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Comparative Study of Alkaline Electrolyzed Water and Tourmaline Water in terms of their Enhancing Effect on the Antioxidant Activity of Ascorbic Acid.

Tongjiao Wu[1], Masahiro Hiroshima[2], Chinatsu Tachibana[2], Mizuki Gaja[2], Ayane Nagahama[2], Yoshinori Kamitani [1,2]*

[1] The United Graduate School of Agricultural Sciences, Kagoshima University and
[2] Graduate School of Agricultural, Forestry and Fisheries, Kagoshima University
1-21-24 Korimoto Kagoshima, 890-0065 Japan

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Abstract: Free radicals are causative factors of lifestyle diseases so that the research on free radical scavengers and the development of their effective utilization methods are particularly necessary. In this context, some functional waters have been reported to possess SOD-like activity, which may remove reactive oxygen species, inhibit tumor angiogenesis, and protect oxidative damage of DNA and RNA. In the present study, alkaline electrolyzed water (AlEW) and tourmaline water (TW) were examined for their enhancing effect on the antioxidant activity of ascorbic acid. Consequently, the enhancing effects of these waters were confirmed. Subsequently, the influence of components of AlEW on the antioxidant activity of ascorbic acid was examined. It turned out that neither EC nor pH showed linear relationship with the SOD activity. Moreover, H_2 bubble water adjusted to the same pH and EC values as those of AlEW showed SOD activity almost equal to that of the control, indicating no significant roles of these factors.

Keywords: Alkaline electrolyzed water; antioxidant effect; SOD activity; tourmaline water

1 Introduction

Lifestyle-related diseases are due to risky behaviors to health such as poor diet, smoking, alcoholism, or lack of exercise[1]. They include hypertension, hyper-lipidemia, hyperuricemia, diabetes, arteriosclerosis as well as cancer. The main reason for lifestyle-related diseases is that an imbalance between generation of reactive oxygen species (ROS) or free radicals and their scavenging system in cells leads to oxidative stress[2]. ROS or free radicals[3], such as singlet oxygen ($^1O_2$), superoxide anion radical ($O^{\cdot}{}_2^-$) hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) are highly reactive and potentially capable of damaging transient chemical species formed in aerobic state, and even cause oxidation of biological macro-molecules such as protein, DNA and enzymes in the body, giving rise to several diseases[4]. On the other hand, there is an antioxidant system that relies on endogenous antioxidant enzymes and antioxidant substances in the organism, that is, free radical scavengers. Among them, ascorbic acid is a cofactor in numerous physiological reactions and acts as an antioxidant in biological systems[5]. It can protect the compounds in the extra-cellular and intracellular spaces, prevent the damage caused by free radicals to DNA, and scavenge free radicals[6]. Hence, it is meaningful to study the activity enhancement of antioxidants and effective scavenging of free radicals.

Electrolyzed water is a functional water generated by the electrolysis of aqueous-solution containing chloride ion (Cl^-). It includes acidic electrolyzed water and alkaline electrolyzed water (AlEW)[7]. The latter is also called electrolyzed reduced water (ERW), is produced at the cathode upon electrolysis and showed capability of extraction and anti-oxidation. Moreover, the AlEW with low oxidation-reduction potential (ORP) has reducibility that might result in scavenging free radicals in biological systems[8]. Shin *et al.*[9] reported that daily drinking of

* Corresponding author: Yoshinori Kamitani (kamitani@agri.kagoshima-u.ac.jp)

alkaline-reduced water (ARW) for eight weeks improved the quality of life in patients with diarrhea-predominant irritable bowel syndrome (IBS). ERW may also inhibit the tumor angiogenesis[10)] and protect the liver from carbon tetrachloride damage[11)]. In 1997, Shirahata *et al.* first reported that ERW had SOD-like activity, and could protect DNA from oxidative damage[12)]. Lee *et al.* reported that ERW could protect against oxidative damage to DNA, RNA and protein[13)]. Hanaoka pointed out that ERW could improve the activity of antioxidants to scavenge free radicals and ROS. Besides, the hydrogen peroxide scavenging activity of ERW may depend on the concentration of activated hydrogen molecules produced in the ERW [14)].

