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

Yoshichika Kitagawa [a,*], Chengwei Liu [a], Xiaodong Ding [b,c,*]

[a] Graduate School of Bioresource Sciences, Akita Prefectural University, Akita 010-0195, Japan
[b] Department of Neurology, The University of Texas Southwestern Medical Center, TX 75390, USA
[c] Former JSPS (Japan Society for the Promotion of Sciences) Fellow in Biotechnology Institute, Akita Prefectural University, 2-2 Minami, Ohgata, Akita 010-0444, Japan

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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

* Corresponding authors. Addresses: Graduate School of Bioresource Sciences, Akita Prefectural University, Shimoshinjo 241-438, Akita 010-0195, Japan. Fax: +81 18 832 3461 (Y. Kitagawa), Department of Neurology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, TX 75390, USA. Fax: +1 214 645 6238 (X. Ding).
E-mail addresses: kitagawayy@kitagawa-inst.com (Y. Kitagawa), xiaodong.ding@utsouthwestern.edu (X. Ding).

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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, 1×10^6 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 (1×10^4 cells/well) and incubated with 2.0×10^5 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

AQP2 which shares 93% identity with human AQP2 as an example, because we could provide the purified AQP2 protein from rat as a positive control and the antibody against rat AQP2. The total membrane proteins were fractionated in SDS–PAGE gel and applied for Western blot using anti-APQ2 antibody. Western blot showed that a major band (25 kDa) was present in both the purified rat AQP2 protein and the oocyte membrane lysate injected with rat AQP2 cRNA but was absent in oocyte membrane lysate injected with water, indicating the tested aquaporin expressed well in the oocytes (Fig. 1A). Under fluorescence microscope, we visualized strong green fluorescence signal in the plasma membrane of oocytes injected with GFP-OsPIP cRNA but no signal in oocytes injected with GFP cRNA. Fig. 1B shows the expression of a representative fusion aquaporin, GFP-OsPIP2;1. These results indicate that the exogenous aquaporins were highly expressed and properly localized in oocytes.

3.2. The effect of mineral water on water permeability of aquaporins

To elucidate how mineral water affects aquaporin permeability, oocytes expressing human aquaporin (AQP1, AQP2, AQP3, AQP4, AQP5, or AQP7) were transfer to 1/3-diluted MBS buffer prepared by Yokohama tap water, Hita tenryosui water, Rokko water, or Evian water. The expansion rates of oocytes expressing each aquaporin in the four kinds of water were recorded and the water permeability was calculated. The results suggested that AQP1, AQP2, AQP4 and AQP5 showed significantly high water permeability in these four water samples, and AQP3 and AQP7 showed moderate to low water permeability (Fig. 2A). Intriguingly, the water permeabilities of all aquaporins except for AQP7 in Hita tenryosui water were higher than those in the other three water samples. Generally, the eggs in Yokohama tap water and Evian water demonstrated relatively low water permeability. For example, the Pf of AQP1 was about 211 μm/s in Yokohama tap water but it was up to 237 μm/s in Hita tenryosui water. Statistically, AQP1 and AQP2 had significant higher water permeabilities in Hita tenryosui water than those in tap water or Evian water ($p < 0.01$ or $p < 0.05$). AQP4 and AQP5 had significant higher water permeabilities in Hita tenryosui water than those in Evian water ($p < 0.05$). At the same time, we examined the water permeabilities of eight plant aquaporins from rice PIP2 subgroup in Yokohama tap water and Hita tenryosui water. The data indicated that all plant aquaporins except for PIP2;7 had relatively higher activities in Hita tenryosui water than those in Yokohama tap water. However, only PIP2;1 had statistically significant difference ($p < 0.05$) (Fig. 2B). All the above data suggest that Hita tenryosui water has special function to promote aquaporin permeabilities compared to the other water samples.

