Participation of Trace-elements in "Function Water" to the restoration of cellular function of Tributyltin chloride intoxicated *Euglena gracilis* Z

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Abstract

In our previous study it has been reported that either Ca or Mg in the "Function Water" prepared by the treatment with high electric field loading in the presence of charcoal or Al-Fe based ceramics should have participated in the restoration of regeneration of flagellum and motility of tributyl tin chloride (TBTCl)-intoxicated *Euglena gracilis* Z. In the present study, the effect of "Function Water" prepared by Al-Fe based ceramics material on the ATPase activity of TBTCl-intoxicated *E. gracilis* Z was examined.

The "Function Water" was prepared by immersing one piece of alumina-iron (Al-Fe) based ceramic in the ultra-pure water overnight before examining its effect. TBTCl-intoxication on the *Euglena* cells was brought about by exposing cells (10^5/ml) to 50 μM of TBTCl for 3 minutes. To examine restoration of motility of TBTCl-intoxicated cells by the "Function Water", the cells were washed and subsequently incubated with the "Function Water". Restoration of the cells was evaluated under the microscope equipped with computer aided image analyzer. Effect of "Function Water" on ATPase activity was evaluated by radio-assay using ^{32}P-labelled ATP and *E. gracilis* Z homogenate prepared from TBTCl-intoxicated *Euglena* cells or non-intoxicated cells.

Exposure of 50 μM TBTCl to *Euglena* cells for 3 minutes inhibited ATPase activity by ca. 40%, and incubation of the cells in Al-Fe ceramics treated water definitely recovered ATPase activity to 90% of the control activity in 4 hours. However, the "Function Water" which was treated with either EGTA or Chelex 100 to trap minerals did not show any restoration effect at all, indicating that minerals should have participated in its restoration. Since treatment with Desferal (deferoxamine) specific to iron chelating did not affect the restoration effect so much, the effect of the Al-Fe based ceramics treated water

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should not be due to iron but either Ca or Mg.

Introduction

Although it is not authorized scientific name yet, "Function Water" or "Physiologically functional aqua-solution" is generic term given to water species that exhibit biological function superior to the control aqua-solution system prepared with distilled water[1]. So-called "Function Water" species are prepared by treatment with high-electric field loading, magnetic field loading[2], contact with ceramics, piezoelectric element[3], mineral concentrate etc. Previously we reported a water species which was prepared by high-electric field loading in the presence of charcoal (wood ceramics) definitely restored the morphology and cell motility of tributyl tin chloride intoxicated *Euglena gracilis* Z[4]. During the course of studies to reveal what made it effective on the restoration of cell morphology and motility, we showed data suggesting that its effect should not be due to water cluster size but minerals (possibly Ca)[5]. Further comparative studies on the effect of restoration of *Euglena* cell morphology and motility after TBTCl intoxication showed that water species prepared by alternate current electric field loading with wood ceramics ("Charged water") and that prepared by immersing alumina-iron ceramic were apparently effective[6].

TBTCl has been known to affect membrane function and ATPases[7,8] which have been regarded as the energy source of morphological restoration and cell motility. In the present study, the authors examined whether or not the restoration of cell motility by "Function Water" species had anything to do with ATPase activity in connection with Ca and/or Mg.

Materials and Methods

"Function Water" material examined

As reported in the previous report[6] water species prepared by the immersion of Al-Fe ceramics exhibited the highest restoration effect among examined, Al-Fe ceramics treated water was chosen to examine its effect on ATPase activity of *E. gracilis* Z. Al-Fe ceramics (Trade name Two Pieces) was purchased from ACM Co., Tokyo. One piece of Al-Fe ceramic was immersed in 300 ml of ultrapure water which was prepared by filtration through hollow fiber tube overnight. Prior to its use the treated water was filtered through membrane filter ($2\,\mu$m). In order to examine the participation of minerals the water species prepared by immersing Al-Fe ceramics was filtered through Chelex 100 column to remove minerals. In case of EGTA treatment EGTA was added to the water at the final concentration of $250\,\mu$M[9] to lower Ca and/or Mg concentration of the water species. To compare the restoration effect on the TBTCl-intoxicated *Euglena* cells, several species were chosen; i.e., mineral concentrate (purchased from Hayakawa Institute), mineral water products which were purchased from market.