Tourmaline stone, a natural borosilicate mineral, is usually expressed as the following chemical formula; $XY_3Z_6Si_6O_{18}(BO_3)_3W_4$, where X is Na^+, Ca^{2+}, K^+, or vacancy; Y is Mg^{2+}, Fe^{2+}, Mn^{2+}, Al^{3+}, Fe^{3+}, Mn^{3+}, Cr^{3+}, Ti^{4+}, or Li^+; Z is Al^{3+}, V^{3+}, Cr^{3+}, or Mg^{2+}; and W is OH^-, F^-, or O^{2-}. The electric property of tourmaline stone is excellent in thermoelectricity and piezoelectricity[15)]. At present, there are many products related to tourmaline stone treated water (hereafter tourmaline water or TW) on the market. They claim that they show weakly alkaline property and capability of good extraction and dissolution similar to these of alkaline electrolyzed water.

The purpose of this research is to compare alkaline electrolyzed water with tourmaline water in terms of their enhancing effect on the anti-oxidant activity of ascorbic acid by using the SOD assay method.

2 Materials and Methods

2.1. Solutions

The following solutions each containing 2 mmol/L ascorbic acid were used for SOD assay.

2.1.1. Alkaline electrolyzed water (AlEW) was generated by the electrolyzed water generator (model ROX-20TA of Hoshizaki Corporation with some modifications by us. The electrolysis voltage was 10V. The physico-chemical properties of AlEW were shown in Table 1. Ec, pH, ORP, EC, DO and DH of AlEW 4A, 8A, 12A and 16A were measured respectively with Electrical Conductivity meter (CUSTOM, CD-6021A), pH meter (TPX-999, Toko Chemical Laboratory), ORP meter (silver-silver chloride electrode: TRX-90, Toko Chemical Laboratory), Dissolved Oxygen meter (Mettler-Toledo, SG9-ELK) and Dissolved Hydrogen meter (TRUSTLEX, ENH-2000). The particle size of dissolved hydrogen in AlEW ranged 20~560μm.

2.1.2. Pure water was generated by pure water generator (Merck Millipore, Elix Essential UV3) with the reverse osmosis membrane and continuous ion exchange so as to get EC value of 2.0μS/cm or less.

2.1.3. pH-adjusted waters with pH of 8.5, 9.5, 10.5 and 11.5 were prepared with 0.1 M NaOH aqueous solution (Nacalai Tesque Co., Ltd.).

2.1.4. EC-adjusted water was obtained by adjusting EC to 1.9 mS /cm with NaCl (Salt Industry Center of Japan) to match EC of AlEW 8A as shown in Table 1. The NaCl concentration of EC-adjusted water was set to 0.03-0.2% (EC 0.6~3.7 mS/cm).

2.1.5. H_2 bubble water (HB) was generated by using the pump swirling shear type bubble generator (Shoko Tsusho Co., Ltd., SK20D). Hydrogen gas was eluted in pure water at a flow rate of 0.4L/min and stirred at 25 ℃ for 30 min.. The particle size distribution of H_2 in hydrogen bubble water was determined as described in previous studies. There were two peaks in the distribution curve. The H_2 existence rate of the first peak was 2%, and the particle size of the hydrogen bubble was 120 μm. The

Table 1. The physico-chemical properties of AlEW

Ec of AlEW	pH	ORP(mV)	EC(mS/cm)	DO(mg/L)	DH(ppm)	Temp(℃)
4A	10.13±0.1	-762±5.7	0.93±0.13	6.0±0.4	0.49±0.7	25.2±0.2
8A	10.92±0.1	-827±7.2	1.91±0.21	3.7±0.6	0.53±0.1	24.8±0.2
12A	11.18±0.1	-848±7.6	2.69±0.30	3.6±0.5	0.54±0.1	24.5±0.4
16A	11.46±0.3	-872±4.0	3.97±0.09	3.3±0.0	0.56±0.1	24.5±0.5

Ec of AlEW is the electrolysis current of the AlEW; ORP is Oxidation-Reduction Potential; EC is electrical conductance; DO is dissolved oxygen concentration; DH is dissolved hydrogen concentration; Temp is the temperature of AlEW. Each value is expressed as the mean value ± standard deviation of three replicates.

