Kangen Water Proof Book

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ACID-ALKALINE BALANCE: ROLE IN CHRONIC DISEASE AND DETOXIFICATION

Deanna M. Minich, PhD, FACN, CNS; Jeffrey S. Bland, PhD, FACN

Deanna M. Minich, PhD, FACN, CNS, is the Director of Information Integration and Innovation at Metagenics, Inc, and a clinical nutritionist at the Functional Medicine Research Center in Gig Harbor, Wash. Jeffrey S. Bland, PhD, FACN, serves as the chief science officer of Metagenics, Inc, and is president of the MetaProteomics Nutrigenomics Research Center.

Several researchers have noted that the contemporary Western diet has increased in net acid load relative to diets of the ancestral pre-agricultural *Homo sapiens*.[1-3] Quite possibly, this shift occurred because of the agricultural revolution and the ubiquity of processed grains and shelf-stable food products devoid of essential nutritional components. In addition to this underlying foundational change in diet, there is the overlay of various nutritional fads that have risen and fallen over the past few decades. Most recently, the latest diet trend has been an interest in high-protein foods accompanied by a compensatory decrease in the phytochemical load from fresh fruits and vegetables. Indeed, high-protein diets increase net dietary acid load and acidify the urine pH.[2-5] Conversely, diets high in fruits and vegetables have been proposed to be associated with a greater degree of alkalinity.[4,6] Remer and Manz calculated the potential renal acid loads of certain food groups and reported that alkaline-forming foods were primarily vegetable and fruits, whereas acid-forming foods were derived from cheese, meat, fish, and grain products (Table 1).[4]

TABLE 1 Average Potential Renal Acid Loads (PRAL) of Specified Foods[4]

Food	PRAL* (mEq)
Fats and oils	0
Fish	7.9
Fruits and fruit juices	-3.1
Grain products	3.5-7.0
Meat and meat products	9.5
Milk and dairy products	1.0-23.6
Vegetables	-2.8

*PRAL = mEq of Cl + PO4 + SO4 – Na – K – Ca – Mg)

Over time, ingestion of a high dietary acid load can progress to a chronic low-grade level of metabolic acidosis. The incidence of low-grade acidosis resulting from our modern diet has been well documented.[1-3,6] A chronic acidic load can cause a number of health conditions such as osteoporosis, kidney disease, and muscle wasting.[1,7] Sebastian et al articulates this cause and effect relationship eloquently: "Increasing evidence . . . suggests that such persisting, albeit low-grade, acidosis, and the relentless operation of responding homeostatic mechanisms, result in numerous injurious effects on the body including dissolution to bone, muscle wasting, kidney stone formation, and damage to the kidney."[1(p1308)]

In order to maintain acid-alkaline balance throughout the various body systems, one system may be required to support another. For example, the bone matrix contains a substantial alkaline reserve such as calcium and magnesium cations that are released from the bone to balance an overly acidic dietary load in the event of inadequate buffering capacity in the blood. However, repeated borrowing of the body's alkaline reserve in response to a consistent increased (dietary) acid load can be potentially detrimental. In humans, hypercalciuria and negative calcium balance due to calcium efflux from bone may lead to metabolic bone disease and calcium nephrolithiasis.[2,8,9] In the chapter titled "Potassium" of its report *Dietary Reference Intakes for Water, Potassium, Sodium, Chloride, and Sulfate,* the Institute of Medicine Food and Nutrition Board states the following:

> *In the setting of an inadequate intake of bicarbonate precursors, buffers in the bone matrix neutralize the excess diet-derived acid, and in the process, bone becomes demineralized. Excess diet-derived acid titrates bone and leads to increased urinary calcium and reduced urinary citrate excretion. The resultant adverse clinical consequences are possibly increased bone demineralization and increased risk of calcium-containing kidney stones.*[7(p187)]

Conversely, dietary modification can positively influence bone metabolism. A diet favoring neutralization of net endogenous acid production increases calcium and phosphate retention, reduces bone resorption markers, and increases markers of

bone formation in postmenopausal women.[10] Furthermore, studies have demonstrated a positive association between a high intake of alkali-rich fruits and vegetables with preservation of bone mineral density.[6,11,12]

PHYSIOLOGY OF ACID-ALKALINE BALANCE

From a physiological perspective, the body has compartmentalized organ systems operating within specific pH ranges (Table 2).[13] The "potential of hydrogen," or "pH," is based on a logarithmic scale, meaning that there is a 10-fold difference between each number going from 1 to 14. The lower numbers (1-6.99) represent the acid (or H+ donating) range, and the higher numbers (7.01-14) represent the alkaline (or H+ accepting) range. For the most part, body tissues remain within the neutral pH of 7. Some body systems such as the blood (7.35-7.45) are more tightly regulated than others (eg, urine pH ranges from 4.5-8.0), and any extended disturbance in acid-alkaline balance may upset cell functioning via its transport and signaling processes.[14-16] The human body has several means whereby it is able to regulate acid-alkaline balance, including the following: (1) at the cellular level via chemical reactions generating or consuming H+; (2) in the blood with the assistance of bicarbonate, amino acids, albumin, globulin, and hemoglobin; and (3) systemically through the release of carbon dioxide from the lungs and hydrogen ions from the kidney.[17]

TABLE 2 pH of Selected Body Tissues[13,17]

Body Tissue	pH
Blood	7.35-7.45
Muscle	6.1
Liver	6.9
Gastric juice	1.2-3.0
Saliva	6.35-6.85
Urine	4.5-8.0
Pancreatic juice	7.8-8.0

CLINICAL DETERMINATION OF ACID-ALKALINE BALANCE

Although it is not common, blood pH levels can shift to the side of excessive acidity or alkalinity, in which case several clinical symptoms will appear. Acidosis can lead to symptoms of lethargy, progressing to stupor and coma, while alkalosis can lead to a host of nervous conditions such as cramps, muscle spasms, irritability, and hyperexcitability. Clinical determination of a high systemic acid load can be accomplished by a number of methods, including a review of the diet diary for at least 3 to 7 days to gauge the degree of processed food and animal protein intake relative to fruit and vegetable consumption and a measurement of urine pH using narrow-range indicator paper. Urine pH is a good indicator of the net dietary acid load, as reported by Remer and Manz, who observed an inverse relationship between the two variables.[4] The urine compartment appears to be well suited for measuring the effect of acute and chronic factors. In our clinical experience, we have noted that the urine pH responds to a dietary intervention in as little as 2 hours. Additionally, the fact that the urine pH range spans a greater continuum (4.5-8.0) indicates that it has more potential to reflect systemic pH changes. From our testing, it was determined that due to the wide pH range of the urine, it is important to take a fasting urine sample and to control for water intake during the fasting period. Intra- and inter-individual variability can be further reduced if the same person is determining the pH readings consistently.

URINARY ALKALINIZATION

The concept of acid-alkaline balance in the field of medicine is not entirely novel, as it has been embraced by several groups within the medical community. Naturopathic medicine has used the acid-alkaline balance as a theoretical model to explain the foundation of many diseases. Allopathic medicine has examined pH modulation in specific organ systems such as the kidney to control the formation of stones and the elimination of toxins. For example, urine alkalinization has been part of the medical protocol for the management and prevention of uric acid stones.[18,19]

Another aspect of the acid-alkaline balance is its role in detoxification, via either the acute removal of a drug or poison due to overdose or a nutritional protocol to support metabolic detoxification and decrease dietary toxins. Urinary pH alkalinization is a method employed under acute medical settings for the enhanced elimination of toxins in the event of a severe overdose. Conversely, acidification of urine also increases the elimination of specific toxins, although to a seemingly lesser degree.[20,21] The method by which urine alkalinization works to enhance toxin elimination is by the medically recognized process of "ion trapping," which is the ability to enhance urinary excretion of weak acids in alkaline urine, preventing the reabsorption of xenobiotics by renal tubules.[22,23] Proudfoot et al published a position paper on urine alkalinization, approved by the American Academy of Clinical Toxicology, which describes the use of urine alkalinization to ≥7.5 via intravenous sodium bicarbonate administration for acute poisoning and toxicity.[22] In this extensive review, the effect of urine alkalinization on the excretion of various pharmaceuticals and environmental toxins is elucidated. This report states that "urine alkalinization increases the urine elimination of chlorpropamide, 2,4-dichlorophenoxyacetic acid, diflunisal, fluoride, mecoprop, methotrexate, phenobarbital, and salicylate."[22] The potential of urine alkalinization to enhance toxin excretion is exemplified by the work of Blank and Wolffram, wherein they modulated urine pH in pigs with 2% dietary sodium bicarbonate, changing the urine pH from 5.7 ± 0.2 to 8.3 ± 0.1, and favorably impacted the excretion of ochratoxin A, a mycotoxin, from 9.3 ± 1.9% to 22.2 ± 4.3% of the dose.[24] Also, experimental and clinical studies confirm that urine alkalinization is effective for salicylate poisoning.[23,25,26] Garrettson and Geller showed in humans that an increase in urine pH from 6.1 to 8.1 changed the renal clearance of salicylate from 0.08 ± 0.08 L/h to 1.41 ± 0.82 L/h.[23]

Therefore, if the rapid removal of toxins can be achieved to a large extent with increasing urine pH 2 points on the pH scale

(which corresponds to a 100-fold decrease in H+ ions), it would follow that smaller quantities of toxins may be removed on a prolonged basis if there were a subtle increase of urine pH in the alkaline direction. Due to the logarithmic pH scale, a small change in urine pH could have a disproportionately large effect on drug and xenobiotic clearance.[22] The concept of "progressive" versus rapid alkalinization of urine may be useful as an adjunct for integrative health approaches employing metabolic detoxification using specific (nutritional) protocols. Traditionally, functional medicine has addressed detoxification or the removal of harmful endo- or exogenous substances, from the aspect of upregulating hepatic phase I and phase II enzymes to enable the chemical biotransformation of toxins into water-soluble metabolites for excretion in the urine. With the added clinical procedure of urine alkalinization, the removal of these compounds from the body is accelerated. There are many dietary agents to assist in progressive alkalinization. Foods that are high in potassium are noteworthy (Table 3).[27] One approach to clinically implementing these strategies for metabolic detoxification involves initiating the patient on an elimination diet high in whole fruits and cruciferous vegetables and low in animal protein. In addition to potassium, cruciferous vegetables contain myriad phytochemicals, such as indole-3-carbinol and sulforaphane, which are essential for facilitating toxin biotransformation.[28-30] Additionally, these vegetables can favorably alkalize urine pH. In a pilot trial with 5 volunteers, we found that a 200 g serving of cooked broccoli, carrots, and cauliflower (with broccoli as the predominant vegetable) resulted in an increase in urine alkalinization for up to 4 hours afterwards (baseline pH = 6.20 ± 0.51; after vegetables = 6.91 ± 0.45, *P*=.01). Thus, the simple instruction to alter diet to include cruciferous vegetables can promote detoxification by upregulating phase II enzymes and by alkalizing urine, resulting in enhanced excretion of toxins.