Euglena cell strain

Euglena gracilis Z (kindly gift from Prof. Y. Nakano, Osaka Pref. Univ. and kept in authors' lab. for 4 years) grown on the Koren-Hutner medium[10] at 28°C under illumination (2800 lux) with 12 hours intervals for 6 days was used for the TBTCl intoxication and subsequent restoration experiments.

Intoxication of *Euglena* cells

After incubating *Euglena gracilis* Z for 6 days cells were harvested and provided for TBTCl intoxication as shown in Scheme 1.

E. gracilis Z grown on Koren-Hutner medium for 6 days at 28°C (150 ml) with aeration
↓
Harvested Cells
- suspended in DDW (100 ml)
- exposed to TBTCl (50 μM final conc.) for 3 min.

Intoxicated Euglena cells
- washed with water species to be examined for 3 times.
- suspended in water species

20 ml Cell suspension
- incubated for 0, 2 and 4 hours at 28°C under illumination (2800 lux)

↓
Measurement of ATPase activity by radio-assay

Scheme 1. Procedure for the intoxidation of *Euglena gracilis* with TBTCl

Euglena cell suspension
- homogenized with 30% sucrose-20mM Hepes buffer (pH 6.8) at 0~3°C

↓
Cell homogenate
- suspended in incubation system comprising:

  200mM NaCl, 50mM KCl, 2mM Dithiothreitol, 20mM $MgSO_4$, 0.5mM EDTA-2Na, 100mM Hepes (pH 7.4)
- add 20 μl 100mM ATP containing γ-^{32}P-ATP (400 μmol)
- incubated for 30 min at 30°C.
- terminated reaction by addition of 1N HCl.
- add 600 μl Norit EXW to adsorb unreacted ATP
- kept in ice for 10 min.
- centrifuged at 9,000× g for 10 min.

↓
Supernatant
↓
Measurement of ^{32}P by Cherenkov method on Liquid Scintillation Counter (Beckman)

Scheme 2. Procedures for measurement of ATPase activity

Confirmation of Restoration of Cell Morphology and Motility

Restoration of cell morphology and motility were examined by video microscope (Argus 100, Hamamatsu Photonics, Hamamatsu, Japan) as reported earlier[4,5].

Procedure for measurement of ATPase activity

Euglena cells with or without TBTCl intoxication and subsequent incubation for 0, 2, and 4 hours were provided for measurement of ATPase activity as presented in Scheme 2. This procedure is according to Schnebli and Abrams[11] with slight modification. Gamma ^{32}P labelled ATP (specific activity: 0.37M Bq/mol) was purchased from Du Pont NEN^{TM} Research Produts.

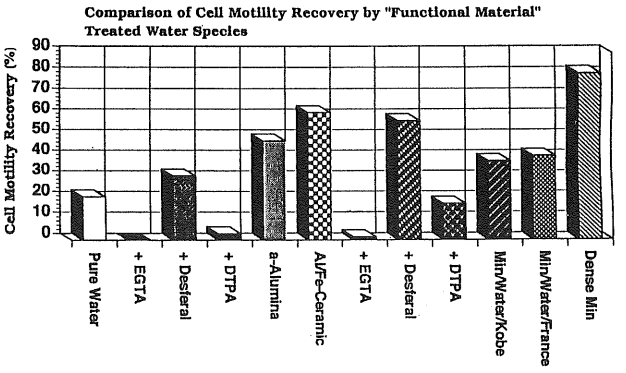

Figure 1. From far left to right column:

Pure Water; doubly distilled water with subsequent filtration through hollow fiber filter (0.22 μm).

+EGTA; water species treated "Pure Water" with 250 μM EGTA and subsequent filtration through 0.22 μm membrane filter.

+Desferal; water species treated "Pure Water" with deferoxamine mesylate (10 ppm as the final concentration) and subsequent filtration through 0.22 μm membrane filter.

+DTPA; water species treated "Pure Water" with diethylenetriaminepentaacetic acid (250 μM as the final concentration) and subsequent filtration through 0.22 μm membrane filter.

a-Alumina; water species prepared by immersing 5% wt. of alpha-alumina granules overnight at 25°C and subsequent filtration through 0.22 μm membrane filter.

