

Effect of sodium arsenite exposure on *Euglena gracilis* SMZ: Inhibition of growth and viability of *E. gracilis* SMZ exposure to sodium arsenite

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Abstract

To estimate the cytotoxicity of sodium arsenite (NaAsO₂) on *Euglena gracilis* SMZ, alternations of cell growth and cell viability were first assessments. Monitoring of cell growth was one of the suitable indicators to appreciate cytotoxicity of NaAsO₂ because of previous reports indicated that growth of *E. gracilis* was inhibited by treatment of Cu, Zn, Hg, Pb and Cd (Devars *et al.*, 1998; Einicker-Lamas *et al.*, 2002).

The author assessed the cell growth to determine the arsenic concentration required for the inhibition of cell proliferates by more than 50 % of the control (IC50) or 50 % lethal concentration (LC50). In our laboratory, it was suggested that NaAsO₂ and dimethylarsinic acid (DMA) (1.75, 17.5, 175, 250, 500, 1000 and 1750 μ M) inhibited cell growth of *E. gracilis* SMZ in Koren-Hutner (K.H.) medium (pH 3.5). On the basis of these results, the author further investigated the effect of NaAsO₂ on cell proliferation and cell viability under different concentrations in K.H. medium (pH 5.5). Exposed of NaAsO₂ higher than 2 mM strongly inhibited the cell growth, however, the viable cell number remained ca. 70 %. Below 750 μ M exposure, as high as more than 90 % cells were found alive suggesting the effect of NaAsO₂ would not be fatal but to lower cell proliferation activity that eventually led cells rest to survive.

Introduction

Arsenic (As) is a ubiquitous element in the environment and its amount is 1.5-2 ppm in the earth crust. Arsenic occurs as a result of several inputs that contain this element in organic and inorganic forms. Arsenic is present in coal, lead, zinc, gold and copper ores in the form of several minerals namely Arsenopyrite (FeAsS), Orpiment (As₂S₃), Realgar (AsS), Arsenolite (As₂O₃) and Lollingite (FeAs₂) (IPCS, 2001). The commonly existing chemical species of As in groundwater are in two forms namely arsenite (As^{III}), which is the reduced state of inorganic arsenic, and arsenate (As^V), which is the oxidized state of inorganic arsenic (Joshi *et al.*, 2003).

It has been known that inorganic arsenic assimilated in mammals was methylated and transformed into DMA via monomethylarsenic acid (MMA) (Vahter, 1999) and glutathione plays an important role in arsenic metabolite pathway which reduces arsenate to arsenite (Yamauchi, 1999).

The rate of methylation varies considerably between arsenical species. The initial step in arsenic detoxification in the prokaryote *Escherichia coli* and the eukaryote *Saccharomyces cerevisiae* are reduction of As^V (Rosen, 2002). Previously, methylation was thought to be the detoxification of As. It has been known for many years that the bio-transformation of inorganic As in mammals is through its progressive reduction/oxidation and methylation path-

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ways resulting to the formation of monomethylated and dimethylated metabolites. This view can no longer be true, since monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) have been identified as intermediates in the metabolic pathway and they are more acutely toxic and more genotoxic than arsenite (Mass *et al.*, 2001; Petrick *et al.*, 2001; Styblo *et al.*, 2000).

Euglena sp. are able to live in environment polluted with heavy metals. *E. gracilis* has been shown as a useful model for the study of oxidative damage induced by a number of metals including Hg, Cu and Cd (Mendoza-Cozatl *et al.*, 2002; Watanabe *et al.*, 2003), but only a few reports published about the effect of arsenic on *E. gracilis* (Blum, 1966). The author undertook research to obtain basic information on the extent *E. gracilis* SMZ as a model organism for the cytotoxicity study of arsenic. Since *E. gracilis* has highly developed subcellular organella, it may have many common characteristics with higher organisms in many cell functional aspects, which makes the organism eligible to be an alternative experimental model organism from the traditional species. It should be an interest in investigating the similarity effect between *E. gracilis* SMZ and other organisms by arsenic. In the present report, the author described the effect of NaAsO₂ on the cell growth, viability, accumulation of arsenic of *E. gracilis* SMZ.

Materials and methods

Reagent

A stock solution of 0.5 % Fluorescein diacetate (FDA, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was prepared by dissolving 50 mg of FDA in 10 ml of dimethylsulfoxide (DMSO), and 0.002 % FDA was prepared by adding 4 μ l of 0.5 % FDA, and diluting with 1.0 ml phosphate buffer saline (PBS) (pH 7.0).

