

Introduction

Tributyltin chloride (TBTCl), one of the organotin compounds, used as anti-fouling agent, is well known to cause endocrine disruption at low concentrations[1)]. Recently, it has been suspected that TBTCl not only disturbs endocrine functions to shellfishes, but also be cytotoxic to small biota at lower concentrations[2)]. The authors have reported the cytotoxicity of TBTCl on *E. gracilis*. Ohta et al. reported the acute effect of TBTCl above 50 μM to *E. gracilis* and detoxification[3)]. However, there is little information concerning the effects of TBTCl at extremely low concentration especially on DNA. Therefore, this study was focused on the cytotoxicity of low level TBTCl at 1 to 4 μM by assessing the effect on cell growth and DNA strand.

Materials and Methods

Cell culture and TBTCl exposure

Photosynthetic *Euglena gracilis* Z and achlorophyllous mutant SMZ used in all experiments were cultured in liquid Koren-Hutner medium[4)] at 28 ℃ under illumination (2800 lx) with 12 h light and 12 h dark cycle. To assess the effect of TBTCl on the cell growth, cells were grown in Koren-Hutner medium containing TBTCl at 1 to 4 μM for 3days. For comet assay and cell viability, cells exposed to TBTCl at 1 μM in same medium for 3 days were used.

Assessment of the effect of TBTCl on the cell growth

The effect of TBTCl on the cell growth was monitored by measuring the turbidity. Briefly, 10^4 cells were inoculated into 5 ml of Koren-Hutner medium containing 1, 2 and 4 μM of TBTCl to incubate for 3 days without aeration. The cell growth was measured by reading the optical density at 610 nm.

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Comet assay

To examine whether TBTCl induces the oxidative damage to DNA, comet assay was carried out. Cells were cultured in Koren-Hutner medium containing 1μM of TBTCl for 3 days, and then subjected to the assay. Microscope slides were precoated with 0.1% low melting point agarose and air-dried. The cell suspension was mixed with the same agarose and spread on the precoated slides. Then the slides were lysed in a cold lysis solution consisting of high salts and detergents for 1 h in the dark. These slides were immersed in alkaline solution (pH > 13.0) to form single-stranded DNA and washed twice in TBE buffer. Then the electrophoresis was carried out in the same buffer using a horizontal electrophoresis apparatus. Thereafter, the slides were immersed in a cold ethanol and air-dried. The cells were stained with SYBER Green® I solution and examined with a fluorescence microscope (excitation; 490 nm, emission; 520 nm) and images were captured by CCD camera. Then the length of DNA migration, the image length and the nuclear sizes were analyzed with NIH image and the tail moment was calculated.

Evaluation of the cell viability

The cell viability was estimated by the Trypan Blue Dye Exclusion Test with 0.04% Trypan Blue[5]. After staining, the cells were washed again with PBS. Cell viability was evaluated under a microscope and expressed as the percentage of viable cells for total cells.

Statistics analysis

Data were expressed as the means ± S.D. Statistical significance was assessed with a one-way ANOVA test. A *P*-value of < 0.05 or 0.01 was considered significant.

Results and Discussion

Effect of TBTCl on the cell growth

The cell growth exposed to 1, 2 and 4μM of TBTCl were monitored for 3 days. In both the strains, the cell growth was inhibited by the TBTCl dose dependently (Fig. 1). In both strains even 1μM of TBTCl caused retardation of the cell growth. A more marked cell growth inhibition was observed in the SMZ strain than Z strain. It is clear that low concentrations of TBTCl as low as 1μM significantly affected the growth of both the strains. The results suggest that the growth of the microalgal would be inhibited by aqueous environment polluted with comparatively low concentrations of TBTCl at several μM.

Considering the fact that microalgae are the primary producers, the inhibitory effects of TBTCl should greatly influence on to the secondary producers, or small fishes and shellfishes, and other higher predators through the food chain or its breakdown, finally adverse effects to the mankind.

Effect of TBTCl on DNA

The TBTCl induces DNA fragmentation by generating reactive oxygen species (ROS)[6]. We also observed intracellular peroxidation of *E. gracilis* cells under TBTCl exposure (Watanabe and Suzuki, unpublished data). Therefore, the effect of low level as 1 μM TBTCl on the DNA in *E. gracilis* was investigated by comet assay. TBTCl exposure significantly increased the comet tail length in both the strains (Fig. 2). However, there was a significant difference in the level of DNA strand breaks between the two strains. Comet tail length was obviously longer in the SMZ strain than the Z strain (Fig. 2) suggesting SMZ strain was more susceptible to DNA strand breaks caused by TBTCl. As described above, the DNA strand breaks caused by TBTCl should be regarded as an oxidative stress, however, the comet assay does not identify which DNA in the cell was damaged by TBTCl exposure. The difference in DNA strand breaks between Z and SMZ was probably due to the difference of cellular response to TBTCl-induced stress. The Z strain that inevitably produces oxygen and ROS during photosynthesis