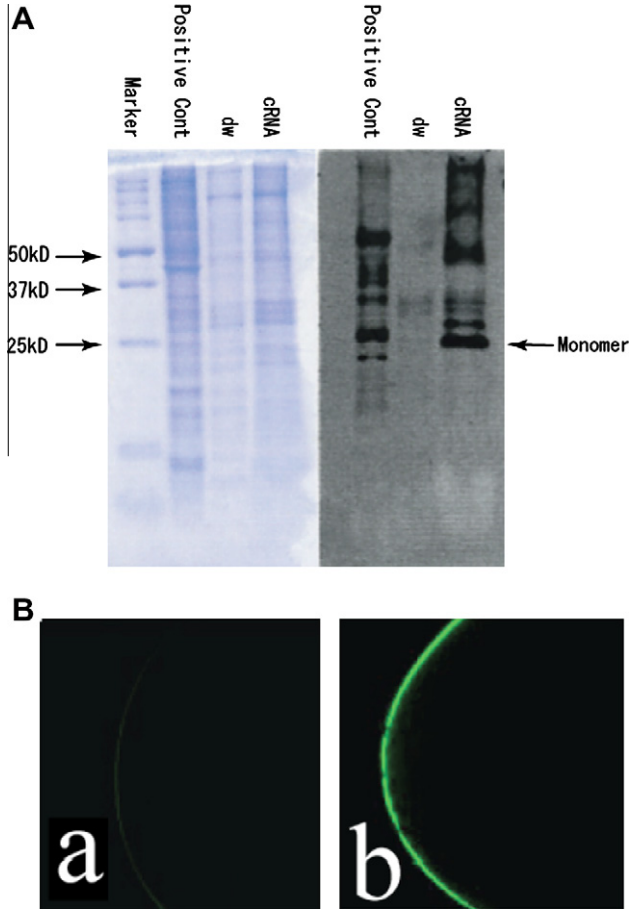

Fig. 1. The expression and water permeability of human and plant aquaporins in oocytes. (A) Western blot of rat AQP2 in the oocytes. The left panel is Coomassie Blue staining of the gel and the right panel is the Western blot probed with anti rat AQP2 antibody. The band indicated by arrow is monomeric AQP2. (B) Expression and plasma membrane localization of PIP2;1. The left panel (a) was the oocyte injected with GFP cRNA and the right panel (b) is the oocyte injected with GFP-PIP2;1 cRNA.