TABLE 3 Potassium Content of Selected Foods[27]

Food	Serving	Potassium (mg)
Potato, baked with skin	1 medium	721
Prunes, dried	½ cup	633
Raisins	½ cup	598
Prune juice	6 fl oz	530
Lima beans, cooked	½ cup	478
Banana	1 medium	467
Acorn squash, cooked	½ cup (cubes)	448
Tomato juice	6 fl oz	400
Orange	1 medium	237

Moreover, alkalinization during metabolic detoxification may be particularly useful, as it is believed that cellular pH and the blood buffering system shift to the acid side of ideal pH reserve during detoxification due to increased circulation of xenobiotics and organic acids (eg, glucuronic acid). Furthermore, organic cation transporters that are responsible for the transport of xenobiotics in and out of the cell are pH-sensitive.[31,32]

ALKALIZING AGENTS

In addition to dietary changes, nutritional supplementation for a short-term course of 3 to 4 weeks with select botanicals can facilitate metabolic detoxification. It would be appropriate to include specific alkalizing agents, such as potassium, within this nutritional regimen (Table 3). Unfortunately, the mainstream American diet is poor in potassium, as it often lacks sufficient fruits and vegetables. The adequate intake (AI) established by the Food and Nutrition Board of the Institute of Medicine for potassium is 4.7 g daily,[7] which is the same amount that is encouraged by the Dietary Approaches to Stop Hypertension (DASH) diet to maintain lower blood pressure levels, decrease the effects of salt intake, decrease the risk of kidney stones, and possibly reduce the incidence of bone loss. Current median intakes of potassium in the United States are roughly 35% and 50% below the AI for men and women, respectively.[7] African Americans would particularly benefit from increased potassium intakes due to their relatively low potassium intakes and high prevalence of elevated blood pressure and salt sensitivity.[7] For the healthy population, intake of potassium at levels higher than the AI is not of particular high risk due to the ability of the kidney to excrete excess amounts.[7] However, potassium intakes should be closely monitored for patients with acute or chronic renal failure and pre-existing heart disease and for those on medications that increase potassium reserves in the body, such as potassium-sparing medications.[7]

Various potassium salts are available to alter urine pH. Studies using sodium bicarbonate administration reveal little effect on urinary calcium excretion in contrast to studies that used potassium bicarbonate or potassium citrate supplementation and found significant reductions.[33,34] Potassium citrate, a therapeutic regimen to prevent kidney stones, can effectively alkalize urine. Doses of 4 to 8 g daily for 2 weeks in patients with homozygous cystinuria have effectively alkalized urine.[35] Additionally, there are a number of studies on the use of potassium citrate to counteract bone resorption caused by chronic acidemia of protein-rich diets.[36-38]

The effects of potassium depend on its accompanying anion.[7] Potassium chloride, commonly used in processed food products, does not appear to have the same alkalizing ability as potassium citrate.[7] In a recent study, Jehle et al demonstrated that potassium citrate was more efficacious than potassium chloride in increasing bone mineral density in postmenopausal women with osteopenia.[39] Furthermore, potassium chloride led to decreased bone mineral density in the lumbar spine. Potassium citrate supplementation in these subjects resulted in a sustained and significant reduction in urinary calcium excretion and an increase in urinary citrate excretion, indicating that alkalinization had occurred.[39,40]

Additionally, the citrate anion may be especially relevant for detoxification since it is an intermediate of the Krebs cycle and can potentially play a role in energy production. As many clinicians acknowledge from their experience, lack of energy is a common side effect of the first stages of metabolic detoxification.

Therefore, eating foods that are high in citrate, such as certain fruits and vegetables, may be beneficial. It is also worth noting that citrate is metabolized to bicarbonate in the body, thereby further adding to the buffering potential.[7]

SUMMARY

In conclusion, the increasing dietary acid load in the contemporary diet can lead to a disruption in acid-alkaline homeostasis in various body compartments and eventually result in chronic disease through repeated borrowing of the body's alkaline reserves. Adjustment of tissue alkalinity, particularly within the kidney proximal tubules, can lead to the more effective excretion of toxins from the body. Metabolic detoxification using a high vegetable diet in conjunction with supplementation of an effective alkalizing compound, such as potassium citrate, may shift the body's reserves to become more alkaline.

Acknowledgments

Special thanks to those individuals who reviewed this manuscript, including Gary Darland, PhD; Bhaskar Shenai, PhD; Robert Lerman, MD, PhD; and Barbara Schiltz, MS, RN, CN.

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Anticancer Effect of Alkaline Reduced Water

Kyu-Jae LEE[1,2], Seung-Kyu PARK[1,2], Jae-Won KIM[1], Gwang-Young KIM[1], Young-Suk RYANG[5], Geun-Ha KIM[1], Hyun-Cheol CHO[3], Soo-Kie KIM[2,3], and Hyun-Won KIM[2,4]

[1] Dept. of Parasitology, [2] Institute of Basic Medical Sciences, [3] Dept. of Microbiology, [4] Dept. of Biochemistry, Wonju College of Medicine, Yonsei Univ. (Wonju , Korea)

[5]Dept. of Biomedical Laboratory Science and Institute of Health Science, College of Health Science, Yonsei Univ. (Wonju , Korea)

Abstract: Certain minerals can produce alkaline reduced water with high pH and low oxidation-reduction potential (ORP) when dissolved in water. Alkaline reduced water (ARW) showed significant anticancer effect. When B16 melanoma cells were inoculated subcutaneously and intra-peritoneally, C56BL/6 mice fed with ARW showed tumor growth delay and the survival span was significantly lengthened. ARW also showed the inhibition of metastasis by reducing the numbers of B16 melanoma colonies when injected through tail vein. The amount of reactive oxygen species (ROS) was very reduced when fed with ARW except for spleen, which is a major organ for immunity. Even for normal mice, ARW intake invoked systemic cytokines, such as, Th1 (IFN-γ, IL-12) and Th2 (IL-4, IL-5), suggesting strong immuno-modulation effect. Both ROS scavenging effect and immuno-modulation effect might be responsible for anticancer effect of alkaline reduced water.

Keyword: alkaline reduced water, anticancer effect, antioxidant, immuno-modulation

1. Introduction

Reactive oxygen species (ROS) or free radicals are one of the major offenders to render oxidative damage to biological macromolecules. These unstable ROS are known to cause or aggravate a variety of incurable diseases such as cancer, cardiovascular diseases, neuro-degenerative diseases as well as aging [1, 2].

The cellular radial-scavengers such as superoxide dismutase, catalase, glutathion peroxidase are natural defense system against ROS. External source of antioxidative protection include antioxidant vitamins C and E, carotene and carotenoids as well as minerals such as selenium and zinc. Great efforts have been made in an attempt to find safe and potent natural antioxidants.

Water consists 70% of Human body. Water reaches every tissue of human body within 30 minutes after drinking. It even flows through blood brain barrier with no obstacle, and has almost no side effect. If water itself could work as a radical scavenger, it would be an ideal antioxidant [3].

Recently, electrolyzed-reduced water with high pH and significant negative redox potential (ORP) was shown to have SOD-like activity and catalase-like activity, and thus, scavenge active oxygen species and protect DNA from damage by oxygen radicals in vitro [4].

We developed a mineral combination to produce alkaline reduced water (ARW) with high pH and low ORP similar to electrolyzed-reduced water. The mineral combination was easy to carry and less expensive than the system to produce electrolyzed-reduced water.

Present article demonstrates anticancer effect of alkaline reduced water in animal model.

Hyun-Won KIM, ph.D., Dept. of Biochemistry, Wonju College of Medicine, Yonsei Univ., Wonju 220-701, Korea.
Phone +82-33-741-0283, Fax. +82-33-743-0411
E-mail kimhwbio@wonju.yonsei.ac.kr

2. Materials & Methods

Alkaline Reduced Water (ARW)

ARW was made by putting mineral combinations into water bottle. The pH of water was increased up to 10.5 and ORP was decreased until -200mv. The mineral contents of ARW were also increased time dependently.

Animals and Cells

C57BL/6 mice (4-5 weeks old) were obtained from the Daehan Bio Link Co., Ltd. (Chungbuk, Korea), and maintained on standard chow and tap water until taking ARW. Mouse B16 melanoma cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 95% air-5% CO_2 at 37°C. The high-metastatic B16 cell line was isolated from the lung of C57BL/6 mouse, which had been inoculated intraperitoneally with B16 cells. Cells were cloned by the limiting dilution methods and selected cloned were inoculated into mice. This method was repeated thrice, and the final clone with high metastastic potential was obtained as B16-BL6 melanoma cells.

In Vivo Evaluation of Antimetastatic Effect

Cultured B16-BL6 melanoma cells were harvested with 2 mM EDTA in PBS, and then washed three times with PBS. 1×10^6 B16-BL6 melanoma cells were intravenously injected in tail vein of control and ARW-administered C57BL/6 mice. After 20 days, mice were sacrificed and the lungs were collected to count the colonies of metastasized B16-BL6 melanoma cells.

In Vivo Suppression of Tumor Growth

Cultured B16-BL6 melanoma cells were harvested with 2 mM EDTA in PBS, then washed three times with PBS. 1×10^6 B16-BL6 melanoma cells were subcutaneouly injected on the back of C57BL/6 mouse. The length of long and short axis of tumor were measured every day and the volumes were calculated using the formula $ab^2/2$, where a is the length of long axis and b the length of short axis. Survival curve was plotted using Kaplan-Meier method.

Cytokine ELISA

Concentrations of cytokines from mice sera were measured by conventional sandwich ELISA method. A coating and biotinylated capture antibody was purchased form Pharmingen (USA): clones JES5-2A5 and JES5-16E3 for IL-4, and clones R4-6A2 and XMG1-2 for IL5, respectively. Coating on 96-well microtiter plate was performed with 250 g coating antibody. Sera sample, biotinylated capture antibody, secondary antibody and then streptavidinylated HRP was sequentially added, incubated and washed. o-phenylenediamine solution was added for development and 2N sulfuric acid for stop the development. The absorbance at 490 nm was measured with ELISA reader at 490 nm.

ROS Assay

Quantitation of cytosolic ROS was measured by oxidation method of 2',7'-dichlorofluroscein-diacetate (DCF-DA) as described in elsewhere[5]. 12.5 M DCFDA was incubated with liver, spleen, lung, or brain homogenate and change of fluorescence was measured at 485 nm of excitation wavelength and 585 nm of emission wavelength. The relative fluorescence unit (RFU) was calculated to 1 mg protein in homogenate.

3. Results

Effect of ARW on tumor growth and survival time

Tumor growth was significantly reduced in ARW fed group. After 10th days from subcutaneous injection of B16 melanoma cells into flank of C57BL/6 mice, tumor mass was palpable and thereafter serially measured. At 10 days tumor size was 0.27 cm^3 for ARW fed group, while that of tap water treated group was 0.48 cm^3. At 19th day tumor size was 3.32 cm^3 for ARW fed group, and 6.02 cm^3 for control group, showing 54% inhibition.

Survival rate was also monitored after intraperitoneal injection of B16 melanoma cells to C57BL/6 mice. ARW lengthened mean survival time from 36 days for control group to 44 days for ARW fed group.