A stock 0.002 % Propidium iodide (PI, Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution was prepared by adding 2 μ l of 10 mg/ml PI containing DMSO, and diluting with 1.0 ml PBS. These solutions were kept in eppendorf tubes at -20 °C until use.

a) Conditions of cell culture and sodium arsenite exposure

Cell culture

Euglena gracilis SMZ strain was grown at 28 °C in a K.H. medium (pH 3.5) under the illumination (2800 lx; 36.4 μ mol/s/m²) with fluorescent light for plant growth on a light/dark cycle 12/12 hours for 5 days until reaching an early stationary phase of growth.

Sodium arsenite exposure

After reaching an early stationary growth phase, cells were collected by centrifugation and NaAsO₂ (Nakarai Chemicals, Ltd., Kyoto, Japan) was added as final concentration of 0, 250, 500 and 750 μ M for 3 and 6 hours in K.H. medium (pH 5.5). The cell suspensions were incubated at 28 °C under illumination for each hour.

After NaAsO₂ exposure, these cells were washed twice with PBS (pH 7.0), collected with centrifuge and frozen at -70 °C until use for measurement of intracellular arsenic concentration.

b) Assessment of growth inhibition by sodium arsenite

To assess the effect of NaAsO₂ on the growth of *E. gracilis* SMZ, cell growth was monitored by comparing cell turbidity at 610 nm.

E. gracilis SMZ cell suspensions (50 μ l) were inoculated into 5 ml of K.H. medium (pH 5.5) containing different concentration of NaAsO₂ as final concentration 0, 250, 500, 750, 1000, 2000 and 3000 μ M, and incubated under the illumination (2800 lx; 36.4 μ mol/s/m²) with fluorescent light. Cell growth was monitored by reading turbidity at 610 nm with a Bausch and Lomb spectrophotometer (Shimadzu, Kyoto, Japan) after incubation for 7 days.

c) Assessment of cell viability

To estimate cell viability, 200 μ l of *E. gracilis* SMZ (containing 10^4 cells) that was exposed to 0, 250, 500 and 750 μ M of NaAsO₂ for 0, 3 and 6 hours and exposed to 1, 2, and 3 mM of NaAsO₂ for 8 days, and then collected by centrifuge at $1000 \times g$ (20°C for 5 min), washed twice with 200 μ l of PBS (pH 7.0). Collected cells were stained with 100 μ l of 20 μ g/ml FDA and 30 μ l of 20 μ g/ml PI at room temperature for 30 min in the dark. After staining, cells were washed again with 200 μ l of PBS solution. The viable and non-viable cells were counted with fluorescence microscope (model BX 51; Olympus, Tokyo, Japan), and cell viability was expressed as percentage of dead cells over total cell number.

d) Measurement of intracellular arsenic

NaAsO₂ exposed *E. gracilis* SMZ cell samples (0.1g w/w) were diluted with 0.4 ml of distilled water (DW). To aliquots of diluted cell samples (0.1 ml) were added 0.9 ml of conc. nitric acid in 10 ml tubes and boiled at 100°C in water bath for 3 hours. Allowed to cool, digested samples were diluted to 10 ml with DW.

Digested samples (1.0 ml) were diluted with 4.0 ml of DW, and added 0.1 ml of 500 ng/ml internal standard mix (prepared in 10 % nitric acid in DW). These samples were analysed using an Agilent 7500CS ICP-MS.

Spiked cell samples were prepared for every treatment doing regime. Digested sample (1.0 ml) was diluted with 1.0 ml of 100 ng/ml stock standard solution (in 2 % nitric acid), 3.0 ml of DW and 0.1 ml of 500 ng/ml internal standard mix.

Results and discussions

Cell growth

We observed a lag phase of cell growth of *E. gracilis* SMZ in K.H. medium (pH 5.5) when compared to K.H. medium (pH 3.5) for the first 7 days. By the 8th day of cultivation in K.H. medium, optical cell density was similar in the two different pH conditions. It was indicative that higher pH delayed cell growth (Fig. 1 - a). The author should make allowance for the effect of medium pH in this study.

The cell growth of *E. gracilis* SMZ exposed to NaAsO₂ in K.H. medium (pH 5.5) is shown Figure 1 - b. There was a slightly growth inhibition observed for the cells incubated in 500 μ M and 750 μ M NaAsO₂, and significant growth inhibition was observed in the cells incubated in higher than 1mM NaAsO₂. For cells incubated in 3 mM of NaAsO₂, there was no growth. The calculated IC₅₀ was 0.95 mM based on the cell growth in various concentration of NaAsO₂ at 7 days (Fig. 2, P = -0.96). These results prove tolerance to arsenite of *E. gracilis* SMZ.

