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Life-prolonging effect of deep sea drinking water in the hypotrichous ciliated protozoan *Euplotes woodruffi*

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Abstract

Deep sea drinking water (DSDW), which is produced by processes of desalination and mineralization of deep sea water, contains various minerals and is drunk to replenish the balance of minerals, particularly Mg. Although the effect of DSDW on living organisms has been well studied, few studies investigating the effect of DSDW on cell lifespan have so far been reported. Therefore, to evaluate the life-prolonging properties of DSDW at a cellular level, we cultured ciliated protozoan *Euplotes woodruffi* in natural mineral water (NMW) and DSDW, and compared the number of cells surviving during the experimental period. Results showed that cells cultured in DSDW lived longer than those cultured in NMW—possibly by consuming a substance richly available in DSDW. To isolate this key element in DSDW, we investigated the effect of various ions on the cell lifespan and found that only cells in the Mg-containing culture water survived as long as cells in DSDW. Judging from the results obtained in the study, we concluded that DSDW can prolong the cell lifespan and that Mg plays a key role in the life-prolonging effect of DSDW.

Introduction

In recent years, deep sea water (DSW) has attracted considerable attention along with the rise of health-consciousness worldwide. DSW is generally defined as sea water that is pumped up from a depth of more than 200 meters and is characterized by a low and uniform temperature, high purity, and a rich mixture of inorganic nutrients [1]. In Japan, the aquamarine research project focusing on the effective utilization of DSW was started in 1985 by the Science and Technology Agency (presently the Ministry of Education, Culture, Sports, Science and Technology) at Muroto City, Kochi Prefecture. Due to its balance of essential minerals, DSW is thought to have potential as a good source of high-mineral content products ranging from health and beauty—such as cosmetics and bathing preparations—to foods and beverages—such as alcohol and drinking water.

Deep sea drinking water (DSDW) has been produced exclusively by processes of desalination and mineralization of DSW to make it suitable for drinking. DSDW contains various minerals and is drunk for the replenishment of the balance of minerals, particularly Mg. Therefore, the effect of DSDW on living organisms has been well studied in human and experimental animals, and recent reports provide accumulating evidence of the therapeutic or preventive effects of DSDW. For

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example, DSDW has the ability to 1) improve cardiovascular hemodynamics in Kurosawa- and Kusanagi-Hypercholesterolemic rabbits [2], 2) improve skin symptoms and mineral imbalances in human atopic eczema/dermatitis syndrome [3,4], 3) prevent hyperlipidemia and atherosclerosis in cholesterol-fed rabbits [5,6], and 4) reduce allergic skin responses and serum levels of cytokines in patients with allergic rhinitis [7]. Moreover, Liao et al. reported that DSDW enhances the longevity of diabetic rats by enhancing cardiac function and that the underlying molecular mechanism includes the suppression of pro-apoptotic proteins and the enhancement of survival proteins [8]. However, few studies investigating the life-prolonging effect of DSDW at the cellular level have so far been reported—possibly due to the fact that an appropriate experimental model for research on the cell lifespan has not yet been established.

In the present study, we investigated the effect of DSDW on the cell lifespan using a free-living unicellular eukaryotic microorganism, *Euplotes woodruffi*, classified into the Phylum Ciliophora and the Kingdom Protista. Usually, *Euplotes* lives in freshwater environments, such as ponds and swamps, but it can be cultured in a laboratory with natural mineral water (NMW), which is normally available on the commercial market [9,10]. The advantages in using the ciliate *E. woodruffi* in the study are that 1) they are large enough to be counted individually under a conventional stereoscopic microscope, 2) dead cells are easily distinguished from live cells because healthy cells move continuously, while dead cells eventually disappear, and 3) they do not rely on feeding on bacteria in order to grow. This third criterion is thought to be the key advantage in the study. The majority of ciliates are heterotrophs and feed on smaller organisms such as bacteria and algae. However, some species of *Euplotes*—including *E. woodruffi*—are unable to survive by feeding only on bacteria. Therefore, in our laboratory, we feed them with separately-cultured algal cells (*Chlorogonium elongatum* or *C. capillatum*) twice a week [9,11]. In other words, if *E. woodruffi* are left in culture water without the addition of food sources, they stop growing and dividing, and finally die and disappear within a few weeks. This property of *E. woodruffi* is convenient for our experiments because it is not necessary to consider the effect of feeding on bacteria on the life-prolonging properties of DSDW during the experimental periods.