Evaluation of antimetastatic activity of ARW on lung metastasis of B16

After intravenous injection of B16 melanoma cells to C57BL/6 mice through tail vein, antimetastatic activity of ARW was evaluated. 15 days after injection, mice were sacrificed. Their lung tissues were removed, and the metastatic lesions were compared. ARW fed group had fewer metastatic lesions. 257 Black colonies were counted on the lungs of ARW group and 145 black spots were shown in the control group, indicating 44% inhibition against metastasis of melanoma cell.

As melanoma cell is know to exhibit increased oxidative stress that could support the progression of metastasis, concentration of ROS was also measured for each organ of the same B16 melanoma injected mice using DCFH-DA (**Fig. 1**). Amount of ROS for lung, liver, and kidney were very low in ARW fed mice compared to that of control. However, the spleen, which is a major organ for immunity, shows very high ROS in ARW fed group. This might suggest the immune boosting effect of ARW.

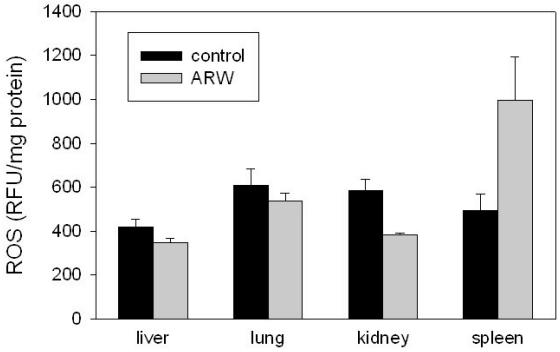

Fig. 1. Effect of ARW on ROS scavenging in mice.

Evaluation of immuno-modulating effect of ARW

ARW intake invoked systemic cytokines, such as, Th1 (IFN-γ, IL-12), cytokines for cellular immunity and Th2 (IL-4, IL-5), cytokines for humoral immunity (**Fig. 2**). This suggests that ARW stimulates both cellular and humoral immunity. Th1 and Th2 reached maximal peak after 2 week of ARW feeding and returned back to baseline. Cytokines levels of tap water fed control remained at the baseline.

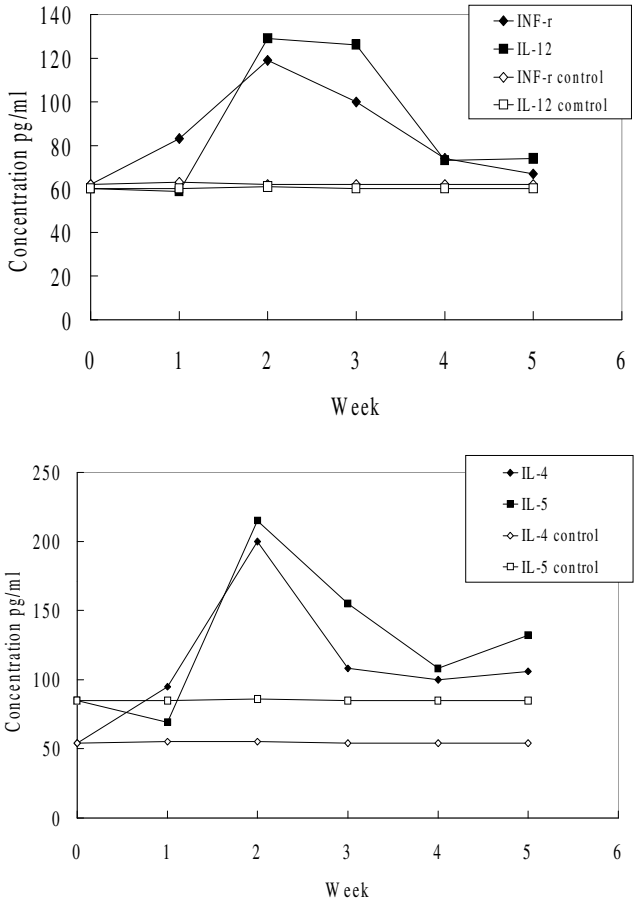

Fig. 2. Time kinetics in serum concentrations of Th1 and Th2 cytokines in ARW or control mice injected with B16-BL6 melanoma cells.

4. Discusssion

Recently, electrolyzed-reduced water with high pH and significant negative redox potential was shown to have SOD-like activity and catalase-like activity, and thus, scavenge ROS and protect DNA from damage by oxygen radicals *in vitro*. If ARW is actually acting as antioxidants and protects DNA from damage, it might be hypothesized that ARW intake would be possible to show anticancer effect.

Present investigation demonstrates the anticancer effect of ARW. ARW intake slowed down tumor growth, and inhibited the intravenous metastasis, leading to prolonged survival span in B16 melanoma injected mice. B16 melanoma cell is one of the most frequent tumors in humans and is characterized by its high capacity for invasion and metastasis[6, 7]. They escape from immune surveillance and spread more rapidly than any other tumors using several mechanisms including MHC down-regulation, increasing reactive oxygen species (ROS) levels, and thus, expedite the progression of metastasis[8]. Our study demonstrated that ARW not only acts as an antioxidant but also acts as a strong immnuo-moduator, both of which could be responsible for the anticancer effect. Thus, ARW is expected to be effective for the various diseases resulting from low immunity and/or high reactive oxygen species as well as for the prevention of cancer.

ARW not only has high pH and low ORP, but also contain some magnesium ion. Recently, magnesium was shown to be effective for the prevention of various diseases[8]. We cannot exclude the possibility that the magnesium in ARW might have contributed to the anticancer effect.

Water reaches every tissue of human body within 30 minutes after drinking. It even flows through blood brain barrier with no obstacle, and has almost no side effect. Taking ARW could be an ideal way to maintain health.

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Effect of Electrolyzed Water on Wound Healing

Artificial Organs 2000; 24:984-987

*Naoki Yahagi, *Masashi Kono, *Masaki Kitahara,
*Akito Ohmura, †Osao Sumita,
‡Toshimasa Hashimoto, ‡Katsuaki Hori,
‡Chen Ning-Juan, §Paul Woodson, ¶Shoji Kubota,
**Arata Murakami, and **Shinichi Takamoto,
*Department of Anesthesiology, Teikyo University Mizonokuchi Hospital; †Coherent Technology Inc.; ‡Faculty of Engineering, Tokyo Institute of Technology; §Basic Sciences Department, Kenmore Bastyr University; ¶Water Design Laboratories, and **Department of Thoracic Surgery, University of Tokyo, Tokyo, Japan

Abstract: Electrolyzed water accelerated the healing of full-thickness cutaneous wounds in rats, but only anode chamber water (acid pH or neutralized) was effective. Hypochlorous acid (HOCl), also produced by electrolysis, was ineffective, suggesting that these types of electrolyzed water enhance wound healing by a mechanism unrelated to the well-known antibacterial action of HOCl. One possibility is that reactive oxygen species, shown to be electron spin resonance spectra present in anode chamber water, might trigger early wound healing through fibroblast migration and proliferation.

Key Words: Wound healing— Electrolyzed water—Anode water—Cathode water—Hypochlorous acid.

Electrolyzed water, in a variety of forms and made by a variety of different processes, is widely used in

Japan as a topical disinfectant (1,2). We describe here our findings that some types of electrolyzed water appear to accelerate the healing of full-thickness cutaneous wounds in rats.

Materials and methods

Six different types of water were made and tested. The first, ultrapure water (resistivity $\simeq 18\ M\Omega \cdot cm$), was produced by a sequence of treatments applied to city tap water: charcoal filtration, reverse osmosis, and ion exchange. One group of experimental animals was treated with this ultrapure, nonelectrolyzed water. Four types of electrolyzed water were made from this ultrapure water. In each case, the water was electrolyzed (voltage gradient 13 V and apparent current density 200 mA/cm^2) while being pumped (500 ml/min) through a 3 chamber device (Coherent Technology, Tokyo, Japan) (3). One chamber contained a platinum-plated titanium anode. Usually, the middle chamber was filled with a saturated sodium chloride solution made with the ultrapure water. The third chamber contained the cathode, also made of platinum-coated titanium. Proprietary ion-exchange membranes separated the chambers. The following 3 types of electrolyzed water were made using the Coherent Technology device in this configuration (all measurements made at 25°C). The first, acid pH water, Ac(+), was taken from the anode chamber [pH 2.50–2.63, oxidation-reduction potential (ORP) 1104–1191 mV, concentration of residual chlorine (determined by the o-toluidine method) 80 to 100 ppm]. Neutral pH (7.40) anode chamber water, N(+), was made by adding NaOH (1N) to the electrolyzed water taken from the anode chamber [pH 7.4, ORP 749–784 mV, concentration of residual chlorine almost the same as in the Ac(+) solution]. Alkaline pH water, Al(−), was taken from the cathode chamber (pH 10.65–10.85, ORP 212–297 mV, residual chlorine only a few ppm). Replacing the platinum cathode in the Coherent Technology device by one made of carbon and replacing the center-chamber solution by 5 *M* citrate (organic acid; citric acid) in ultrapure water allowed us to make a fourth type of electrolyzed water. This acidic water [Ac(−)] was taken from the cathode chamber (pH 3.86–3.87, ORP 212–297 mV, no residual chlorine). Finally, hypochlorous acid (HOCl) solution was made by electrolyzing 0.45% NaCl in a single-chamber device (Omuko Co. Ltd., Tokyo, Japan) (Voltage gradient, apparent current density, and pump rate were the same as for the Coherent Technology device.). The pH and ORP of this solution were 7.45 and 780 mV, respectively. When stored in PET bottles, the pH and ORP of all 6 types of water remained stable for at least a month.

Forty-two Wistar rats (8 weeks old) were randomly assigned to 6 experimental groups, housed in individual metabolic cages at 25°C, and fed rodent chow and water ad libitum. Under pentobarbital anesthesia, the back was shaved and two 1.0 cm square, full-thickness cutaneous wounds were made, one behind the other and 1.5 cm apart, on the back of each animal. In each rat, 1 wound (selected randomly) was treated twice a day for 7 days with 1 of the 6 types of water described previously; the other wound was left untreated. The rat was observed carefully until the water was absorbed by the wound to ensure that no spillage occurred. The first treatment was administered immediately after surgery. All wounds were allowed to heal without dressings. For 17 days after surgery, wound areas were measured daily by planimetry, using digital video camera images displayed via a personal computer.

Acid and neutralized anode waters were studied by electron spin resonance (ESR; JEOL-JES-RE2X; Nihon Denshi, Tokyo, Japan) with the addition of a spin trapping agent [5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Sigma, St. Louis, MO, U.S.A.)] using a flat cell of 1 mm width (4). This was carried out 24 hr after the preparation of the electrolyzed water. The duration of the incubation of the electrolyzed water with DMPO was 2 min. The magnetic field was of 335 ± 5 mT. Signal strength was compared with the signal derived from Mn^{2+} (as a control).

All protocols were approved by the Animal Research Review Committee of Teikyo University Medical School.

For each group, the results are presented as mean wound area ± SD. The comparison between the areas of water-treated and nontreated wounds was performed using a paired Student's *t* test. The comparisons among the experimental groups were made using a one-way analysis of variance. When statistical significant was detected, Dunnett's test was used to determine which values differed significantly from those obtained using the nonelectrolyzed ultrapure water. Values of $p < 0.05$ were considered significant.