Table 2. The physico-chemical properties of tourmaline water

Temp of TS	pH	ORP(mV)	EC(μS/cm)	DO(mg/L)	DH(ppm)	Temp(℃)
25℃	7.3±0.3	216.3±4.9	154.1±6.4	7.1±0.7	0.0	25.4±0.1
50℃	7.4±0.1	223.7±9.3	150.3±5.4	8.1±0.7	0.0	25.4±0.1
75℃	7.4±0.1	253.7±57	149.3±6.4	8.8±0.8	0.0	25.3±0.1
100℃	7.3±0.1	255.0±56	145.8±12	7.6±0.3	0.0	25.4±0.2

Temp of TS is the temperature of tourmaline stone; ORP is Oxidation-Reduction Potential; EC is electrical conductance DO is dissolved oxygen concentration; DH is dissolved hydrogen concentration; Temp is the temperature of tourmaline water. Each value is expressed as the mean value ± standard deviation of three replicates.

second peak showed 12% in existence rate, and the particle size of the hydrogen bubble was 677 μm. H_2 bubble water was adjusted to pH 10.5 and EC 1.9mS/cm similer to these properties of AlEW 8A.

2.1.6. Tourmaline water (TW) was prepared as follows. 770 g of Brazilian tourmaline stone was heated at 25, 50, 75 and 100 ℃ for 30 min. in a water bath and 7.7 L of pure water was added and circulated for 30 min. at 2.2~ 2.4L/min with magnet pump. Physico-chemical properties of TW were summarized in Table 2.

2.1.7. Tourmaline-adjusted water was prepared as follows. Tourmaline water (TW) was adjusted to pH 10.5 and EC 1.9mS/cm to match the properties of AlEW as shown in Table 1.

2.2. SOD Assay method

In order to examine the above solutions for their enhancing effect on the anti-oxidant activity of ascorbic acid, the SOD Assay Kit-WST (Dojindo) was employed. In this assay system, superoxide radical-producing reaction by xanthine oxidase (XO) is coupled with the reducing reaction by WST-1 (tetrazolium salts), resulting in the formation of WST-1 formazan (yellow color) and O_2. However, coexistence of SOD, which is capable of converting superoxide radicals to O_2 and H_2O_2, inhibits the above coupling reaction. In addition, if any compound with radical scavenging activity such as ascorbic acid coexists, the SOD activity should be apparently increased.

In our study, ascorbic acid was used as the control and the enhancing effect of AlEW and tourmaline water were examined. Actually, the following experimental procedure was carried out.

First, 20 μl each of sample solutions and the control solution (PW) was filled in wells of the 96-well plate. Subsequently, 200 μl of WST working solution was added to each well and mixed well by pipetting or with plate mixer, followed by addition of 20 μl of Enzyme working solution. Then, plates were incubated at 37°C for 20 minutes in order to measure the absorbance at 450 nm with a plate reader. The SOD Activity (Inhibition Rate %) was calculated by following the supplier's instruction.

2.3 Statistical analysis

Each measurement was carried out three times. The results were analyzed by using the One-Way ANOVA of SPSS (Statistical Package for the Social Sciences; SPSS, Inc, Chicago, IL) software. Statistical significance was set at a value of $P < 0.05$.

3 Results

3.1 Effect of electrolysis current of AlEW on SOD activity

The effect of AlEW with different EC on the SOD activity was shown in Fig.1 (A). AlEW4A and AlEW8A enhanced markedly the activity of SOD. The highest SOD activity was 89.7% in AlEW8A. AlEW12A and AlEW16A showed significantly lower SOD activities, compared with PW (control).

These results might be due to that high current electrolysis might cause the reduction or loss of the antioxidant ability of ascorbic acid itself.

3.2 Effect of NaCl concentration on SOD activity

Fig. 1(B) shows the effect of NaCl concentrations ranging 0.03-0.2% (EC 0.6-3.7 mS / cm), which were equivalent to those of weakly alkaline to strongly alkaline electrolyzed waters. Within this range, the SOD activity was 80% or higher, indicating the enhancing effect in all NaCl concentrations, although no linear relationship between SOD activity enhancement and NaCl concentration was observed. Additionally, there was no significant difference in comparison with the control (PW).

