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# The influence of natural mineral water on aquaporin water permeability and human natural killer cell activity

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ABSTRACT

Aquaporins are the intrinsic membrane proteins functioning as water channel to transport water and/or mineral nutrients across the biological membrane systems. In this research, we aimed to clarify if the selected mineral water can affect aquaporin functions *in vitro* and the assumption of the mineral water can modify aquaporin expression and activate natural killer cell activity in human body. First, we expressed six human and eight plant aquaporin genes in oocytes and compared the effect of different kinds of natural mineral water on aquaporin activity. The oocyte assay data show that Hita tenryosui water could promote water permeability of almost all human and plant aquaporins in varying degrees, and freeze-dry and organic solvent extraction could reduce AQP2 activity but pH change and boiling could not. Second, each volunteer in two groups (10 in one group) received an oral Hita tenryosui or tap water load of 1000 ml/day for total four weeks. We found that these two kinds of water did not directly affect the relative expression levels of AQP1 and AQP9 in the blood cells, but intriguingly, the natural killer cell activities of the volunteers drinking Hita tenryosui water were significantly improved, suggesting that Hita tenryosui water has obvious health function, which opens a new and interesting field of investigation related to the link between mineral water consumption and human health and the therapies for some chronic diseases.

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## 1. Introduction

Aquaporin was first found as water channel in human blood cells [1]. There are one or two isoforms in microorganisms, 13 isoforms in human and up to decades in plants such as 36 in rice and 38 isoforms in Arabidopsis genome. Aquaporins are present in plasma membrane, vacuole, ER membrane of the cells from microorganisms to higher organisms and have narrow holes of about 3 Å which contribute directly water entrance and exit to maintain cellular water balance [2–4]. Besides water molecules, aquaporins can also conduct a wide range of nonpolar solutes, such as urea or glycerol and even more unconventional permeants, such as the nonpolar gases carbon dioxide and nitric oxide, the polar gas ammonia, the reactive oxygen species hydrogen peroxide and the metalloids antimonite, arsenite, boron and silicon [5,6]. The permeability of most aquaporins is dynamically regulated at different levels. The factors affecting the gating behavior possibly involve phosphorylation, heteromerization, pH, $Ca^{2+}$, pressure, solute gradients, temperature and nutritional conditions [7,8].

Water is a critical component for all living cells. Information largely from aquaporin knockout mice has implicated key roles of aquaporin-facilitated water transport in transepithelial fluid transport (urinary concentrating, gland fluid secretion), water movement into and out of the brain, cell migration (angiogenesis, tumor metastasis, wound healing) and neural function (sensory signaling, seizures) [9]. Mineral water is characterized by its purity at source, its content in minerals, trace elements and other constituents, its conservation and its healing properties recognized by clinical and pharmacological trials. And different kinds of mineral water with different features (e.g. mineral contents, pH) greatly affect human health [10]. Increasingly science is providing evidence linking the health of people with water [11]. For example, certain mineral water which could enhance immune activity in humans and anti-cancer immunity in mice raises activity of natural killer cells [12] and certain mineral water or electro-reduced water could improve atopic dermatitis and life-style related diseases [13]. In the present study, we compared the effect of mineral water from different sources on the water permeability of human and plant aquaporins and aimed to test whether the assumption of water

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with different mineral contents could lead the changes in aquaporin expression levels in human blood cells and the natural killer cell activity.

## 2. Materials and methods

### 2.1. Water samples and treatments

Tap water from Yokohama city in Japan (Yokohama tap water), natural mineral water from Hita city in Japan (Hita tenryosui water), delicious natural water from Kobe city in Japan (Rokko water), and natural mineral water from Evian-les-Bains in France (Evian water) were obtained or purchased for the experiments. Yokohama tap water and Hita tenryosui water were treated with freeze-dry, heating, pH change, and organic solvent extraction. The water samples were freeze-dried in Labconco freeze drying FZ-12 (Labconco, USA) and the lyophilized residuals were re-dissolved in Milli-Q water to the original volumes. The pH of the obtained water samples was respectively adjusted to original Yokohama tap water pH at 7.0 and Hita tenryosui water pH at 8.2. Heating treatment was performed in boiled water bath for 10 min. pH change was performed by using the diluted HCl or NaOH to adjust the water samples to 6.0, 7.0, 8.0 or 9.0. Equal volume of organic solvent, hexane or ethyl acetate, was respectively mixed with water and the mixture was vigorously agitated for 10 min at room temperature to extract the organic dissolved substances. After extraction, the organic phases were removed and the left aqueous phases were vacuumed for 1 h to remove residual organic solvent in the water. Milli-Q water was added to recover the volume if necessary.