Results

As shown in Table 1, both types of anode chamber water [acidic and neutral: Ac(+) and N(+), respectively] accelerated wound healing (when compared to the effect of nonelectrolyzed ultrapure water) ($p < 0.05$). This acceleration of wound healing was evident from as early as postoperative Day (POD) 1 in Ac(+) or POD 2 in N(+). The alkaline water from the cathode chamber [Al(−)] showed a slight, but

TABLE 1. *Comparison between the areas of water-treated and nontreated wounds, and comparison between experimental groups*

POD	0	1	2	3	4	5	7	9	11	14	17
		b	*b*		*b*	*b*	*b*	*b*	*b*	*b*	*b*
Water (n = 7)											
Mean	1.0399	1.3117	1.3082	1.1325	1.0894	0.8760	0.6588	0.3165	0.2280	0.1525	0.0839
SD	0.0777	0.1889	0.1559	0.2135	0.1194	0.1133	0.1203	0.0348	0.0801	0.0159	0.0202
NT											
Mean	1.0931	1.1958	1.1007	1.0242	0.9849	0.8509	0.6558	0.3208	0.2000	0.1685	0.0795
SD	0.0839	0.2697	0.2186	0.2407	0.1832	0.1154	0.1554	0.1189	0.0664	0.0609	0.0416
Ac(+) (n = 9)		*a,c*	*a*	*a*	*a,c*	*a,c*	*a,c*	*a,c*	*a,c*	*a,c*	*a,c*
Mean	1.0347	0.9213	1.0904	0.9001	0.7937	0.6248	0.3709	0.1597	0.0878	0.0225	0.0040
SD	0.1105	0.2005	0.2187	0.1868	0.1832	0.1578	0.1783	0.0868	0.0330	0.0225	0.0083
NT											
Mean	1.0876	1.1913	1.3229	1.2836	1.1969	1.0530	0.7255	0.3509	0.2114	0.1225	0.0430
SD	0.1815	0.2506	0.3939	0.3362	0.3535	0.2524	0.2738	0.1552	0.0967	0.0399	0.0351
N(+) (n = 6)		*a*	*a*	*a*	*a,c*	*a,c*	*a,c*	*a,c*	*a,c*	*a,c*	*a,c*
Mean	1.0457	1.1577	0.9918	0.8878	0.7846	0.6214	0.3754	0.1960	0.1122	0.0230	0.0151
SD	0.1076	0.2757	0.1751	0.1926	0.1775	0.1438	0.1273	0.0957	0.0700	0.0239	0.0371
NT											
Mean	1.0972	1.4260	1.3473	1.3465	1.2990	1.1729	0.8549	0.4755	0.3371	0.1447	0.0811
SD	0.0494	0.2773	0.1889	0.1699	0.1379	0.1614	0.2917	0.1421	0.1781	0.1191	0.0719
Al(−) (n = 6)		*c*			*a*	*a*				*c*	*c*
Mean	1.0151	0.9611	0.9970	0.8861	0.8052	0.7372	0.4776	0.2692	0.1221	0.0368	0.0305
SD	0.0595	0.1277	0.0876	0.1099	0.1335	0.1400	0.1461	0.1524	0.0280	0.0227	0.0251
NT											
Mean	1.0488	1.0272	1.1619	1.0939	1.0963	0.9781	0.7044	0.3958	0.2025	0.1406	0.0720
SD	0.0643	0.1376	0.3234	0.3316	0.2564	0.2191	0.2431	0.1335	0.0843	0.0510	0.0442
Ac(−) (n = 6)			*c*								
Mean	0.9723	1.1173	0.9656	0.9082	0.8789	0.7429	0.5517	0.2316	0.1471	0.0979	0.0763
SD	0.0642	0.2219	0.3055	0.2881	0.2423	0.2130	0.2021	0.0842	0.0584	0.0482	0.0311
NT											
Mean	0.9374	1.0732	1.0655	1.0394	1.0151	0.8215	0.6636	0.3369	0.1941	0.1110	0.0795
SD	0.0682	0.1278	0.1326	0.1958	0.2027	0.1632	0.1818	0.1109	0.0440	0.0487	0.0318
HOCl (n = 8)											*c*
Mean	1.1368	1.1986	1.2423	1.1403	0.9823	0.8702	0.6797	0.3585	0.1802	0.0961	0.0509
SD	0.1941	0.2300	0.3154	0.2580	0.2814	0.2474	0.2628	0.1830	0.1005	0.0563	0.0407
NT											
Mean	1.1909	1.1885	1.3253	1.3231	1.2036	1.1455	0.8468	0.3892	0.1901	0.1488	0.0660
SD	0.2448	0.3144	0.3970	0.3555	0.3412	0.2983	0.2766	0.1555	0.1103	0.1155	0.0559

[a] $p < 0.05$ vs the area of the NT wound (paired Student's *t* test).
[b] $p < 0.05$ among the groups (one-way ANOVA).
[c] $p < 0.05$ versus water (Dunnett).
Values are cm^2. POD: postoperative day (wounds were made on Day 0), Water: nonelectrolyzed pure water, NT: nontreated wounds, Ac(+): acid pH anode water, N(+): neutral pH anode water, Al(−): alkaline pH cathode water, Ac(−): acid pH cathode water, HOCl: hypochloric acid.

insignificant, tendency to increase healing (compared with the ultrapure water), but the acidic water from the cathode chamber [Ac(−)] and the HOCl solution were both ineffective. All the wounds in all animals were free from signs of infection.

As shown in Fig. 1, no significant ESR spectrum was observed for untreated acidic anode chamber water. However, when we added a small amount of ferric salt to the anode water (5) to mimic the in vivo experiment, 4 lines suggestive of hydroxyl radicals were observed in the spectrum. Similar ESR spectra were observed for neutralized anode chamber water (in the absence or presence of ferric salt, respectively).

Discussion

The bactericidal action of various types of anode chamber water in the past has been attributed to their HOCl content (1). Since our HOCl solution was without effect on wound healing, we suggest that the anode chamber waters tested here may have a mode of action unrelated to any antibacterial HOCl produced by the electrolysis. One possibility is that free radicals, generated in the anode chamber waters by contact with the wound (Fig. 1), may be responsible for the very early effect on wound healing seen in this model. This interpretation is supported by the known effects of free radicals on inflammation as well as by what is known of the role of inflammation

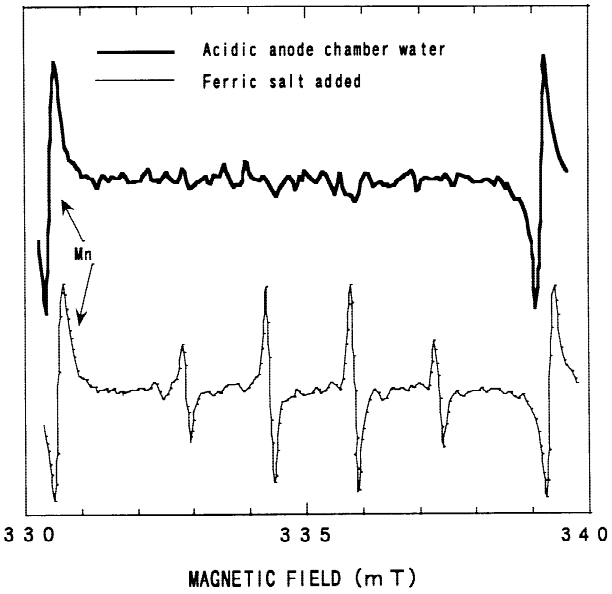

FIG. 1. Shown are electromagnetic spectra of acidic anode chamber water. The upper line is for untreated acidic anode chamber water. There was no significant ESR spectrum. Note that the lower line, the spectrum of the same water with ferric salt added, shows 4 lines suggestive of hydroxyl radicals.

in the initiation of healing (6) in this model. In our study, differences in pH could not account for the significant difference in the acceleration of wound healing between acidic (pH about 2.5) and neutralized (pH 7.4) anode chamber waters on the one hand, and acidic cathode chamber water (pH about 3.8) on the other. Furthermore, there was no difference in pH or ORP between neutralized anode chamber water (pH 7.4, ORP 749–784 mV) and HOCl (pH 7.45, ORP 780 mV). Thus, ORP presumably did not substantially affect wound healing in our experiment.

Reactive oxygen species were shown to affect DNA synthesis and proliferation in fibroblasts. A low level of these species stimulates DNA synthesis and cell division while a high level inhibits DNA synthesis (the cytotoxicity induced being proportional to the level of reactive oxygen species) (7–9). Moreover, in small amounts, reactive oxygen species were shown to be involved in the mechanism underlying fibroblast chemotaxis into sites of injury or inflammation (10).

To our knowledge, there is no report clearly explaining the direct beneficial effect of HOCl on wound healing. HOCl generates not only chlorine gas, which is the main cause of the bactericidal effect of HOCl, but also singlet oxygen, which is a reactive oxygen species whose physiological effect is still unknown (11). Kozol et al. demonstrated that sodium hypochlorite had toxic effects on wound modules (e.g., on neutrophils, fibroblasts, and endothelial cells even at dilute concentrations of 2.5×10^{-2}% to 2.5×1^{-4}% (12). Furthermore, wounds created on mouse ears and treated with 0.25% sodium hypochlorite showed delayed epithelialization and neovascularization (13). This might be the reason that HOCl was without effect in our wound healing model.

In conclusion, acid and neutral anode chamber waters accelerated the healing of full-thickness cutaneous wounds in rats. This effect might be caused by the generation of reactive oxygen species; however, further study is necessary for the elucidation of the mechanism underlying the effects of electrolyzed water on wound healing.

Acknowledgement: The authors owe grateful thanks to Dr. Yuko Nishimoto for valuable discussion.

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Inhibitory Effect of Electrolyzed Reduced Water on Tumor Angiogenesis

Jun YE,[a,b] Yuping LI,[a,c] Takeki HAMASAKI,[d] Noboru NAKAMICHI,[e] Takaaki KOMATSU,[d] Taichi KASHIWAGI,[d] Kiichiro TERUYA,[a,d] Ryuhei NISHIKAWA,[d] Takeshi KAWAHARA,[d] Kazuhiro OSADA,[d] Kazuko TOH,[d] Masumi ABE,[d] Huaize TIAN,[d] Shigeru KABAYAMA,[f] Kazumichi OTSUBO,[f] Shinkatsu MORISAWA,[f] Yoshinori KATAKURA,[a,d] and Sanetaka SHIRAHATA*,[a,d]

[a] *Graduate School of Systems Life Sciences, Kyushu University;* [d] *Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University; 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812–8581, Japan:* [b] *Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Science, Xiamen University; Fujian, 361005 P.R. China:* [c] *School of Life Sciences, Nanchang University of Science and Technology; Nanchang 330006, P.R. China:* [e] *Functional Water Cell Analysis Center Co., Ltd.; Fukuoka 812–0053, Japan: and* [f] *Nihon Trim Co., Ltd.; 1–8–34 Oyodonaka, Kita-ku, Osaka 531–0076, Japan.*

Received June 9, 2007; accepted October 16, 2007; published online October 17, 2007

Vascular endothelial growth factor (VEGF) is a key mediator of tumor angiogenesis. Tumor cells are exposed to higher oxidative stress compared to normal cells. Numerous reports have demonstrated that the intracellular redox (oxidation/reduction) state is closely associated with the pattern of VEGF expression. Electrolyzed reduced water (ERW) produced near the cathode during the electrolysis of water scavenged intracellular H_2O_2 and decreased the release of H_2O_2 from a human lung adenocarcinoma cell line, A549, and down-regulated both VEGF transcription and protein secretion in a time-dependent manner. To investigate the signal transduction pathway involved in regulating VEGF expression, mitogen-activated kinase (MAPK) specific inhibitors, SB203580 (p38 MAPK inhibitor), PD98059 (ERK1/2 inhibitor) and JNKi (c-Jun N-terminal protein kinase inhibitor) were applied. The results showed that only PD98059 blocks VEGF expression, suggesting an important role for ERK1/2 in regulating VEGF expression in A549 cells. As well, ERW inhibited the activation of extracellular signal-regulated kinase (ERK) in a time-dependent manner. Co-culture experiments to analyze *in vitro* tubule formation assay revealed that A549 cell-derived conditioned medium significantly stimulated the formation of vascular tubules in all analyzed parameters; tubule total area, tubule junction, number of tubules, and total tubule length. ERW counteracted the effect of A549 cell-conditioned medium and decreased total tube length ($p<0.01$). The present study demonstrated that ERW down-regulated VEGF gene transcription and protein secretion through inactivation of ERK.