We cultured *Euplotes* cells with both NMW and DSDW, and compared the lifespan of these cells. Our results indicated that DSDW possesses life-prolonging properties and that Mg plays a key role in the beneficial effect of DSDW.

Materials and Methods

The ciliated protozoan *Euplotes woodruffi* was obtained from the Iwakuni City Micro-life Museum (Yamaguchi, Japan), and was cultured in NMW (Delicious water Rokko, Asahi Soft Drinks Co., Ltd., Tokyo, Japan) at 25°C with *Chlorogonium capillatum* added as a food source. *C. capillatum* was cultured axenically in a medium (1 mg/ml sodium acetate and 5 mg/ml yeast extract) at room temperature under continuous lighting conditions. In the present study, to investigate the life-prolonging properties of DSDW, we used desalinated Muroto Deep-Seawater (Amami's Water, AKO KASEI Co., Ltd., Kochi, Japan). The water hardness of NMW and DSDW was calculated to be 48.4 and 1001, respectively, by the following equation: hardness (mg/l) = Ca (mg/l) × 2.497 + Mg (mg/l) × 4.118. Therefore, NMW is considered to be soft water and DSDW is considered to be hard water. The elements and hardness of these waters are listed in Table 1. For cell survival experiments, the culture of *E. woodruffi* in NMW was first starved for more than 3 days after its last feeding to obtain cells in a uniform condition. Cells were then collected by centrifugation, resuspended in fresh culture water (NMW, DSDW, or conditioned water), seeded into wells of a plastic plate, and cultured in a temperature-controlled incubator at 25°C. The

Table 1. Levels of elements and hardness of natural mineral water (NMW) and deep sea drinking water (DSDW).

Elements	Content in water (mg/l)	
	NMW	DSDW
Na	29	74
Ca	9.5	71
Mg	6	200
K	1	69
Hardness	48.4	1001

Water hardness was calculated by the following equation: hardness (mg/l) = Ca (mg/l) × 2.497 + Mg (mg/l) × 4.118.

number of surviving cells was counted daily under a stereoscopic microscope and continuously recorded until all cells had died. No food was supplied during the experimental period. The detailed procedures of each experiment are as follows.

Experiment 1: To see the difference in culturing cells in NMW and DSDW, cells were cultured in both water types, and counted the number of surviving cells daily.

Experiment 2: To investigate in detail the life-prolonging effect of DSDW on the cell lifespan, cells were cultured in DSDW diluted with deionized water at different dilution rates or in NMW and DSDW with different cell concentrations.

Experiment 3: To characterize the life-prolonging effect of DSDW, the culture water was exchanged at the middle of the culture period.

Experiment 4: To investigate the effect of ions contained in DSDW on the cell lifespan, the conditioned water was prepared by the addition of Na, Ca, Mg, and K—each alone and in various combinations—to control NMW. The concentration of each ion in the conditioned water was identical to that in DSDW or adjusted to between 1 mM and 5 mM.

The experiment was performed in duplicate or triplicate and only the representative result was shown. For nonparametric comparison between two groups, as shown in Fig. 1, a Mann–Whitney *U* test was performed. *P* values < 0.05 were considered statistically significant.

Results and Discussion

Experiment 1

The objective of the present study was to evaluate the life-prolonging effects of DSDW. As shown in Fig. 1, the number of cells cultured in NMW (open circles) drastically decreased at 4 days, and all cells had died at 6 days after the onset of the experiment. On the contrary, the number of cells cultured in DSDW (filled circles) remained unchanged up to 8 days, and only after 11 days all cells had died. Cells cultured in DSDW survived significantly longer than those cultured in NMW, indicating that DSDW possesses life-prolonging properties.