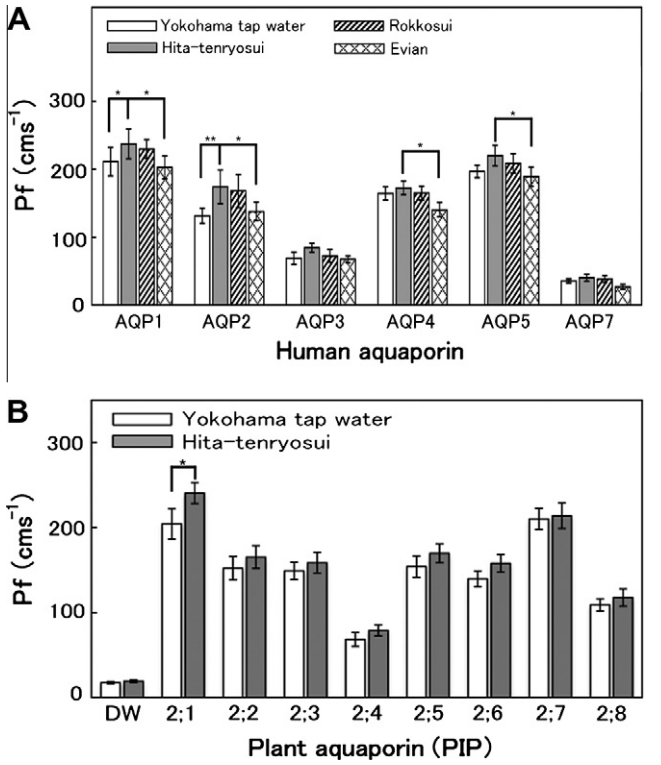

Fig. 2. The water permeability of aquaporins in the different kinds of mineral water. (A) Water permeability of human aquaporins in the oocytes. The water permeability of the oocytes injected with the cRNA of the indicated aquaporins were determined in Yokohama tap water, Hita tenryosui water, Rokko water, or Evian water (10–15 eggs were injected and measured for each aquaporin in each water samples. $^{*}p < 0.05$, $^{**}p < 0.01$). (B) Water permeability of plant aquaporins in the oocytes. The water permeability of the oocytes injected with the cRNA of the indicated aquaporins were determined in Yokohama tap water, Hita tenryosui water (10–15 eggs were injected and measured for each aquaporin in each water samples. $^{*}p < 0.05$).

3.3. Influence of the treated mineral water on AQP2 water permeability

Since the water samples from different sources have different physical and chemical features, these differences may potentially affect aquaporin activity. In here, we investigated the effect of pH, temperature, boiling, organic solvent extraction and freeze-dry on aquaporin (AQP2) permeability.

3.3.1. pH

In natural condition, Yokohama tap water has neutral pH at 7.2, and Hita tenryosui water has slight alkaline pH at 8.2. We adjusted the pH values of Yokohama tap water and Hita tenryosui water to 6.0, 7.0, 8.0 or 9.0 by using diluted HCl or NaOH. The resulted water samples were used for AQP2 activity assays. The data showed that AQP2 had the highest activity in both water samples with pH at 7.0. Except in weak acidic condition (pH 6.0), AQP2 has slightly higher activity in Hita tenryosui water than Yokohama tap water at pH from 7.0 to 9.0. However, none reach statistical significance (Fig. S1), suggesting that AQP2 activity is probably not regulated by pH in oocytes.

3.3.2. Temperature

The activity of some aquaporins can be regulated by temperature [15]. To know if AQP2 is sensitive to temperature or not, we examined AQP2 activity in Hita tenryosui water and Yokohama tap water at 37 °C, 20 °C, 15 °C and 4 °C, respectively. AQP2 demonstrated highest activity at 37 °C and then decreased with temperature dropping. At 37 °C, AQP2 demonstrated significant higher activity in Hita tenryosui water than in tap water ($p < 0.05$) (Fig. 3A).

3.3.3. Boiling

Hita tenryosui water and Yokohama tap water was boiled for 10 min and then cooled to 20 °C. The water permeability of oocyte expressing AQP2 was compared in unboiled or boiled water samples. We found AQP2 activity in Hita tenryosui water was higher than in tap water but boiling treatment did not cause significant change of AQP2 permeability (Fig. S2).

3.3.4. Organic solvent extraction

To further elucidate the importance of the minor components in Hita tenryosui water for aquaporin activity, we performed organic solvent extraction on water samples. Water permeability of AQP2 was dissected in extracted or unextracted water. Interestingly, AQP2 activity was significantly inhibited in the extracted Hita tenryosui water compared to the unextracted water ($p < 0.05$) but was not in the extracted or unextracted tap water (Fig. 3B), further confirming that some important minor components influencing water permeability of aquaporin were removed from Hita tenryosui water by organic solvent extraction.

3.3.5. Freeze-dry

Hita tenryosui water and Yokohama tap water were freeze-dried, and the lyophilized residues were re-dissolved in original volume of Milli-Q water. The water permeability of oocytes expressing AQP2 indicates that freeze-dry treatment of Hita tenryosui water significantly inhibited water channel activity ($p < 0.05$) (Fig. 3C), implicating that the components which can activate the water permeability of aquaporins in Hita tenryosui water were probably precipitated or were chemically changed by freeze-drying process and subsequently AQP2 activity was partially arrested.