Key words electrolyzed reduced water; angiogenesis; oxidative stress; vascular endothelial growth factor; extracellular signal-regulated kinase; A549 cell-conditioned medium

Tumor angiogenesis, the formation of new blood capillaries by vascular endothelial cells from existing vessels, is an important mechanism for supplying nutrients, oxygen, growth factors and others to tumor cells. Tumor cells trigger angiogenesis by secreting angiogenic factors, especially vascular endothelial growth factor (VEGF-A),[1] which plays an important role in the regulation of normal and abnormal angiogenesis.[2]

VEGF-A (commonly known as VEGF) was first reported as a vascular permeability-inducing factor secreted by tumor cells, and referred to as vascular permeability factor (VPF).[3] VEGF gene expression is initiated by extracellular signals including growth factors, mitogens, phorbol ester, cytokines and extracellular stresses. The first three of these exogenous signals activate the Ras-Raf-MEK-ERK pathway that transduces mitogenic signals regulating cell proliferation or differentiation. The other extracellular signals activate the JNK/SAPK and p38 pathways that regulate cellular inflammatory or stress responses.[4] VEGF is overexpressed at both mRNA and protein levels in a high percentage of malignant animal and human tumors, as well as in many immortalized and transformed cell lines.[5—7] The VEGF-A gene transcript undergoes alternative splicing to yield mature isoforms of 121, 165, 189, and 206 amino acids, with $VEGF_{165}$ appearing to be quantitatively and functionally predominant in most angiogenic states.[8] $VEGF_{121}$ and $VEGF_{165}$ are secreted as soluble compounds, whereas $VEGF_{189}$ and $VEGF_{206}$ remain cell surface associated or are primarily deposited in the extracellular matrix.[9]

Reactive oxygen species (ROS) are suggested to play an important role in angiogenesis.[10] Furthermore, there is increasing evidence of the involvement of H_2O_2 in the regulation of angiogenesis.[9,11—13] As well, a variety of cell lines derived from human tumors has been shown to produce large amounts of H_2O_2.[14] Constitutive surveillance for cellular protection against oxidative stress is conferred by intracellular antioxidative agents.[15] Excess amounts of ROS are toxic and cause a reduction of intracellular antioxidant levels.[16] It has been reported that pretreatment of the heart with exogenous antioxidants improved its condition as a result of reducing ROS production.[17] The VEGF-A gene is one that has its expression regulated by ROS, especially by H_2O_2. Additional data support that VEGF-A mRNA is up-regulated by H_2O_2 in a dose- and time-dependent manner.[18,19] Taken together, these suggest that some endogenous as well as exogenous antioxidative agents can be used to regulate VEGF-A gene expression and/or H_2O_2 production for therapeutic purposes.

Electrolyzed reduced water (ERW) has attracted much attention because of its antioxidative potential. Water electrolysis typically produces two forms of water: reduced or alkaline (high pH) water near the cathode and an oxidized or acid (low pH) water near the anode. Applications of oxidized

* To whom correspondence should be addressed. e-mail: sirahata@grt.kyushu-u.ac.jp

water have frequently been reported.[20—22] In Japan, ERW produced from tap water by house-use electrolyzing purifiers is popular as it is thought to have health benefits. ERW has been shown to be clinically effective in the treatment of patients with irritable bowel syndrome or non-ulcer dyspepsia.[23] Shirahata *et al.* first demonstrated that ERW not only exhibited high pH, low dissolved oxygen, extremely high dissolved molecular hydrogen, but most importantly, showed ROS scavenging activity and protective effects against oxidative damage to DNA.[24] Thereafter, the inhibitory effects of ERW on alloxan-induced pancreatic cell damage[25] and on hemodialysis-induced oxidative stress in end-stage renal disease (ESRD) patients[26,27] were reported. Kim and Kim reported that ERW derived from tap water exhibited an anti-type 2 diabetic effect in animal experiments.[28]

Although the data accumulated so far suggest that ERW could be a useful antioxidative agent, further studies are required to elucidate the mechanisms of its actions in cells. To this end, we hypothesized that ERW could regulate VEGF-A gene expression to exert antiangiogenic effects *via* scavenging ROS, in particular H_2O_2. We carried out a series of experiments as a first step to uncover the mechanisms involved.

Here we present evidence that ERW attenuates both the release of H_2O_2 and the secretion of VEGF. This then leads to the suppression of angiogenesis induced by tumor cells.

MATERIALS AND METHODS

Preparation of Electrolyzed Reduced Water (ERW) ERW (oxidation reduction potential, −600 mV; pH 11) was prepared by electrolyzing ultra pure water containing 0.002 M NaOH at 100 V for 60 min using an electrolyzing device equipped with platinum-coated titanium electrodes (TI-200s, Nihon Trim Co., Osaka, Japan), and typically contains 0.2 ppb Pt Nps when assayed with ICP-MS spectrometer (unpublished data). A batch type electrolyzing device was used. It consisted of a 4-l vessel (190 mm length×210 mm width×140 mm height) divided by a semi-permeable membrane (190 mm width×130 mm height, 0.22 mm thickness, pore size is not disclosed, Yuasa Membrane System Co., Osaka Japan). Two electrodes (70 mm width×110 mm length) were placed at a distance of 55 mm from each side of the semi-permeable membrane.

Cell Culture and Reagents All electrolyzed alkaline ERW was neutralized by adding 1 ml of 10× minimum Eagle's medium (MEM) (pH 7) and 0.2 ml of 1 M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 5.3) to 9 ml of ERW (pH 11) before use. Human lung adenocarcinoma, A549 cells and human diploid embryonic lung fibroblast, TIG-1 cells were obtained from the Health Science Research Resources Bank and maintained in MEM supplemented with 10% fetal bovine serum (FBS) designated as 10% FBS/MEM (Biowest, France). During the experiments, A549 cells were cultured with MEM (no FBS) prepared by dilution of 10× MEM with Milli Q water which designated as serum-free MEM/Milli Q or cultured with MEM (no FBS) prepared by dilution of 10× MEM with ERW which designated as serum-free MEM/ERW. In a preliminary experiment done in the past, we had compared two MEM media prepared either with 0.002 M NaOH aqueous solution or with Milli Q water to examine whether MEM media with addition of NaOH could scavenge intracellular ROS or not, and such effect was not observed. Also, these MEM media were applied to human fibrosarcoma HT1080 cells and measured matrix metalloproteinase (MMP) gene expressions. We did not observe any difference in the levels of MMP expression between HT1080 cells cultured with the two MEM media (unpublished observation). Together with these observations and the knowledge that both MMP and VEGF are redox-sensitive genes, we judged that an addition of NaOH into culture media has no effect on intracellular redox state and related genes expression. We therefore used MEM media prepared with Milli Q water as a control in subsequent experiments.

Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex and cultured in EGM-2 medium (Cambrex, MD, U.S.A.). Homovanillic acid (HVA) and horseradish peroxidase type VI were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SB203580, PD98059 and c-Jun N-terminal protein kinases inhibitor (JNKi) were purchased from Calbiochem (CA, U.S.A.). The Quantikine kit (Human VEGF Immunoassay, Catalog Number DVE00) was obtained from R&D Systems, Inc. (Minneapolis, MN, U.S.A.). The Quantikine VEGF Immunoassay kit is designed to measure $VEGF_{165}$ levels in cell culture supernates. An Angiogenesis Tubule Staining Kit (for staining CD31) was obtained from TCS Cellworks (Buckingham, U.K.). Total and phospho-ERK mitogen-activated protein kinase (MAPK) antibody was purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.).

Measurement of Intracellular H_2O_2 Scavenging Activity by ERW H_2O_2 produced in A549 cells was measured using DCFH-DA. A549 cells were pretreated with serum-free MEM/ERW for 30 min, and then incubated with 5 μM DCFH-DA for 30 min at 37 °C. DCFH-DA diffused freely into cells and was then hydrolyzed by cellular esterases to DCFH, which was trapped within the cell. This non-fluorescent molecule was then oxidized to fluorescent dichlorofluorescein (DCF) by the action of intracellular H_2O_2. Cells were washed with phosphate-buffered saline (PBS, pH 7.4) to remove the DCFH-DA. H_2O_2 levels were measured using flow cytometry (EPICS XL System II; Beckman Coulter, U.S.A.) by determining the intensity of the fluorescence relative to that of control cells.

Measurement of H_2O_2 Release H_2O_2 release from A549 cells into the culture medium was assayed by a published method.[29] Briefly, A549 cells were cultured in a 24-well plate with serum-free MEM/Milli Q or serum-free MEM/ERW for 24 h. The cells were washed with PBS and then incubated with an 800 μl reaction buffer (100 μM HVA, 5 units/ml horseradish peroxidase type VI, and 1 mM HEPES in Hanks balanced salt solution without phenol red, pH 7.4). The reaction buffer without cells was treated in the same way, as a control. This solution was then collected after incubation for 30 min, pH was adjusted to 10.0 with 0.1 M glycine–NaOH buffer, and fluorescence was then measured using a fluorescence spectrophotometer (F-2500, Hitachi, Japan) at excitation and emission wavelengths of 321 nm and 421 nm, respectively.