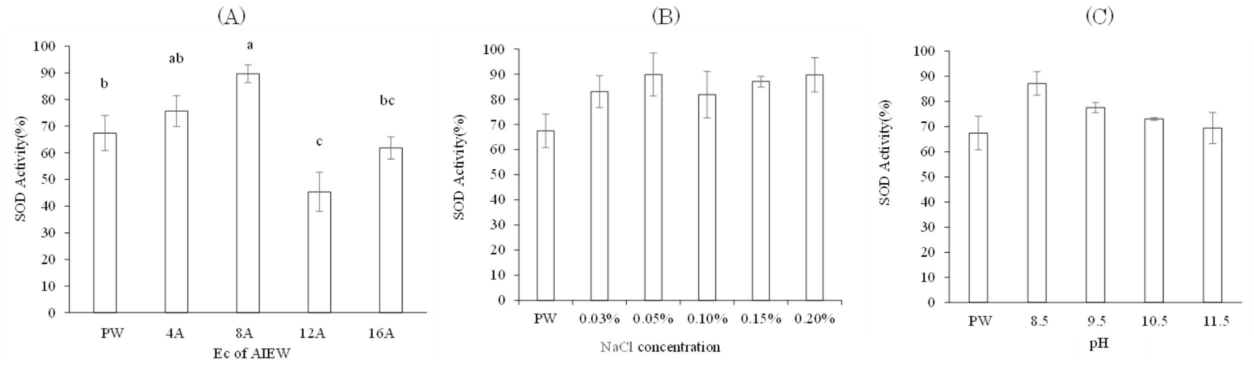

Fig.1. Influence of electrolytic current (A), NaCl concentration (B) and pH (C) on SOD activity.
PW: the control. (A): 4A, 8A, 12A and 16A are Ec. of AlEW (refer to Table 1).
The different letters in (A) indicate significance ($p < 0.05$). Each value is expressed as the mean value ± standard devitation of three replicates.

3.3 Effect of pH on SOD activity

The effect of pH on SOD activity was shown in Fig. 1(C). In this experiment, the pH of pH-adjusted water was targeted at the range of weakly alkaline to strongly alkaline electrolyzed water. It turned out that the lower the pH was, the higher the SOD activity. The SOD activity was highest (87%?) at pH8.5 and lowest (70%) at pH 11.5 that was equivalent to that of PW (control), indicating no enhancement effect on the SOD activity. There was no significant difference between pH8.5 and PW (control).

It was thus unlikely that alkaline pH was not the indicator of the antioxidant ability enhancing effect of AlEW.

3.4 Effect of hydrogen bubble on SOD activity

Based on that AlEW 8A (Table1) showed the highest enhancement of SOD activity, hydrogen bubble water (HB-A) with physico-chemical properties similar to those of AlEW 8A was prepared; namely, the pH 10.5, EC 1.9 ms/cm and ORP -557.6±3.2mV. As shown in Fig. 2 SOD activities were 67.3%, 67.8% and 89.7% in PW (control), HB-A and AlEW 8A, respectively. Statistical analysis indicated that the SOD activities of HB-A and AlEW 8A were significantly different, whereas no substantial difference was observed between PW (control) and HB-A, indicating that hydrogen bubble has no substantial effect as an antioxidant.

On the other hand, Fig.3 showed the change of SOD activity and dissolved hydrogen concentration during the storage of hydrogen bubble water. This was to test the

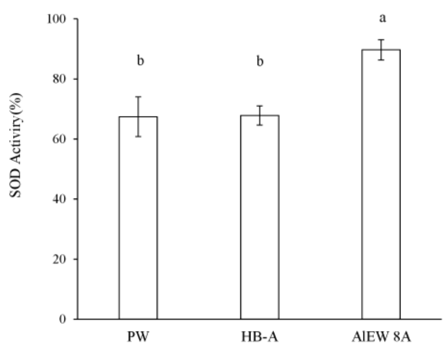

Fig.2. SOD activities of PW, hydrogen bubble water (HB-A) and AlEW 8A.

stability of the HB-A. Dissolved hydrogen (DH) concentration decreased gradually during the storage time of hydrogen bubble water. However, there was no significant difference in the SOD activity. Therefore, in this study, hydrogen bubble water was used after operating for 30 minutes at the time of generation.