### 2.2. In vitro transcription of aquaporin genes, microinjection of Xenopus oocytes and measurement of water permeability

Rice aquaporin genes (OsPIP2;1, OsPIP2;2, OsPIP2;3, OsPIP2;4, OsPIP2;5, OsPIP2;6, OsPIP2;7, OsPIP2;8) were cloned by using the primers listed in Table S1 and were inserted into *Bgl*II site of pXβG-ev1. The human aquaporin genes (AQP1, AQP2, AQP3, AQP4, AQP5 and AQP7) and rat AQP2 gene inserted in pXβG-ev1 were gifted from Dr. Ishibashi, K., Dr. Yasui, M., and Dr. Sasaki, S. The obtained constructs were applied for RNA *in vitro* transcription. The capped complementary RNA (cRNA) was synthesized using T3 RNA polymerase of the mMESSAG EmMACHINE High Yield Capped RNA Transcription Kit (cat no: AM1348, Ambion, USA) after linearization of the aquaporin pXβG-ev1 constructs. The synthesized RNA samples were purified and the concentrations were measured. The oocytes selection, treatment, injection and water permeability calculation were performed according to Preston et al. [1].

### 2.3. Western blot analysis

Cruel membrane proteins were extracted from oocytes using ReadyPrep Protein Extraction kit Membrane II (Bio-Rad, USA), and protein concentrations were determined using Quickstart Bradford Dye Reagent (Bio-Rad, USA). The membrane protein samples were denatured at 100 °C for 10 min. 1.5 μg of each membrane protein samples and the purified standard rat AQP2 protein (gifted from Dr. S. Sasaki) were separated in 12% SDS–PAGE gel. The gel was pre-stained in Coomassie Blue dye and then was transferred to nitrocellulose membrane for Western blot using anti-AQP2 antibody.

### 2.4. Expression of GFP-PIP2;1 fusion protein in oocyte

The above eight rice PIP genes were inserted in the downstream of GFP sequence in 35S-GFP-NOS3/pUC18 (gifted from Dr. Shimamoto, K.). GFP or GFP-OsPIP fusion fragment was swapped into *Bgl*II site of pXβG-ev1 vector for RNA *in vitro* transcription and 50 ng of the purified cRNA was injected into oocytes. The expression of GFP-OsPIP was visualized under the fluorescence microscope.

### 2.5. Preparation of granular leukocyte from volunteers drinking Yokohama tap water or Hita tenryosui water

We selected 20 volunteers with normal health condition and divided them into two groups with 10 members in a group. Each person in one group drank 330 ml of Yokohama tap water and in another group each person drank the same volume of Hita tenryosui water for three times (total 1000 ml/day) for total four weeks. All examinees were provided with the same food and lived common lives during the experiment. Blood samples were taken from each examinee on the starting day and finishing day. The granular leukocytes were isolated by BD Bakyutina blood-collecting vessel (Becton, Dickinson and Company).

### 2.6. Aquaporin gene expression by real-time RT-PCR

Total RNA was extracted from the obtained granular leukocytes using an RNeasy Plus Kits (Qiagen, Tokyo) and treated with DNase I for 30 min. The cDNA was synthesized from total RNA with Primescript RT regent kit (Takara Bio., Shiga, Japan). Quantitative real-time RT-PCR was performed using cDNA as template and SYBR Premix Ex Taq (Takara Bio, Japan). The specific primers for each gene were listed in Table S1. The real-time PCR reactions were carried out in a Thermal Cycler Dice™ Real Time System (TP800, Takara Bio).

### 2.7. Natural killer (NK) cell activity assay

Natural killer cell activity of the obtained granulocytes was measured according to the protocol described by Kobayashi et al. [14]. Briefly, $1 \times 10^6$ cells of the tumor-derived cell line K562 (Rockville, MD) as target cells were labeled with 100 μCi $^{51}Cr$ ($Na_2Cr^{51}O_4$ from New England Nuclear) for 60 min at 37 °C and were washed for three times to remove unincorporated isotope. Labeled targets were added to 96-well U-bottom plates ($1 \times 10^4$ cells/well) and incubated with $2.0 \times 10^5$ of the granular leukocytes as effector cells for 4 h at 37 °C in a 5% $CO_2$ atmosphere. Supernatants were assayed for $^{51}Cr$ release in a gamma counter. Spontaneous release of $^{51}Cr$ was assessed by the incubation of targets in the absence of effectors, and maximum release of $^{51}Cr$ was determined by incubation of targets in 0.1% Triton X-100. Percentage of specific $^{51}Cr$ release was determined using the following equation: % specific Cr release = [(experimental release − spontaneous release)/(maximum release − spontaneous release) × 100].

## 3. Results

### 3.1. Expression of aquaporins in oocytes

To investigate the aquaporin activity in different water samples, we injected AQP cRNA into oocytes and incubated the injected eggs in 1 × MBS for 48 h at 20 °C. During the incubation, the exogenous proteins are expected to properly express in the oocytes. We extracted the total membrane protein from oocytes injected with rat AQP2 cRNA or water. In this experiment, we used the rat