Cell viability

The cell viability of *E. gracilis* SMZ cultured with long-term NaAsO₂ exposure for 8 days was shown at Table 1. The cell viability of *E. gracilis* SMZ exposed to 1, 2 and 3 mM of NaAsO₂ was 10.0, 28.5 and 25.1 %, respectively. While cell viability increased significantly in 2 mM of NaAsO₂, no additional increase of cell viability was observed in 3 mM of NaAsO₂ exposure. Cell death was increased in every concentration of NaAsO₂ for 3 hours short-term exposure, and no significant difference between control cells and 250 and 500 μ M exposures cells for 6 hours (Table 2).

The delay of cell proliferation was not observed and the cell death was only 25 % of total cell number, therefore it was considered that NaAsO₂ does not have a LC₅₀ value against *E. gracilis* SMZ.

Einicker-Lamas *et al.* (2002) had observed LC₅₀ for Cu²⁺ was 0.22 mM and for Zn²⁺ it was 0.88 mM. The minimal CuCl₂ concentration (0.02 mM) tested was sufficient to impair cell division, while Zn²⁺ was able to increase cell proliferation even at 0.1 mM. It is suggested that *E. gracilis* have a Cu and Zn uptake systems, because these metals are essential elements for *E. gracilis*. Although arsenic is not considered to be an essential element, it is accumulated in the cells, and therefore it should be metabolized as xenobiotics.

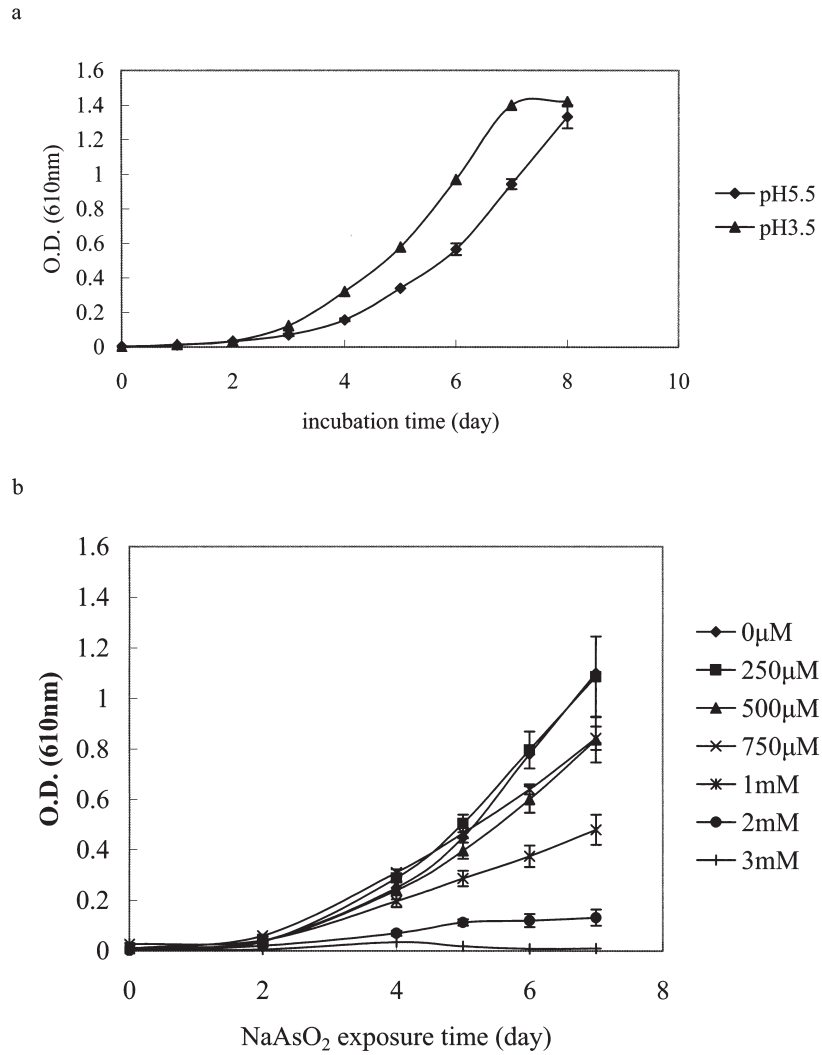


Fig. 1 Growth of *E. gracilis* SMZ under exposed to various concentrations of NaAsO₂.
 a; control in K.H. pH 3.5 and 5.5
 b; 0–3mM NaAsO₂ in K.H. pH 5.5