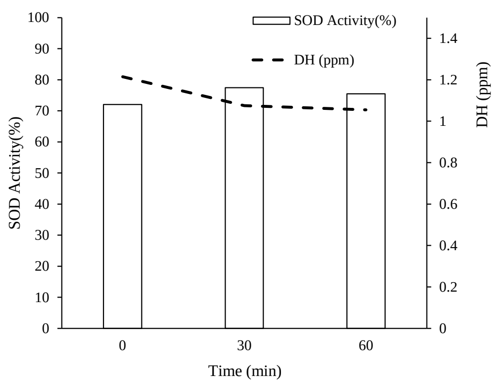

Fig.3. Stability of SOD activity and dissolved hydrogen concentration of H_2 bubble adjusted water (HB-A).
DH: Dissolved hydrogen concentration, expressed as the mean value ± standard deviation of three replicates.

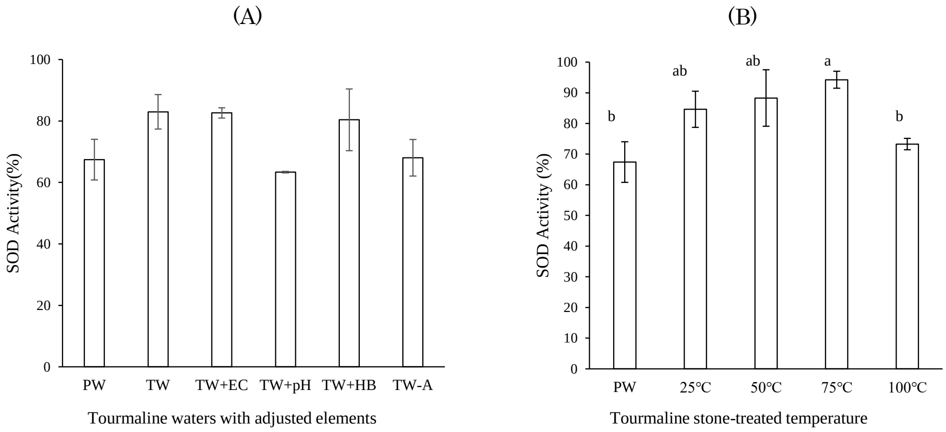

Fig.4. SOD activity of tourmaline waters with various conditions.

(A) PW: control, TW: Tourmaline water produced by treating TS at 25℃, TW+EC: TW with EC-adjusted to that of AlEW, TW+pH: TW with pH adjusted that of AlEW, TW+HB: H_2 bubble adjusted TW with pH 10.5, EC 1.9 mS/cm, TW-A: H_2 added TW with the same pH and EC values as those of AlEW.
The different letters indicated significance ($P < 0.05$). Each value is expressed as the mean value ± standard deviation of three replicates.
(B) PW: control, 25 ℃~100℃: temperatures for tourmaline stone (TS) treatment for 30 min. to prepare tourmaline waters.

3.5 Effect of tourmaline water on SOD activity

The effect of tourmaline water with various elements on SOD activity were shown in the Fig.4 (A). Tourmaline water (TW), tourmaline EC-adjusted water (TW+EC), and tourmaline H_2 bubble water (TW+HB) showed SOD activity (80.4%) higher than that of the PW (control). On the other hand, the SOD activity of tourmaline-adjusted water (TW-A) was 68.05%, while tourmaline pH-adjusted water showed the lowest SOD activity. It was therefore suggested that tourmaline water had an enhancing effect on the anti-oxidant activity of ascorbic acid. However the effect was lowered at the alkaline pH.

The SOD activity of tourmaline water produced by treatment at different temperatures of tourmaline stone was shown in Fig. 4 (B). SOD activity increased and then decreased with the increase of tourmaline treatment temperature. Based on the results of the significance analysis it seemed likely that the SOD activity of TWs (excepted for 100°C) were significantly higher than that of the control (PW) group. The peak value 94.25% was reached with TW 75℃. However, the SOD activity of TW 100 °C was 73.27% indicating no significant difference from the control group (PW).

3.6 SOD activity comparison of AlEW and TW

As summarized in Fig. 5, both AlEW and TW enhanced the SOD activity or the antioxidant ability of ascorbic acid. The enhancement effect is significantly different from that of PW (control). Moreover, the SOD activity of HB-A was significantly lower than that of AlEW and tourmaline water.