Experiment 2

Next, we cultured cells under various conditions. As shown in Fig. 2a, cells cultured in NMW survived for 9 days (open bar). On the

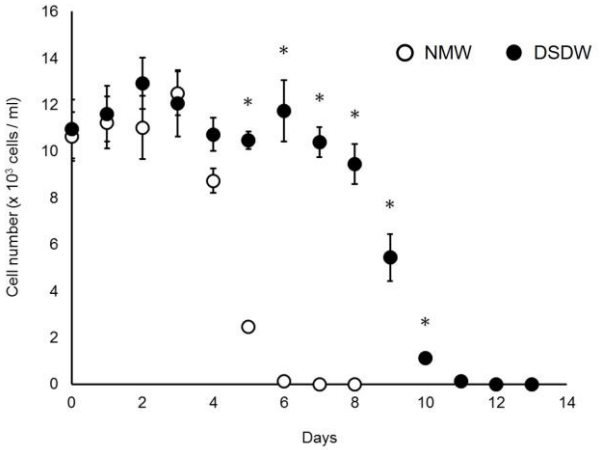

Fig. 1. Survival curves of *Euplotes woodruffi* cultured in natural mineral water (NMW, open circles) and deep sea drinking water (DSDW, filled circles). Cells cultured in DSDW survived significantly longer than those cultured in NMW. Bars represent standard errors. Asterisks indicate significant difference at $p < 0.05$ between NMW and DSDW (Mann–Whitney *U* test).

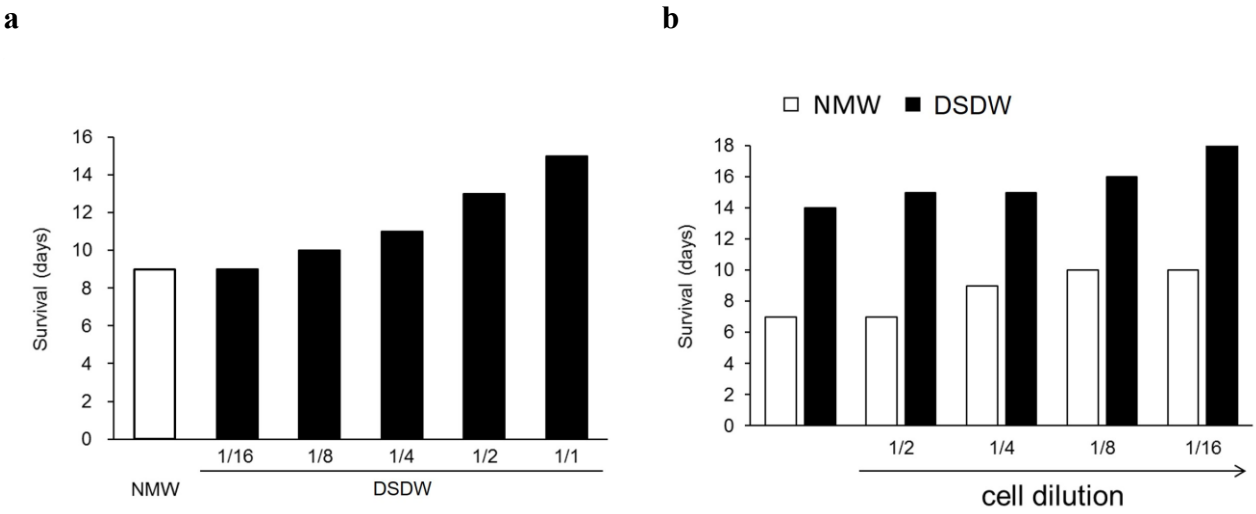

Fig. 2. Survival days of cells cultured in NMW and DSDW under various culture conditions. (a) Cells were cultured in NMW (open bar) and DSDW diluted at different dilution rate (filled bars). The life-prolonging effect of DSDW was decreased with an increase in the dilution rate. (b) Cells were cultured in NMW (open bars) and DSDW (filled bars) with different cell concentrations. The life-prolonging effect of DSDW was observed under all culture conditions, independently of cell concentrations. Moreover, the survival days of cells increased with a decrease in the cell concentration regardless of the water types.