Semiquantitative Reverse Transcription-Polymerase

Chain Reaction (RT-PCR) Total RNA was isolated using a GenElute™ Mammalian Total RNA isolation kit (Sigma Chemical Co., St. Louis, MO, U.S.A.) and following the protocol provided by the supplier. The primer sequences for glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) are 5′ACCACAGTCCATGCCATCAC3′ (forward) and 5′TCCACCACCCTGTTGCTGTA-3′ (reverse), which amplify a 512 bp segment (NCBI Acc#: NM 002046). The common primer sequences for VEGF transcripts are 5′GGGCCTCCGAAACCATGAAC3′ (forward) and 5′CTGGTTCCCGAAACCCTGAG3′ (reverse), which differentiate alternatively spliced $VEGF_{165}$ and $VEGF_{121}$ transcripts by generating 625 bp and 495 bp fragments, respectively.[8,30] PCR amplification for VEGF was carried out at 94 °C for 45 s of denaturing, annealing for 45 s at 60 °C, and extension for 1 min at 72 °C for 35 cycles using *Taq* polymerase (Takara). Likewise, PCR amplification for GAPDH was carried out at 94 °C for 3.5 min of denaturing, annealing for 30 s at 58 °C, and extension for 1 min at 72 °C for 30 cycles. The semi-quantitative RT-PCR products were not saturated under the conditions used in the present experiments. Amplified products were resolved by agarose gel electrophoresis and then photographed with a digital camera (ATTO, Tokyo). For densitometric analysis, recorded images were analyzed by an NIH image analyzer program (Image 1.62f) using a personal computer. Values below the panel were normalized by arbitrarily setting the density of the $VEGF_{165}$ and $VEGF_{121}$ bands of untreated A549 cells to 1.0. GAPDH transcripts were used as an internal control for cellular activity.

Measurement of VEGF Secreted into the Culture Medium A549 cells (5×10^4 cells/well) were seeded in 24-well plates with 10% FBS/MEM and cultured overnight. The medium was replaced with serum-free MEM/ERW and incubated for another 24 h. The conditioned medium was collected to measure secreted VEGF, which was measured according to the manufacturer's protocol.

Preparation of Conditioned Medium and Tubule Formation Assay A549 cells (1×10^6 cells) were seeded in a 90 mm dish with 10% FBS/MEM and incubated overnight. The medium was replaced with serum-free MEM/Milli Q or serum-free MEM/ERW and cultured for 24 h. The conditioned medium was collected and filtered with a 0.2 μm filter. Aliquots were stored in a −80 °C deep-freezer. Tubule formation assay was performed with a co-culture system. HUVEC were mixed with TIG-1 cells at 1 : 40, seeded in 24-well plates, and cultured in EGM-2 medium overnight. The medium was removed and a mixture of A549 cell conditioned and EGM-2 media mixed at 2 : 1 was added. The conditioned medium was changed every 2 d. Tubules formed with different media were detected with HUVEC-specific markers CD31 (PECAM-1). Briefly, at day 11, the medium was completely removed, and the co-culture plate was fixed for 30 min with 70% ethanol solution. After incubation with PBS containing 1% bovine serum albumin (BSA), the co-culture plate was incubated with a mouse anti-human CD31 antibody (1 : 4000) for 60 min, followed by another 60 min incubation with a secondary goat anti-mouse IgG antibody conjugated with alkaline phosphatase (both antibodies were included in the Tubule Staining Kit). After washing the culture plate, 5-bromo-4-chloro-3-indolylphosphate toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) substrate was added until tubules developed a dark purple color. Co-cultures were then dried and analyzed. Tubule formations in the co-culture system were observed by phase-contrast microscopy and photomicrographs were documented with a digital camera (Olympus, Japan). Recorded images were analyzed by Angiogenesis Image Analysis Software (AngioSys 1.0, TCS, Cellworks, U.K.). Twelve random fields per well were photographed for tubule formation assessment.

Western Blot Analysis Appropriately treated cells were washed with PBS, and incubated with extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM PMSF, 1% NP-40, 0.1% SDS, 10 μg/ml aprotinin and 10 mM EDTA) on ice. Cells were collected with a scraper. The lysate was then centrifuged at $12000\times\boldsymbol{g}$ for 5 min. Thirty micrograms of protein samples were boiled in a ratio of 3 : 1 with sample buffer (250 mM Tris–HCl pH 6.8, 40% glycerol, 20% β-mercaptoethanol, 8% SDS and 0.04% bromophenol blue), and electrophoresed in SDS-PAGE. Resolved proteins were then transferred onto Hybond-ECL membranes (Amersham Bioscience, U.K.), which were blocked with 0.05% Tween 20-PBS (T-PBS) containing 10% skim milk powder (Wako, Osaka, Japan) and probed with primary and secondary antibodies coupled with peroxidase. After washing three times with T-PBS, the bound antibody was developed using an ECL plus Western Blotting Detection System (Amersham Biosciences, U.K.).

RESULTS

ERW Scavenges Intracellular H_2O_2 and Decreases the Release of H_2O_2 from A549 Cells It has been reported that cancer cells produce high amounts of ROS, including H_2O_2,[14] and that exogenous ROS stimulates induction of VEGF in various cell types.[18,31] ERW has been shown to effectively scavenge intracellular ROS in HIT-T15 cells (a hamster pancreatic cell line).[25] These data together suggest that ERW might regulate VEGF expression by way of ROS. To test this idea and to ascertain if the ROS scavenging activity of ERW is applicable to other cell types, we began by examining the scavenging effect of ERW in A549 cells. A549 cells were treated with MEM containing ERW and then incubated with DCFH-DA. Intracellular H_2O_2 levels were measured using flow cytometry by determining the intensity of the fluorescence relative to that of control cells, as detailed in the Materials and Methods. The results showed a reduction of intracellular H_2O_2, as the signal curve obtained from ERW-treated A549 cells (designated as "ERW") was shifted to the left compared with untreated A549 cells (designated as "Control") (Fig. 1A). This shift of the signal curve would indicate scavenging of H_2O_2. Thus ERW was suggested to scavenge intracellular H_2O_2 in A549 cells. To test the ROS scavenging activity of ERW, we examined the effect of ERW on the release of H_2O_2 from A549 cells. Our test method was based on the conversion of homovanillic acid, a substituted phenol, to its fluorescent dimer in the presence of H_2O_2 and horseradish peroxidase. As shown in Fig. 1B, when A549 cells were pre-treated with ERW for 24 h, the release of H_2O_2 from A549 cells decreased to approximately 40% compared to non-treated control ($p<0.05$). Thus, the results confirmed a previous report.[25]

The present results from two different assays capable of

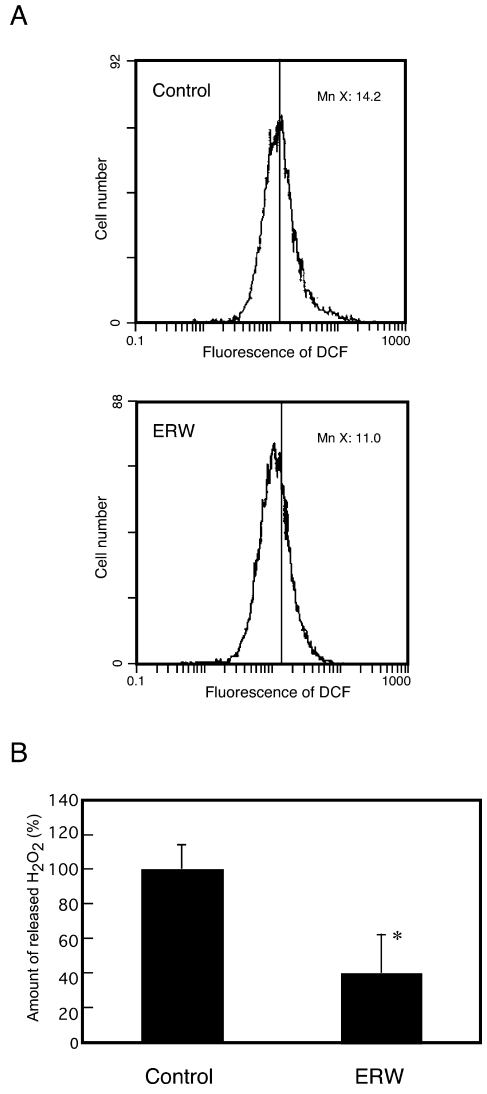

Fig. 1. Intracellular H_2O_2 Scavenging Activity of ERW (A) and Suppression of H_2O_2 Release from A549 Cells by ERW (B)

(A) Cultured A549 cells were pretreated for 30 min with 10% FBS/MEM/ERW, then incubated with 5 μM DCFH-DA for 30 min at 37 °C. The fluorescence intensity of DCFH was measured with a flow cytometer. The fluorescence intensity relative to that of control cells is presented as curves. The curve designated as "Control" is the fluorescence intensity obtained from control A549 cells. The curve designated as "ERW" is the fluorescence intensity obtained from A549 cells treated with ERW. H_2O_2 scavenging activity was judged positive, as the ERW-treatment curve (ERW) was shifted to the left compared with the control curve (Control). Mn X in the ERW and Control panels means the mean of fluorescence intensity. A representative result is shown from three independent experiments. (B) ERW was added to A549 cells in culture followed by further 24 h incubation. Released H_2O_2 in the culture media was measured as described in Materials and Methods. Differences were analyzed by Student's t test (values are the mean±S.D., n=3). An asterisk represents a significant difference compared with controls ($*p<0.05$) and p values of <0.05 are considered statistically significant.

measuring endogenous and exogenous H_2O_2 levels clearly demonstrated that ERW has the potential to reduce and/or scavenge H_2O_2.

ERW Inhibits Both VEGF Gene Expression and Extracellular Secretion in A549 Cells As we had confirmed that ERW reduces H_2O_2 production from A549 cells, we investigated using an RT-PCR method to determine if H_2O_2 and VEGF levels are coordinately regulated by ERW in A549 cells.

Primers were designed to amplify a 495 bp product for the $VEGF_{121}$ transcript and a 625 bp product for the $VEGF_{165}$ transcript. Agarose gel electrophoresis was performed to dissolve RT-PCR products (Fig. 2A). Ratios of band intensities between different incubation periods for GAPDH and those for the two VEGF isoform products were used to compare time dependent transcription levels (Fig. 2). The results showed that ERW treatment down-regulated transcriptions of $VEGF_{165}$ and $VEGF_{121}$ in a time-dependent manner. Notably, when the cells were treated with ERW for 24 h, VEGF transcription was significantly suppressed, while that of GAPDH changed little; indicating that the results were not due to the cytotoxic effects of ERW (Fig. 2A).