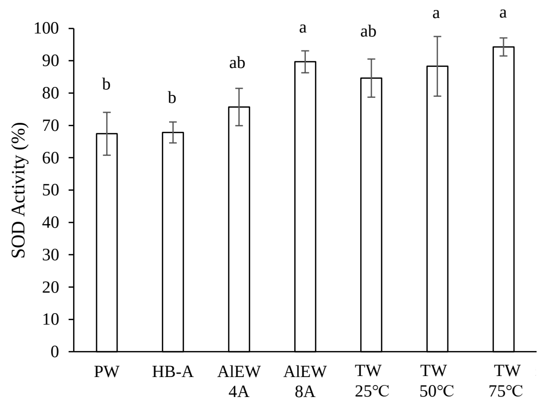

Fig. 5. SOD activities in H_2 bubble adjusted water (HB-A), AlEW and tourmaline waters (TW).

HB-A: H_2 bubble adjusted water with pH10.5, EC1.9 mS/cm. AlEW 4A and 8A: AlEW generated under electrolysis current of 4A and 8A, respectively. TW25~75℃: tourmaline water (TW) produced by heating TS at 25~75℃ for 30 min. The different letters indicated significance ($P < 0.05$). Each value was expressed as the mean value ± standard deviation of three replicates.

4. Discussion

In this study, the enhancing effect of AlEW and tourmaline water on the antioxidant activity of ascorbic acid was examined. The influence of their constituent factors on the antioxidant activity was also analyzed. Consequently, it turned out that both AlEW and tourmaline water were capable of enhancing the anti-oxidant activity. The SOD activity-enhancing effect of AlEW increased firstly and then decreased with the increase of electrolytic intensity. Similar results were obtained in the experiment using tourmaline water that may be weakly alkaline. So far, there are several hypotheses about the reasons for such function of tourmaline water, in spite of that they have not been scientifically proven. Nevertheless, "tourmaline water is similar to AlEW and is beneficial for health" is being advertised for some products. However, based on our research, it should be remarked that AlEW had higher pH, EC, DH values and lower ORP value than those of tourmaline water, as shown in Tables 1 and 2. On the contrary, when the physical properties of tourmaline water were compared with those of pure water without tourmaline treatment, no marked difference was observed except for the EC value. Interestingly, the SOD activity-enhancing effect of tourmaline water and AlEW was similar.

Another our concern is that the tourmaline water without addition of any substance showed the same SOD activity-enhancing effect. It should be noted that the conductivity of the original water is almost 0 so that it would be hard to be electrolyzed. Therefore, it is necessary to add NaCl as the auxiliary electrolysis agent to the water. This was the reason why we studied the SOD activity-enhancing effect of alkaline electrolyzed water in order to elucidate the mechanism. pH, EC and DH are characteristic parameters of AlEW. it was verified whether alkalinity, NaCl and dissolved hydrogen might enhance the antioxidant activity of ascorbic acid.

The concentration of NaCl influences electrical conductivity (EC)[16], which is one of the physical indicators during electrolysis. In our study, similar EC data (EC 0.6~3.7mS/cm) were obtained in the range of the NaCl concentration (0.03~0.2%) of AlEW. Although the SOD activity-enhancing effect of EC adjusted water with a specific EC value was higher than that of pure water, the difference was not significant (Fig. 1(B)). Therefore, it was conclusive that the EC value has no effect on the SOD activity of EC-adjusted water. In order to ensure the consistency of ion species in the solution, NaOH was added to pure water as a pH adjuster. A similar conclusion in terms of the EC value was obtained. The alkalinity of the solutions tested was not a factor for AlEW to enhance the antioxidant activity of ascorbic acid.