contrary, cells cultured in non-diluted DSDW survived for 15 days (filled bar labeled as 1/1), indicating the life-prolonging effect of DSDW. When cells were cultured in DSDW diluted with deionized water at different dilution rates, survival time was decreased with an increase in the dilution rate (filled bars labeled as 1/2, 1/4, 1/8, and 1/16). Next, we cultured cells in NMW and DSDW with different cell concentrations. As shown in Fig. 2b, cells cultured in NMW survived for 7 days (extreme left open bar), whereas cells cultured in DSDW survived for 14 days (extreme left filled bar), indicating again the life-prolonging effect of DSDW. The effect was observed under all culture conditions independently of cell concentrations. Furthermore, the lower the cell concentration in culture was, the longer cells survived, regardless of the water types. These results suggest the possibility that cells consume a substance contained in the culture water during the survival period and DSDW contains the substance to a greater degree than does NMW, so that cells cultured in DSDW lived longer than those cultured in NMW.

Experiment 3

Then, we conducted an experiment in which the culture water was exchanged 4 days after the onset of the experiment. As shown in Fig. 3, cells cultured in NMW (open bar labeled as NMW–NMW) and in DSDW (filled bar labeled as DSDW–DSDW) survived for 8 days and 17 days, respectively. In both cases, the culture water was exchanged with a new sample of the same water. The life-prolonging effect of DSDW was confirmed in the experiment. Cells cultured in NMW followed by DSDW survived for 15 days (gradient bar labeled as NMW–DSDW), indicating that cells which would otherwise have died at 8 days survived for additional 7 days. It was therefore clearly demonstrated that DSDW prolonged the cell lifespan. In contrast, cells cultured in DSDW, which survive for 17 days, survived for only 12 days when cultured in DSDW followed by NMW (gradient bar labeled as DSDW–NMW); thus, their lifespan was shortened by 5 days. The cell lifespan was demonstrably shortened by the exchange of culture water from DSDW to NMW. The period when cells were cultured in DSDW was 0 days in NMW–NMW, 4 days

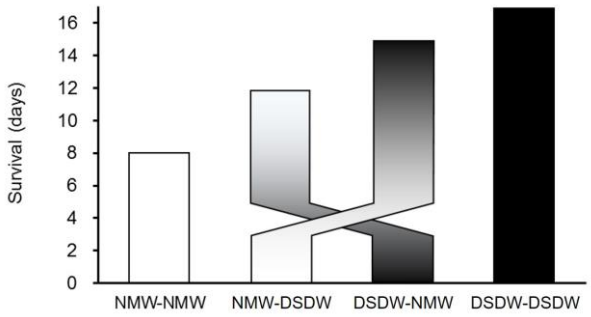

Fig. 3. The effect of exchange of the culture water on survival days of cells. At the middle of the culture period, the culture water was exchanged from NMW to NMW (NMW–NMW), from NMW to DSDW (NMW–DSDW), from DSDW to NMW (DSDW–NMW), and from DSDW to DSDW (DSDW–DSDW). The survival days increased with an increase of the period in which cells were cultured in DSDW.

in DSDW–NMW, 11 days in NMW–DSDW, and 17 days in DSDW–DSDW, and the cell lifespan was 8 days in NMW–NMW, 12 days in DSDW–NMW, 15 days in NMW–DSDW, and 17 days in DSDW–DSDW. These results indicate the dependence of the life-prolonging effect of DSDW on the period over which cells are cultured in DSDW.