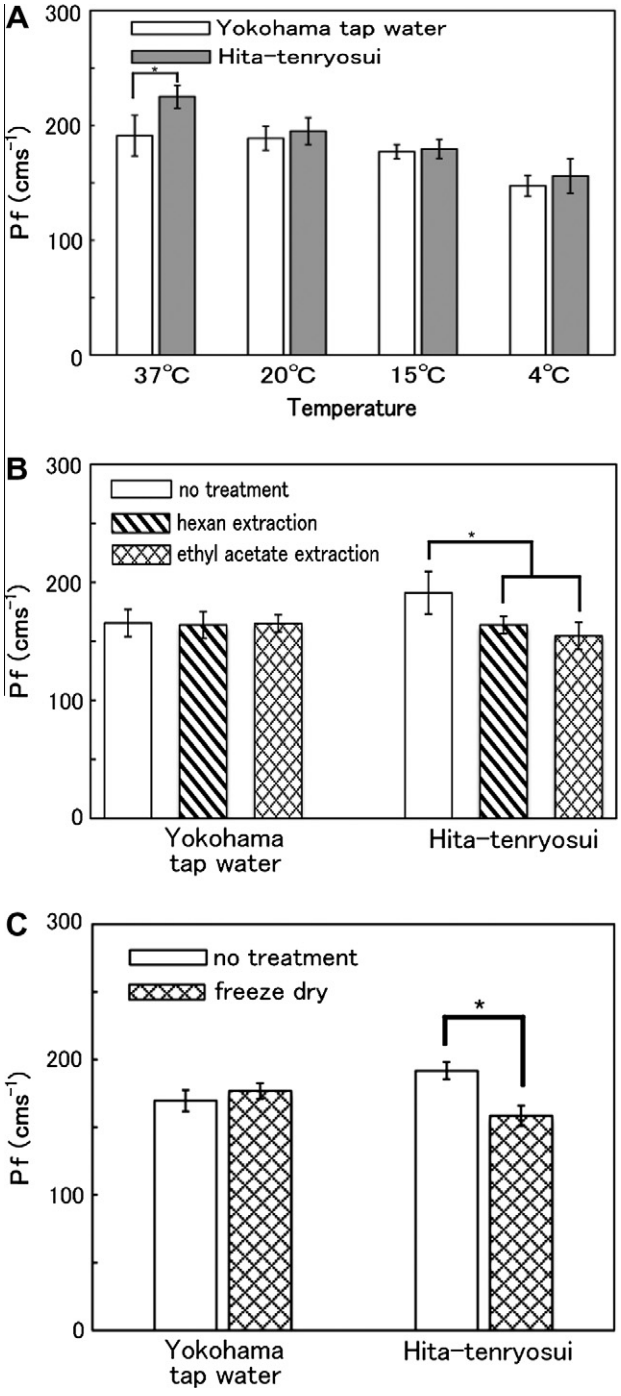

Fig. 3. Influence of mineral water treatments on AQP2 water permeability. (A) Temperature change (*$p < 0.05$). (B) Organic solvent extraction treatment (*$p < 0.05$). (C) Freeze-dry treatment (*$p < 0.05$).

3.4. Effects of Hita tenryosui water consumption on human aquaporin gene expression and NK cell activity

Since aquaporin isoforms, e.g. AQP1, 4, and 9 highly express in hematic granular leukocytes [15], we tried to determine the expression levels of AQP 1, 4, and 9 mRNA in the hematic granular leukocytes collected from the volunteers by real time RT-PCR. In our experiments, we failed to detect AQP4, but we found the expression levels of AQP 1 and AQP 9 have decreasing tendency from before to after drinking tap water or Hita tenryosui water but all have not statistical difference (Fig. S3). However, NK cell