Al/Fe-Ceramic; water species prepared by immersing 5% wt of Al-Fe based ceramics overnight at 25°C and subsequent filtration through 0.22 μm membrane filter.

+EGTA, +Desferal, DTPA; water species treated Al/Fe-Ceramic water with EGTA or Desferal or DTPA as described for "Pure Water".

Min/Water/Kobe; mineral water species with trade name, "Rokko Water" which originates in spring water in Rokko Mts., Kobe, Japan.

Min/Water/France; mineral water species purchased from France.

Dense Min; mineral concentrate prepared by Hayakawa Res. Lab., Ibaragi pref., Japan Final concentration was 230 ppm.

Data represent restoration of cell motility after 4 hours of incubation.

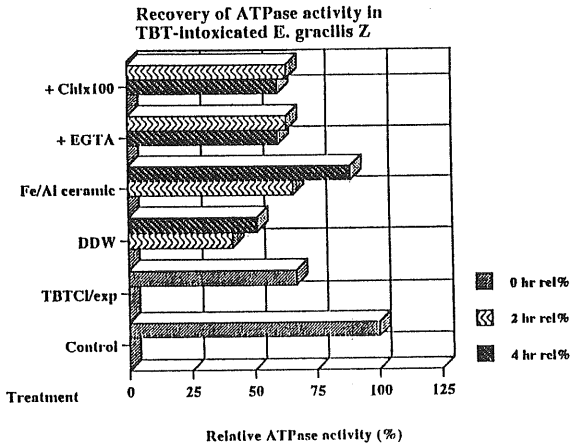

Figure 2. Recovery of ATPase activity in TBTCl-intoxicated *E. gracilis* Z
Data are expressed by percentage activity to control sample.
From bottom to top
Control; ATPase activity without TBTCl intoxication
TBTCl/exp; ATPase activity of *Euglena gracilis* Z intoxicated by 50 μM TBTCl exposure for 3 minutes.
DDW; ATPase activity of *Euglena gracilis* Z incubated in doubly distilled water for 2 and 4 hours after TBTCl intoxication.
Fe/Al ceramic; ATPase activity of *Euglena gracilis* Z incubated in iron aluminum-based ceramic treated water for 2 and 4 hours after TBTCl intoxication.
+EGTA; ATPase activity of *Euglena gracilis* Z incubated in Fe/Al ceramic water which was chelated by 250 μM EGTA for 2 and 4 hours after intoxication.
+Chlx100; ATPase activity of *Euglena gracilis* Z incubated in Fe/Al ceramic water which was filtered through Chelex 100 for 2 and 4 hours after intoxication.

Results and Discussion

As reported in our previous report[6] intoxicated *Euglena* cells of cyst form restored their form by 60% after incubation with Al-Fe ceramics treated water for 4 hours (data not shown). In Figure 1 comparative data on the restoration of cell motility of TBTCl-intoxicated *Euglena* cells are shown. It is quite obvious that Al-Fe ceramics treated water gave high restoration effect along with electric field loaded water and mineral concentrate. It is also clear that its restoration effect is mineral dependent.

In Figure 2 ATPase activities of *Euglena* cells with or without TBTCl intoxication are presented. Data are presented as relative values to that of control as 100%. By exposing to 50 μM of TBTCl for 3 min ATPase activity of *Euglena* was inhibited by 40%; namely its activity decreased to 60% of the control sample. Incubation with Al-Fe ceramic treated water for 2 hours did not show significant restoration of ATPase activity, however, 4 hours incubation restored its activity almost 90% of the control. On the other hand, treatment of the water species with chelator; i.e., Chelex 100 or EGTA, blocked its effect. These results indicate that the ATPase is mineral dependent, probably Ca-linked ATPase. However, as

reported in the previous paper[6] the addition of Mg and/or Ca to the incubation medium did not restore the cell motility. Therefore, some unknown factors besides Ca and/or Mg would participate in the restoration of the cell motility; i.e., detoxication of TBTCl, regeneration of flagellum, restoration of cell shape to recover its maneuverability.

Although the detailed mechanism how the "Function Water" affects the cell function has remained unrevealed, participation of Ca and/or Mg should be quite clear. To reveal its effect in detail, studies on the stereochemical investigation on mineral, water molecules and membrane transport systems in combination with calmodulin should be essential.

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