According to the generation principle of electrolyzed water, hydrogen is co-produced and dissolved in water during electrolysis, resulting in the formation of AlEW having increased DH and lowered ORP values[12]. Therefore, in this study, hydrogen bubble water was used to simulate the dissolved hydrogen in AlEW, and the influence of ORP on SOD activity was also analyzed. The SOD activity of HB-A was approximately the same as that of PW (pure water), indicating that hydrogen could not be the factor to enhance the antioxidant activity. Compared with pure water, AlEW had significantly enhanced SOD activity (Fig.2). Hanaoka reported that the behavior of H_2 in reduced water, which was activated by a platinum electrode, was different from that of H_2 introduced by bubbling of hydrogen gas[14]. Alkaline electrolyzed water and HB-AW had approximately the same pH, EC, and H_2 bubbles. Meanwhile, comparing the ORP values of AlEW, HB-AW and tourmaline water, they were -827±7.2, -557.6±3.2 and 253.7±57mV, respectively. However, the SOD activity of AlEW and tourmaline water was significantly higher than that of HB-AW. In other words, ORP was not a factor for AlEW to enhance the SOD activity.

Alkaline electrolyzed water, as a kind of drinking water, had received a big attention. According to Item 360 of The Ministry of Health, Labor and Welfare No. 112 in Japan, the consecutive daily drinking of AlEW produced by “Water electrolyzer for home use” (JIS T 2004) would improve gastro-intestinal symptoms such as indigestion, excessive stomach acid[17] and chronic diarrhea[9]. Moreover, Hanaoka postulated that electrolyzed-reduced water (AlEW) showed increased dissolving activity[14]. Thus, at the same temperature, compared with ordinal daily drinking water, AlEW might dissolve the drug better, and therefore may maximize the effect of the drug. Our present study demonstrated that AlEW enhanced the antioxidant effect of ascorbic acid. Hence, it might be possible that drinking AlEW might promote scavenging free radicals in human body so as to improve human health.

Further study should be necessary to analyze the influence of temperature changes and other factors on the antioxidant activity-enhancing effect of AlEW in order to elaborate the basic mechanism and to expand the application of AlEW.

5 Conclusion

In this research, AlEW and tourmaline water were examined for their effect on antioxidant activity of ascorbic acid. As results, both functional waters enhanced significantly the antioxidant ability of ascorbic acid. Their enhancement was stimulated with increase of Ec (Peak stimulation was 8A) of AlEW and treatment temperature (Peak stimulation was 75℃) of tourmaline stone.

Subsequently, the physical properties of these two functional waters were compared in order to clarify factors that brought the difference in EC, pH, and ORP, using NaCl, NaOH and H_2 bubble as candidate regulators. It turned out that neither EC nor pH value had no linear relationship with the SOD activity-enhancing effect of the adjusted water. In addition, although HB-AW showed the same pH, EC value and hydrogen bubble as AlEW, the SOD activity was almost equal to that of PW. Based on these results, pH, EC and H_2 bubbles were not the main factors that enhanced the activity of SOD.

Conflict of interest

The authors have nothing to declare.

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アルカリ性電解水とトルマリン水によるアスコルビン酸の SOD 活性増強効果の比較検討

呉彤嬌[1]、廣島誠浩[2]、立花知奈津[2]、我謝瑞希[2]、長濱綾音[2]、紙谷喜則[1,2*]

1鹿児島大学連合大学院大学　２農林水産学連合講座

体内発生する活性酸素種は、高血圧、高脂血症、高尿酸血症、糖尿病、動脈硬化症、癌などの生活習慣病の一因であると報告されている。そのため、活性酸素種のスカベンジャー物質の探究や効果的な利用方法の開発が求められている。機能水の中には、活性酸素種を除去し、腫瘍の血管新生を阻害し、酸化的ダメージから細胞内の DNA、RNA を守ることができる SOD 様活性を持つことが報告されている。本研究では、L-アスコルビン酸（0.2mM）の抗酸化活性に対するアルカリ性電解水とトルマリン水の増強効果について比較研究した。また、抗酸化活性に対するアルカリ性電解水の構成要素の影響を解析した。その結果、水酸化ナトリウム濃度（pH 値）、塩分濃度（EC 値）は SOD 活性に濃度依存的な影響を示さなかった。さらに、アルカリイオン水と同様の pH、EC および水素バブルをもつように調整した水素バブル水の SOD 活性は純水(PW)と変わらなかった。以上のことからこれらの因子は、SOD 活性の増強要因ではないと判断された。結論として、アルカリ性電解水とトルマリン水においてアスコルビン酸の抗酸化作用に対する増強効果がみられた。

* 連絡先：紙谷喜則 (kamitani@agri.kagoshima-u.ac.jp)