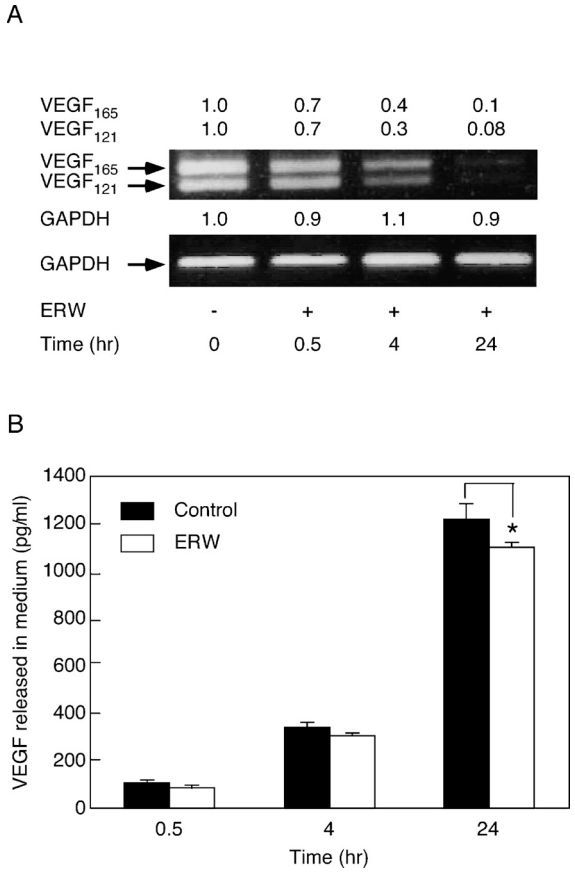

Fig. 2. ERW Down-Regulates VEGF Transcription and Secretion

(A) Four sets of A549 cells were treated with ERW for 0.5, 4 and 24 h. A549 cells treated for designated time periods were used to isolate total RNAs. VEGF and GAPDH transcripts were detected by RT-PCR with an appropriate set of primers, as shown in Materials and Methods. Values above the panel were normalized by arbitrarily setting the densitometry of $VEGF_{165}$ and $VEGF_{121}$ bands at time zero to 1.0. A GAPDH transcript was used as an internal control for cellular activity. (B) A549 (5×10^5 cells) cells/well were seeded in 24-well plates with 10% FBS/MEM for 0.5, 4 and 24 h. The medium was replaced with serum-free MEM/ERW for the indicated time periods. The medium was collected to measure an amount of VEGF secreted by A549 cells, as described in Materials and Methods. Filled columns (■), controls cultured in serum-free MEM/Milli Q; open columns (□), tests cultured in serum-free MEM/ERW. The results of 3 independent experiments were analyzed by Student's t test (values are the mean±S.D., n=3). An asterisk represents a significant difference compared with the control ($*p<0.05$) and p values of <0.05 are considered statistically significant.

VEGF is known to be secreted outside tumor cells to exert its angiogenic effect by stimulating proliferation and migration of endothelial cells.[18] Therefore, the effect of ERW on the secretion of VEGF in A549 cells was tested. The secretion of VEGF from control cells increased in a time-dependent manner, whereas ERW gradually suppressed the increase in the VEGF secretion (Fig. 2B). A significant difference in the secreted VEGF accumulations between control (1217.94±61.83 pg/ml) and treated samples (1095.53±21.50 pg/ml) was only observed when A549 cells were

treated with ERW for 24 h ($p<0.05$, Fig. 2B). This delayed response of VEGF protein secretion compared to VEGF gene transcription level, which after 4 h treatment reduced to approximately 30—40%, may be attributable to assay point differences, *i.e.*, transcription and accumulated protein levels because RT-PCR detects specific transcripts directly at specific time points while VEGF assay detects accumulated total $VEGF_{165}$ protein during the incubation periods indicated.

ERW Inactivates ERK1/2 VEGF gene transcription was demonstrated to be regulated by ERW, suggesting that its action point is at a gene transcription level and/or at signal transduction pathway levels upstream to transcription initiation complexes. As an initial step, the signal transduction pathway involved in regulating VEGF gene expression was investigated using MAPK specific inhibitors, SB203580 (p38 MAPK inhibitor), PD98059 (MEK inhibitor which is an upstream kinase of the ERK pathway) and JNKi (JNK inhibitor). For this purpose, the same RT-PCR assay system and analysis methods, as in Fig. 2A, were used to quantify VEGF transcripts (Fig. 3A). The results showed that only PD98059 blocked VEGF expression, suggesting an important role for the Ras-Raf-MEK-ERK pathway, particularly ERK1/2 factor, in regulating VEGF expression in A549 cells (Fig. 3A). Other inhibitors, SB203580 and JNKi, did not show any significant inhibitory effect on VEGF gene transcription, indicating that the JNK/stress activated protein kinase (SAPK) and p38 pathways were not directly involved in VEGF gene transcription.

Further experiments were performed to determine whether ERW-induced down-regulation of VEGF expression is due to the suppression of ERK1/2 phosphorylation. Western blot analyses showed that phosphorylation of ERK decreased in a time-dependent manner from 0.5 to 4 h. After 4 h, the phospho-ERK level remained low for up to 24 h, even after extended ERW treatment. The total amount of ERK MAPK protein was unaffected by ERW treatment (Fig. 3B). These results further strengthened the results shown in Fig. 3A. They also suggested that MEK is involved in the regulation of VEGF transcription via ERK phosphorylation.

Effect of ERW on Vascular Tubule Formation Induced by A549 Cells Exogenous ROS is known to stimulate VEGF production[18] and to promote tubular morphogenesis in endothelial cells.[32] To evaluate the effect of ERW on tubule formation, four parameters; tubule area, number of junctions, number of tubules, and total tube length, were measured. For this, a co-culture of HUVEC and TIG cells was incubated with mixtures of EGM-2 medium and non-conditioned MEM (Fig. 4A, Control), A549 conditioned MEM (Fig. 4B, A549 CM), and ERW-treated A549 conditioned MEM (Fig. 4C, ERW-A549 CM) at a ratio of 1 : 2, respectively. Co-cultures treated with the A549 conditioned MEM significantly increased the formation of vascular tubules in all analyzed parameters in comparison with control; that is, a 76% increase on total tubule areas, a 200% increase of the number of tubule junctions, a 179% increase of

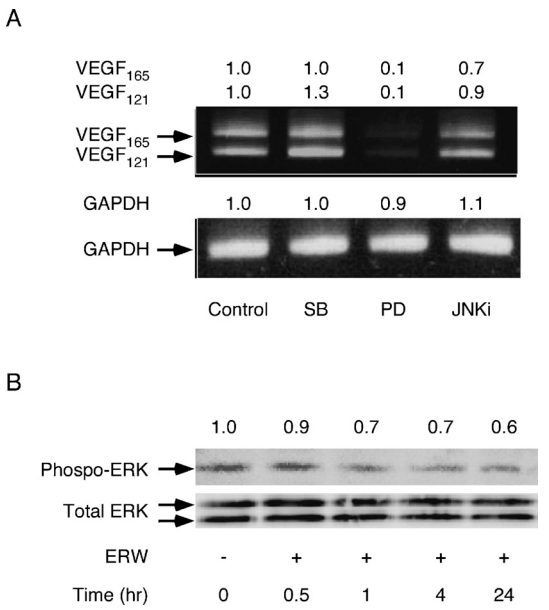

Fig. 3. VEGF Expression Is Down-Regulated *via* Inactivation of the ERK Pathway

(A) A549 cells were treated with serum-free MEM containing SB203580 (10 μM), PD98059 (20 μM) or JNK inhibitor II (40 nM) for 24 h and total RNAs isolated. Total RNAs were used to amplify $VEGF_{165}$ and $VEGF_{121}$ transcripts by RT-PCR with respective primer sets. Amplified products were resolved in an agarose gel electrophoresis and bands were photographed and documented using a digital camera. Recorded images were analyzed by an NIH image analyzer program (Image 1.62f) using a personal computer. Values above the panel were normalized by arbitrarily setting the densitometry of $VEGF_{165}$ and $VEGF_{121}$ bands at time zero to 1.0. A GAPDH transcript was used as an internal control for cellular activity. Control: no inhibitor, SB: SB203580 (p38 MAPK inhibitor), PD: PD98059 (MEK inhibitor), JNKi: JNK inhibitor. (B) A549 cells were incubated in serum-free MEM/ERW for the indicated time periods. Cell lysates were prepared and 30 μg proteins from each lysate were resolved by SDS-PAGE and used for Western-blot analysis as described in Materials and Methods. Total- and phospho-ERK were detected using Total- and phospho-ERK MAPK antibodies. Values above the panel were normalized by arbitrarily setting the densitometry of phospho-ERK band to 1.0. Total ERK was used to monitor cellular activity.

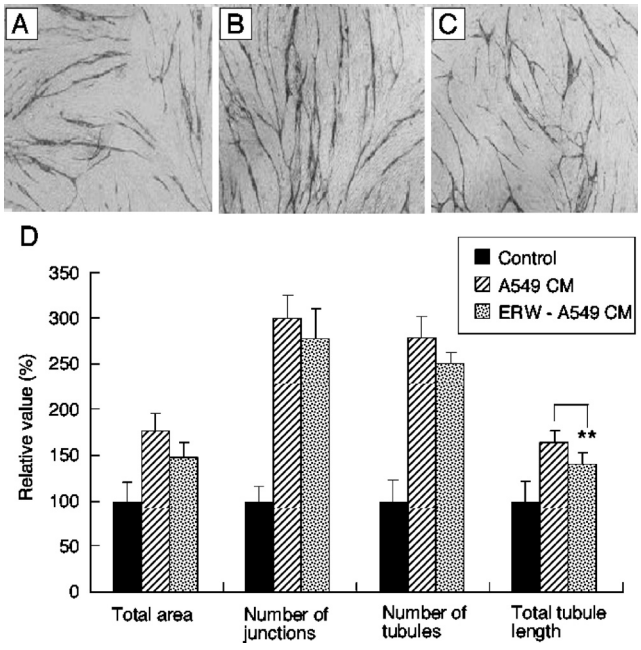

Fig. 4. Effects of ERW on A549 Cell Conditioned Medium-Induced Vascular Tubule Formation

HUVEC/TIG-1 co-culture in a 24-well plate was challenged with mixtures of EGM-2 medium and non-conditioned MEM (A. Control), A549 cell conditioned MEM (B. A549 CM) and ERW-treated A549 cell conditioned MEM (C. ERW-A549 CM) mixed at 1 : 2, respectively. Media were changed every 2 d. At day 11, tubule formations were detected with a Tubule Staining Kit and visualized by phase-contrast microscopy. Photomicrographs from a digital camera were used to characterize tubules. Recorded images were analyzed by angiogenesis quantification software (D). Twelve random fields per well were pictured for tubule formation assessment. Data are expressed as a percentage of the total area, the number of junctions, the number of tubules and the total tube length in untreated control cells (mean±S.E.). Asterisks represent a significant difference compared with controls (** $p<0.01$). p values of <0.05 were considered statistically significant.

the number of tubules, and a 65% increase of total tubule length, respectively (Fig. 4D: compares control and A549 CM). Taking these results into consideration, conditioned medium derived from A549 cells cultured with ERW (ERW-A549 CM) was used to see how ERW influences the tubule formation parameters. The results showed that ERW treatment affected only the total tubule length, decreasing it at a statistically significant level compared to A549 CM treated cells ($p<0.01$, Fig. 4D: see total tubule length). Although, the total tubule length parameter is commonly used for quantitative analysis of tubule formation, other parameters were also measured. ERW was shown to exert its influence by marginally suppressing the other three parameters, though not to a statistically significant level (Fig. 4D). These results strongly suggested that ERW exerts an inhibitory effect on tumor-induced angiogenesis by way of down-regulating H_2O_2 release and VEGF secretion from A549 cells (Fig. 4).

