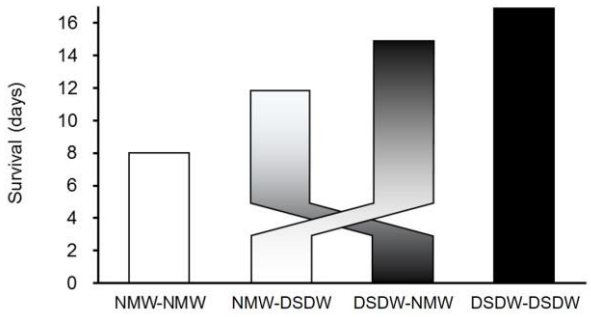

Fig. 3. The effect of exchange of the culture water on survival days of cells. At the middle of the culture period, the culture water was exchanged from NMW to NMW (NMW–NMW), from NMW to DSDW (NMW–DSDW), from DSDW to NMW (DSDW–NMW), and from DSDW to DSDW (DSDW–DSDW). The survival days increased with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

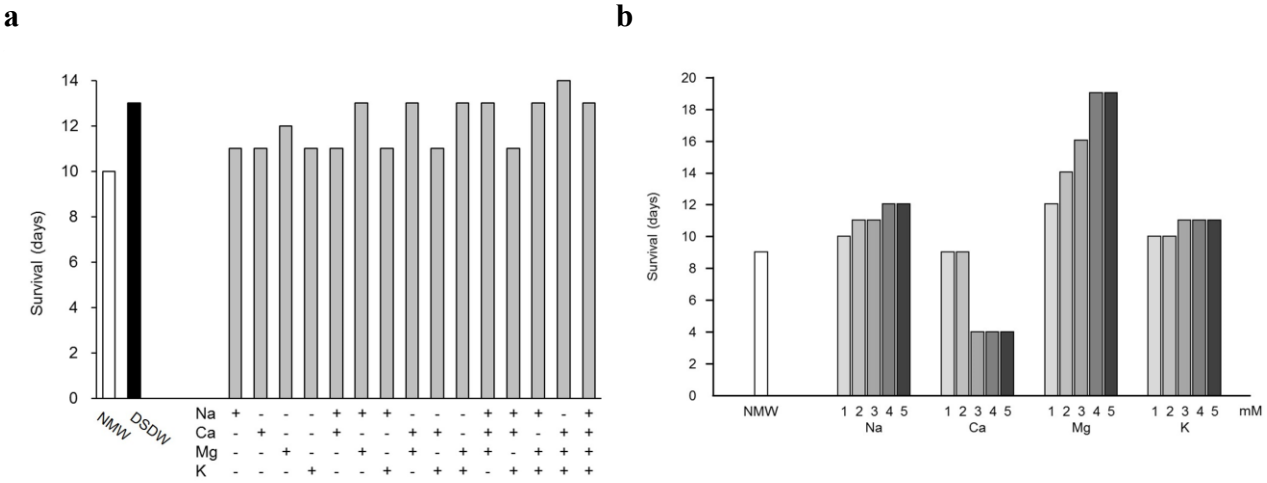

Fig. 4. The effect of elements in the culture water on the cell survival. (a) Cells were cultured in the conditioned water (gray bars) containing various ions. Compared with the control NMW (open bar), only cells cultured in the Mg-containing conditioned water survived as long as did cells cultured in 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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