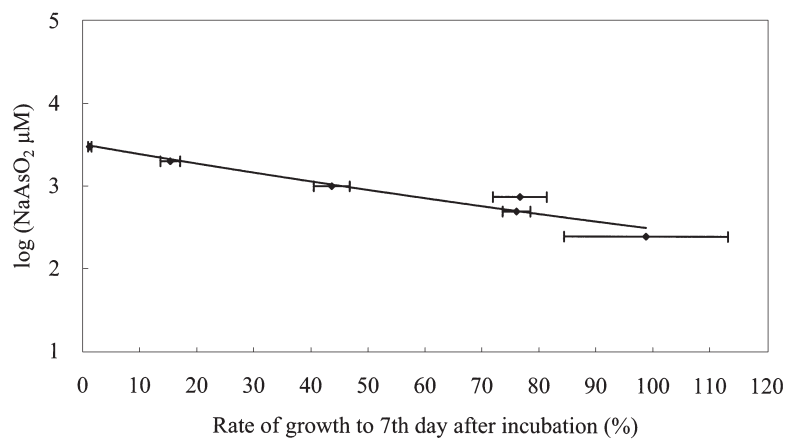


Fig. 2 The interrelation between the exposure concentration of NaAsO₂ and cell growth.

Table 1 Effect of 8 days of NaAsO₂ exposure on the cell viability of *E. gracilis* SMZ

NaAsO ₂ concentration (mM)	0	1	2	3
dead cell (%)	3.85 ± 0.8	10.0 ± 1.2	28.5 ± 2.4	25.1 ± 1.4

Table 2 Effect of short-term NaAsO₂ exposure on the cell viability of *E. gracilis* SMZ

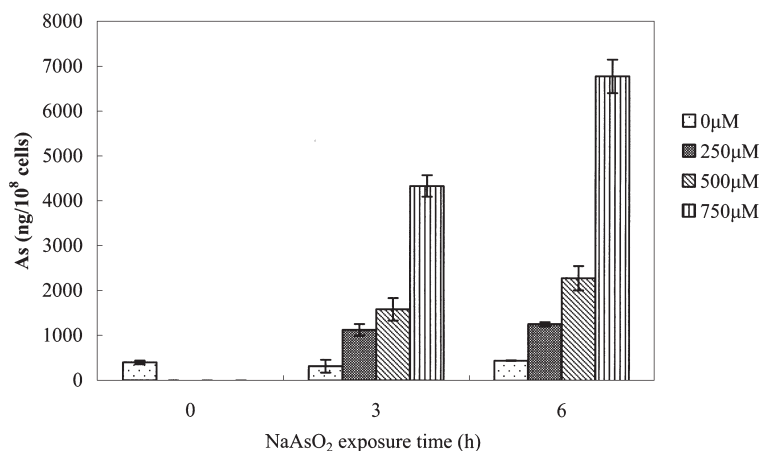
Exposure time (h)	NaAsO ₂ exposure concentration (μM)			
	0	250	500	750
	(%)	(%)	(%)	(%)
0	2.5 ± 0.36			
3	5.7 ± 0.70	9.3 ± 1.11	8.1 ± 1.04	9.1 ± 0.78
6	3.8 ± 0.71	3.4 ± 0.46	4.9 ± 0.70	9.1 ± 1.43

Total intracellular arsenic

For the uptake study of NaAsO₂ by *E. gracilis* SMZ, intracellular arsenic content was measured after 3 and 6 hours of exposure. Figure 3 shows the quantity of NaAsO₂ absorption by *E. gracilis* exposed to 250, 500 and 750 μM NaAsO₂ for 0, 3 and 6 hours measured by ICP-MS following digestion of cells harvested. The concentrations of As in *E. gracilis* SMZ increased with exposure time and in a dose dependent manner. Intracellular As increased drastically up to 4.3 μg/10⁸ cells when exposed to 750 μM NaAsO₂ for 3 hours and 6.8 μg/10⁸ cells when exposed to 750 μM for 6 hours. These data suggest the accumulation of As in the *E. gracilis* SMZ.

Blum (1966) reported that arsenate inhibited phosphate uptake in *E. gracilis* var. bacillaris SMLI, however the phosphate transport system in *E. gracilis* differs from the phosphate transport system in yeast. Accumulation of arsenate by *E. gracilis* was less than that of phosphate, whereas in yeast the rate of arsenate uptake is almost the same rate of phosphate uptake. The mechanisms of arsenic accumulation were not clear in *E. gracilis* and should be studied in the future.

The authors also found with fluorescence microscopic analysis that NaAsO₂ exposure to *E. gracilis* SMZ brought about enhancement of intracellular oxidant level, which suggest generation of reactive oxygen took place accompanied by metabolism of NaAsO₂. However, the detailed mechanism has remained unclear. For further discussion on the toxicity of As and its metabolite in *E. gracilis* SMZ, speciation of arsenite metabolite is essential. The authors have succeeded in the speciation of As metabolites. The detailed information shall be reported soon elsewhere.

**Fig. 3** Arsenic incorporation into *E. gracilis* SMZ under NaAsO₂ exposure.

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