In the present study, the survival days of cells were different among the experiments. For example, cells cultured in NMW survived for 6 days (Fig. 1) and for 9 days (Fig. 2a). Similarly, cells cultured in DSDW survived for 11 days (Fig. 1) and for 15 days (Fig. 2a). This is due to the difference between the periods in which cells were cultured in the control NMW before the onset of the experiments. Whether the period in which cells were cultured in NMW before the onset of the experiments affects the life-prolonging effect of DSDW is unclear. However, cells cultured in DSDW nevertheless survived longer than those cultured in NMW, indicating the life-prolonging effects of DSDW. Moreover, the experiment in which culture water was exchanged in the middle of the culture period revealed that the longer cells were cultured in DSDW, the longer they survived (Fig. 3). This indicates that the life-prolonging effect of DSDW depends on how long cells are cultured in this medium.

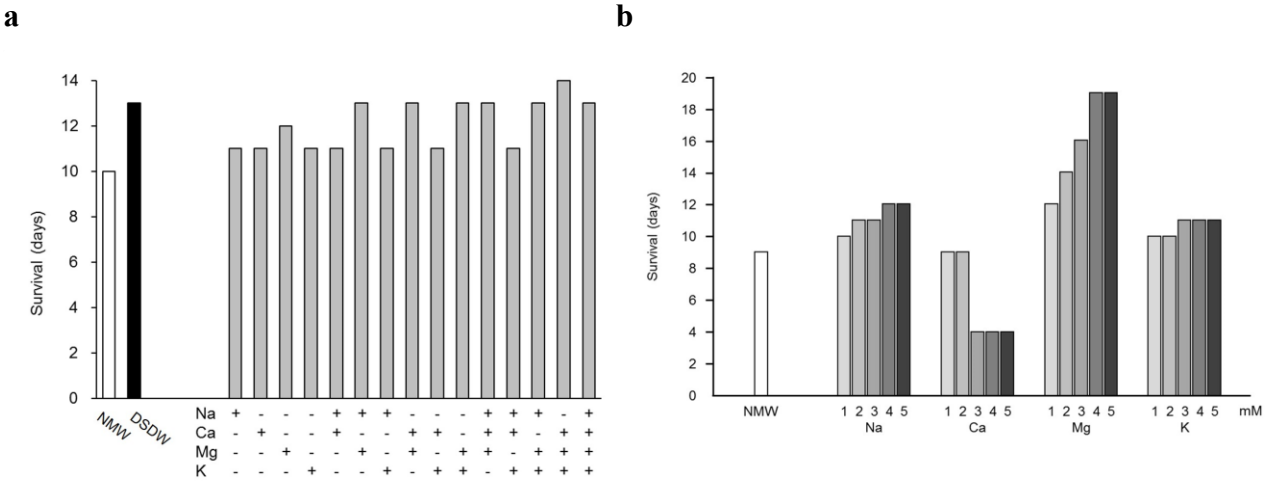

Fig. 4. The effect of elements in the culture water on the cell survival. (a) Cells were cultured in the conditioned water (gray bars) containing various ions. Compared with the control NMW (open bar), only cells cultured in the Mg-containing conditioned water survived as long as did cells cultured in DSDW (filled bar). (b) Cells were cultured in the conditioned water (gray bars) containing high concentrations of Na, Ca, Mg, or K ions. Although high concentrations of Ca are lethal, cells cultured in the conditioned water survived longer than those cultured in the control NMW (open bar) in a concentration-dependent manner. Among the ions tested, Mg showed the greatest life-prolonging effect.