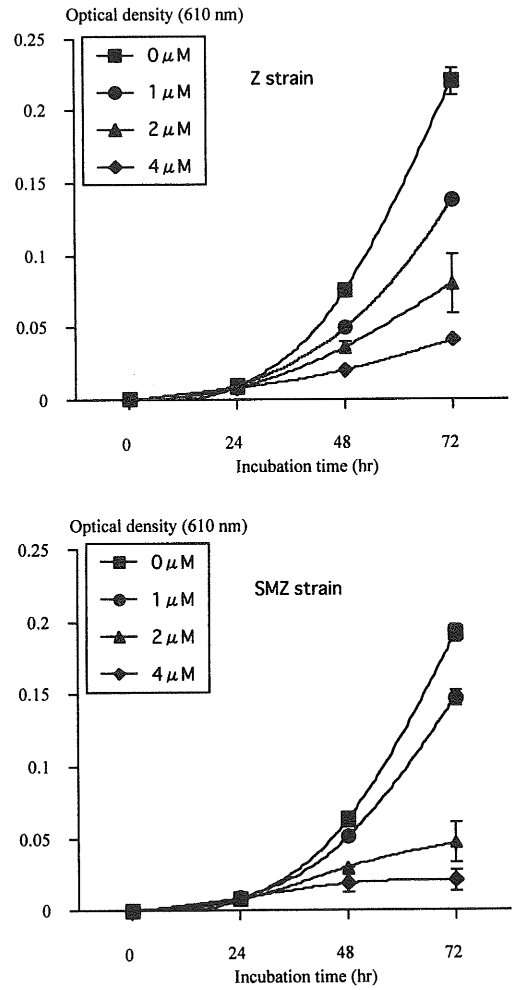

Fig. 1 The growth curve of *E. gracilis* Z and SMZ exposed to different concentrations of TBTCl. The results are expressed as means ± S.D (n=3). TBTCl concentrations ■: 0μM TBTCl ●: 1μM TBTCl ▲: 2μM TBTCl ◆: 4μM TBTCl

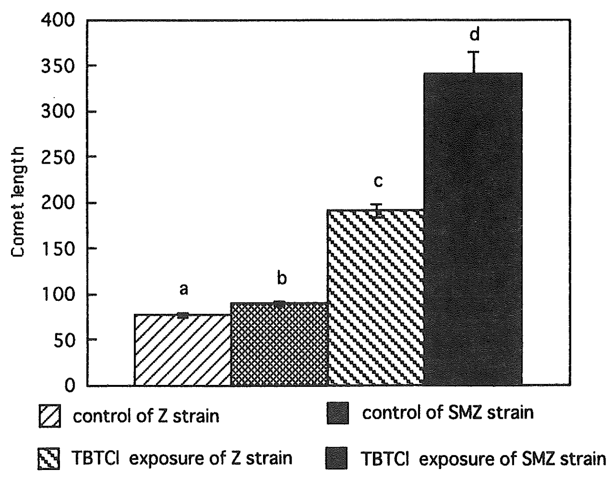

Fig. 2 Comet length of *E. gracilis* exposed to 1 μM of TBTCl for 3 days. The results are expressed as means ± S.E.M. (n = 75). A statistical analysis was carried out using an ANOVA test. Symbols a, b, c, d indicate a significant difference between each group ($P < 0.01$).

has greater ROS scavenging capacity, or antioxidative potential in the cell such as antioxidative substances and enzymes[7]. While the SMZ strain that does not photosynthesize would hold less ROS scavenging capacity than the Z strain. These reasons to elucidate the higher DNA damage in the SMZ strain must be verified by the future studies.

Effect of TBTCl on the cell viability

Cell viability was 97.98 ± 0.46% in Z strain and 98.33 ± 0.65% in SMZ strain under the control condition (Table 1). On the other hand, after TBTCl exposure, it was 96.61 ± 0.64% and 98.14 ± 0.57, respectively (Table 1). For both the strains, there was no significant decrease in cell viability compared with the control and TBTCl-exposed cells. These results demonstrated that both the strains were still viable after 1 μM of TBTCl exposure.

Table 1 Cell viability of *E. gracilis* exposed to 1 μM of TBTCl for 3 days.

TBTCl concentration	% cell viability	
	Z strain	SMZ strain
0 μM	97.98 ± 0.46	98.33 ± 0.65
1 μM	96.61 ± 0.64^{N}	98.14 ± 0.57^{N}

The data are expressed as means ± S.D (n=4). Statistical analysis was carried out using a one-way ANOVA test. N: no significance between the control (0 μM) and experimental groups.

The lower cell growth in the KH medium containing low concentrations of TBTCl is elucidated by the growth suppression or retardation. An interesting fact is the similarity of episode taking place in the higher organism and a unicellular eukaryote, ***E. gracilis*** cells; that is, low dose exposure of TBTCl to higher organisms, e.g., shellfish does not lead to cell death, but endocrine disruption. Teratogenicity caused by long term low dose TBTCl to shellfish should affect gene expression and regulation. Interestingly, low dose exposure of TBTCl to ***E. gracilis*** cells also causes DNA strand breaks that could lead to cellular dysfunction. Low dose TBTCl as 0.4 μM to ***E. gracilis*** led incompletely proliferated starfish-like cells[8]. The data suggest what happening in higher multicellular organisms can happen in lower unicellular eukaryote, and the cellular dysfunction mechanism may be essentially the same.

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