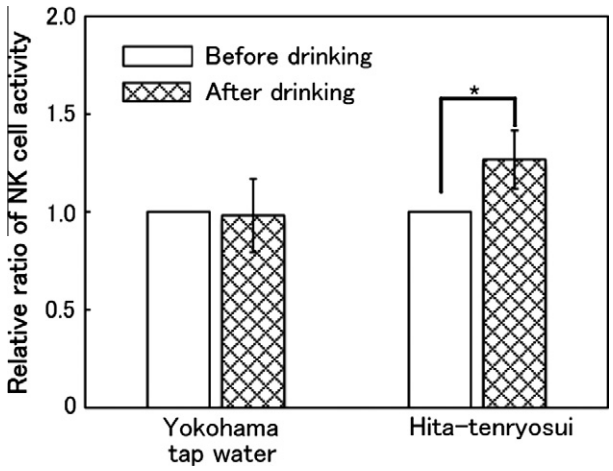

Fig. 4. NK cell activity of hematic granular leukocytes. The relative after-drinking values were calculated based on the average before-drinking values (*$p < 0.05$).

activity assays show that NK activities of blood cells from the volunteers drinking Hita tenryosui water were significantly increased from before to after the drinking ($p < 0.05$), but were not changed from the volunteers drinking tap water (Fig. 4), suggesting the consumption of Hita tenryosui water is able to improve the human immune system.

4. Discussion

Water is the most important component for all organisms. The gain or lose of water in the cells is predominately mediated by membrane-localizing aquaporins. Not only do aquaporins facilitate the transcellular movement of water, but, in some cases, also the flux of small neutral solutes across a cellular membrane. Aquaporin activity can be regulated by many factors including pH and Ca^{2+} [16]. In our present study, we found two human (AQP1 and AQP2) and one plant (PIP2;1) aquaporins but not all demonstrated different water permeability in the different kinds of water. All these three aquaporins had higher activity in Hita tenryosui mineral water than in the other water, suggesting Hita tenryosui water contains special components (e.g. Ca^{2+} and/or the others) to promote aquaporin activities. And we found the phenomena that freeze-dry and organic solvent extraction significantly reduced AQP2 activity in Hita tenryosui water although we did not detect the alteration of AQP2 activity by pH change and boiling, further confirming that in Hita tenryosui water, some dissolved trace substances which were removed or precipitated by the two treatments are critical to promote AQP2 activity. The mystery of what the substances are and the mechanism of how they work still keep for further investigation. Actually, many mammalian aquaporins, such as AQP0, AQP2, AQP3, AQP4 and AQP6, and plant PIPs permeability could be tightly regulated by ions, heavy metals, nutrient, temperature and reactive oxygen species [15–18]. For example, human AQP2 urinary excretion was significantly reduced after water assumption with respect to the basal level and it was lower after low mineral than high mineral water oral drinking [19]. In plant, the expression of aquaporin isoforms could also be responsive to salt and water stresses [20,21]. In our case, we observed that AQP2 had the highest activity in Hita tenryosui water at 37 °C and demonstrated significantly higher permeability than in tap water, implicating that some components in Hita tenryosui water can actively promote AQP2 activity at this healthy human body temperature. Although we did not found that Hita tenryosui water could induce the expression of aquaporins (AQP1 and AQP9) in blood cells, we did see Hita tenryosui water significantly improved the natural killer cell activity. How does Hita tenryosui water do this? We hypothesize that although Hita tenryosui water did not elevate the expression of aquaporins, some components in the water can directly or indirectly promote the activity of aquaporins and subsequently trigger the cell immune responses.

Hita tenryosui water is a kind of natural mineral water drawn from deep underground in Hita city in Japan. This natural reduced water can scavenge intracellular ROS and suppress tumor angiogenesis [22] and oxidative damage of pancreatic β cells induced by alloxan, a type I diabetes inducer and skin diseases and it can alleviate the symptoms of alloxan-induced type I diabetes model mice [23,24]. The disruption or loss-of-function mutation of AQP2 could result in renal failure and nephrogenic diabetes in mice [25]. So in all, our *in vitro* data that Hita tenryosui water can promote AQP2 permeability propose a strong connection of consumption of this natural mineral water and human diseases like cancer and diabetes, providing a clue for the auxiliary treatment of some chronic diseases by water consumption.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.102.

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