Experiment 4

To identify the key element that provides cells with an additional lifespan, cells were cultured in the conditioned water which was prepared based on the composition and the concentration of DSDW listed in Table 1. In the experiment, as shown in Fig. 4a, cells cultured in NMW (open bar) and DSDW (filled bar) survived for 10 days and 13 days, respectively. Among cells cultured in the conditioned water (gray bars), on the other hand, only cells cultured in the Mg-containing conditioned water survived as long as cells cultured in DSDW. Next, we investigated the effect of high concentrations of ions on the cell lifespan (Fig. 4b). Cells cultured in NMW survived for 9 days (open bar). Although high concentrations of Ca are lethal, cells cultured in the conditioned water containing high concentrations of Na, Mg, or K nevertheless survived for longer periods compared with cells in the control NMW. Moreover, the high concentrations of ions prolonged the cell lifespan in a concentration-dependent manner. Among the ions tested, Mg showed the greatest effect on the cell lifespan. Cells cultured in the conditioned water containing the highest concentration of Mg (5 mM) survived over two times longer than the control cells after the onset of the experiment. These results indicate that Mg plays the most important role in the life-prolonging effect of DSDW. Moreover, these results indicate two possibilities: either that not only Mg but also high Na and high K have life-prolonging properties, or that cells survive long in high ionic strength conditions. Judging from the fact, as shown in Fig. 4a, that only cells cultured in the conditioned water containing Mg survived longer than those cultured in NMW, only Mg, and not Na or K, might play a key role in the life-prolonging effect of DSDW. A high ionic strength condition might also have a life-prolonging effect, but it cannot be compared with the effect of Mg alone. Since the concentration of Mg in DSDW is calculated to be approximately 2 mM, higher concentrations of Mg are expected to have a stronger life-prolonging effect. Further experiments to investigate whether the cell lifespan is affected by the ionic strength of the culture medium are needed.

As mentioned above, among ions contained in DSDW, Mg is the most important element in the life-prolonging effect of DSDW. However, the mechanism by which Mg affects cells is still unknown. Mg has so far been reported to support a number of significant functions in the human body. For example, Mg inhibits platelet aggregation and suppresses thrombus formation [12,13]. Mg lowers blood pressure by improving blood fluidity and decreasing peripheral vessel resistance [14]. Mg is also found to have a significant influence on bone metabolism [15-17]. The fact that a shortage of Mg causes various illnesses—including ischemic heart diseases [18,19]—indicates that Mg has important biological functions and is involved in the physiological regulation of tissues and organs. On the other hand, studies at the cellular and molecular levels have revealed that Mg acts as an important modulator of cell functions. For example, in Mg-deficient rats, various inflammatory cytokines and excessive amounts of free radicals are released by leukocytes and macrophages [20], and reactive oxygen species are produced by neutrophils [21]. On the contrary, a high concentration of Mg reduces the production of superoxide anion by eosinophils, of sulphidoleukotrienes by leukocytes, and of proinflammatory cytokines by human blood cells [22,23]. These studies suggest that Mg plays an essential role in a wide range of fundamental cellular reactions. We demonstrated that cells cultured in DSDW survived longer, possibly through their effective utilization of Mg. Mg is quickly captured and smoothly accumulated in cells [24]. However, in terms of the life-prolonging properties of DSDW, how Mg works after being taken into cells is still unknown. Further studies are necessary to clarify whether 1) Mg attenuates the activity of cellular metabolism, 2) Mg improves the efficiency of ATP synthesis, 3) Mg downregulates ATP consumption, or 4) Mg increases the number of mitochondria. Results from such experiments would guide the

understanding of how Mg provides cells with tolerance against unfavorable environmental conditions. Judging from the findings of the present study, it can also be said that Mg inhibits cell aging. In this case, we must consider carefully the term "aging" separately from the technical term "clonal aging" because, in the present study, *Euplotes* cells cultured in the mineral water showed neither division nor proliferation during the experimental period. We reported that DSDW prolonged the cell lifespan, or to be exact, prolonged the survival period of cells cultured without food supply. Although what occurs intracellularly in cells cultured in DSDW is still unknown, culturing the ciliated protozoan *E. woodruffi* in conditioned mineral water can be a useful experimental model for the study of the possible anti-"aging" effect of Mg or of DSDW.

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Conflict of Interest

The authors declare no conflict of interest.

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