DISCUSSION

Our findings suggest that ERW reduces H_2O_2 induced VEGF expression in a human lung adenocarcinoma cell line, A549. As cancer cells produce ROS, including H_2O_2, the release of H_2O_2 could be a trigger for the angiogenic process in those cancer cells.[9,11,12,14] As well, H_2O_2 has also been shown to induce significant VEGF expression in various cell types.[33—35] As well, tumor vascularity has been shown to be directly correlated with VEGF production by tumors.[36—41] Blockade of tumor secreted VEGF by an anti-VEGF antibody caused significant damage on endothelial cells.[42] These results together strongly support the hypothesis that the blockade of H_2O_2 release and VEGF secretion from cancer cells has therapeutic value by conferring an antiangiogenic effect. Along this line, antioxidants such as *N*-acetylcystein, vitamin E, catechins, and natural polyphenols from red wine have been evaluated for their efficacy, with positive results.[42—47]

The signal transduction pathway for VEGF expression is highly divergent and is cell type dependent. Involvement of both phosphatidylinositol 3′-kinase and MAPK/ERK kinase 1/2 in the regulation of VEGF expression is reported in astrocytomas[48] and head and neck squamous cell carcinoma,[49] while phosphatidylinositol 3′-kinase pathway, but not ERK MAPK regulated VEGF expression is involved in hepatocellular carcinoma.[50] As well, p38 MAPK is reported to affect VEGF expression in vascular smooth muscle,[43] and breast cancer[51] cells, while ERK MAPK does so in fibroblasts,[52] and colon carcinoma.[53] In the present study, at least ERK was proven to be involved in regulation of VEGF expression in lung adenocarcinoma A549 cells, because only PD98059 blocked VEGF expression in the cells (Fig. 3).

ERK activation is sensitive to redox stress.[54—58] Thus, the reduction of redox stress induced in cells or the neutralization of exogenous oxidative stress may block activation of ERK MAPK, which can lead to an alteration of the target gene expression. Epigallocatechin gallate, an antioxidant contained in green tea, inhibited VEGF expression *via* the suppression of ERK activation in HT29 human colon cancer cells.[59] In the current study, ERW inactivated ERK in a time-dependent manner, within 4 h, after which ERW showed no further effect on ERK activation. This indicated that the efficiency of ERW on ERK activation is only short-term. The inhibition of constitutive VEGF expression in A549 cells can be partially ascribed to the blockade of ERK activation by ERW. Also, we considered possible transcription factor(s) involved in regulating VEGF gene transcription in relation to ROS. Exogenous stimulation of cultured cells by hydrogen peroxide (H_2O_2) was shown to up-regulate VEGF mRNA in a dose- and time-dependent manner. VEGF mRNA activation was also shown to correlate with an enhanced binding of AP-1 and NF-κB.[60] NF-κB resides in the cytoplasm complexed with the inhibitor protein I-κB masking the nuclear localization signal of NF-κB. NF-κB is activated by H_2O_2 treatment through phosphorylating I-κB to release NF-κB for nuclear translocation *via* the Ras mitogen-activated protein kinase (MAPK) pathway.[61] Our present results with specific inhibitors showed that MAPK pathway (p38 and JNK) is not involved (Fig. 3) and thus VEGF mRNA activation by NF-κB is excluded. On the other hand, AP-1 is considered as a redox-sensitive transcription factor[62] and thus VEGF mRNA up-regulation by H_2O_2 is likely to involve AP-1. Another transcription factor, ETS-1, is up-regulated by H_2O_2 *via* HIF-1α which is stabilized by H_2O_2.[63,64] The HIF-1α (120 kDa) is complexed with HIF-1β (94 kDa) subunit forming functional HIF-1 (hypoxia-inducible factor-1). HIF-1α is the rate limiting subunit which determines the activity of the HIF-1 complex.[65] HIF-1α is a short lived protein that is maintained at low and often undetectable levels in normoxia, whereas it is strongly induced in hypoxic cells.[66] We showed that ERW scavenges endogenous as well as exogenous H_2O_2 (Fig. 1) suggesting that HIF-1 regulated ETS-1 involvement is less likely. However, it has been shown that ETS-1 promoter contains several transcription factor binding sites including AP-1[67] and AP-1 is considered as a redox-sensitive transcription factor.[62] Taking all these information into considerations, we deduced AP-1 as the prime candidate for up-regulating VEGF transcription.

Present results uncovered that mRNA levels were dramatically decreased in the cells treated by ERW while secreted protein levels decreased rather slowly. Drastic mRNA decline could be interpreted as that the half-life of VEGF mRNA is 42 min[68] and 43 ± 6 min[69] under normoxic conditions, while the average half-life of eukaryotic mRNA is 10—12 h.[70] Furthermore, the VEGF mRNA contains destabilizing elements in its 5′UTR, coding region and 3′UTR, and three elements act additively to execute rapid degradation under normoxic conditions.[71] Our experiments were carried out under normoxic conditions and therefore mRNA is considered to be short lived. In addition, the activities of hydrogen peroxide (H_2O_2) regulated transcription factors would also be decreased due to lowered H_2O_2 levels by scavenging activity of ERW. Considering incubation times (0.5, 4.0, 24 h) used and short-half life of VEGF mRNA as well as lowered transcription factor(s) together would explain the drastic decrease of VEGF mRNA levels in the present experimental conditions (Fig. 3A). VEGF protein secreted in the medium is not drastically decreased as mRNA does. Our interpretation is that the levels of VEGF protein assayed are cumulative instead of time point levels. Thus the amount of protein will be accumulated as incubation time is extended up to 24 h. At 24 h point, control medium contains 1217.94 ± 61.83 pg/ml while ERW treated medium contains 1095.53 ± 21.50 pg/ml and

thus ERW treated medium still contains *ca.* 85% of VEGF protein (Fig. 2B). Then, one would expect that more VEGF protein exist in the conditioned medium, more tubule formation will result. ERW could have reduced *ca.* 15% VEGF protein compared to control after 24 h incubation and thus the tubule formation is exerted by *ca.* 85% VEGF protein. Three criteria show the suppressive tendencies though not statistically significant levels, and showed significant reduction in the total tubule length only (Fig. 4D). Therefore, vascular tubule formation assay is in accordance with the protein levels detected in Fig. 2B (Fig. 4D).

The mechanism underlying how ERW effectively scavenges intracellular H_2O_2 remains to be clarified in more detail. ERW contains a high concentration of hydrogen molecule, however, hydrogen molecule is chemically inert at room temperature. In an attempt to overcome this challenging problem, Shirahata *et al.* proposed an active hydrogen hypothesis of reduced water in which active hydrogen with a high reducing potential was produced in ERW by electrolysis and played a key role in scavenging ROS.[24,72] Active hydrogen can be produced from hydrogen molecule by catalysis action of noble metal nanoparticles like platinum nanoparticles.[73] Transition metal nanoparticles such as Pd, Pt, Ni, and Cu are produced during the process of electrolysis.[74] Platinum nanoparticles have also been demonstrated to adsorb active hydrogen.[75] Synthetic platinum nanoparticles were shown to scavenge superoxide radicals.[76] There is a possibility that platinum nanoparticles derived from platinum-coated titanium electrode used here during electrolysis and metal nanoparticls of 1—10 nm size can stably exist in solution for a long time.[74] Taken together, here we propose a new hypothesis of reduced water containing metal nanoparticles and hydrogen molecule as follows: (1) Electrolysis produces active hydrogen on the cathodic platinum electrode and hypersaturated hydrogen molecule in ERW. (2) Metal ions in the solution are reduced to metal nanoparticles on the electrode or in hydrogen-rich ERW. (3) Transition metal nanoparticles with low ionic tendencies adsorbed or absorbed active hydrogen can exist stably in ERW but other metal nanoparticles with high ionic tendencies such as Na and K disappear in ERW soon. (4) Hydrogen molecule is constantly converted to active hydrogen, which can scavenge ROS, by the catalysis of metal nanoparticles. Some kinds of metal nanoparticles like platinum nanoparticles can also directly scavenge ROS without hydrogen molecule.

On the other hand, Kikuchi *et al.* hypothesized, in attempting to elucidate the ROS scavenging substance(s) in ERW, that activated molecular hydrogen or hydrogen nanobubbles are responsible for the reducibility of ERW.[77—81] Recently, molecular hydrogen was demonstrated to act as a selective antioxidant against cytotoxic oxygen radicals like hydroxyl radical and peroxynitrite.[82] Hiraoka *et al.* reported that ERW and several natural mineral waters also possess reducing activities and hypothesized that it is due to molecular hydrogen and/or reductive vanadium ions.[83,84] Hanaoka *et al.* suggested that the enhancement of superoxide anion radical dismutation activity can be explained by changes in the ionic product of water in ERW.[85,86]

Major drawbacks of cancer chemotherapy are the various side-effects of the drugs used and the resistance that can be developing to these drugs. To resolve these problems, an understanding of the biological differences between cancer and normal cells is necessary. It will also be necessary to seek out appropriate therapeutic agents that can block the biological events critical for cancer cells, but not those for normal cells. Cancer cells, as compared to normal ones, are exposed to higher oxidative stresses associated with oncogenic transformation, alterations in metabolic activity, and increased generation of ROS. ERW possesses an advantage over many other antioxidants in that cancer cells with higher oxidative stress are more likely to be affected by ERW, whereas normal cells are not.

Taken together, we demonstrated here for the first time that ERW can suppress angiogenesis induced by A549 cells through down-regulating both H_2O_2 release and VEGF expression. Moreover, our study suggested that ERK MAPK plays a critical role in regulating VEGF expression in A549 cells, and that inhibition of VEGF by ERW partially correlated with inactivation of ERK MAPK.

While the present results have pointed out the intracellular target sites regulated by ERW, what component(s) in ERW actually scavenged intracellular H_2O_2 remains to be elucidated. Future investigations need to be directed to clarifying the reducing agent(s) in ERW. Also, our future studies could be directed to perform similar experiments under normoxic and hypoxic conditions to learn ERW effects at the gene expression levels which include confirmation of AP-1 involvement.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1999, p. 4276–4279 Vol. 65, No. 9
0099-2240/99/$04.00+0

Efficacy of Electrolyzed Oxidizing Water for Inactivating *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes*

KUMAR S. VENKITANARAYANAN,[1] GABRIEL O. EZEIKE,[2] YEN-CON HUNG,[2] AND MICHAEL P. DOYLE[2]*

Department of Animal Science, University of Connecticut, Storrs, Connecticut 06269,[1] and Center for Food Safety and Quality Enhancement, College of Agricultural and Environmental Sciences, University of Georgia, Griffin, Georgia 30223-1797[2]

Received 14 December 1998/Accepted 18 June 1999

The efficacy of electrolyzed oxidizing water for inactivating *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes* was evaluated. A five-strain mixture of *E. coli* O157:H7, *S. enteritidis*, or *L. monocytogenes* of approximately 10^8 CFU/ml was inoculated in 9 ml of electrolyzed oxidizing water (treatment) or 9 ml of sterile, deionized water (control) and incubated at 4 or 23°C for 0, 5, 10, and 15 min; at 35°C for 0, 2, 4, and 6 min; or at 45°C for 0, 1, 3, and 5 min. The surviving population of each pathogen at each sampling time was determined on tryptic soy agar. At 4 or 23°C, an exposure time of 5 min reduced the populations of all three pathogens in the treatment samples by approximately 7 log CFU/ml, with complete inactivation by 10 min of exposure. A reduction of ≥7 log CFU/ml in the levels of the three pathogens occurred in the treatment samples incubated for 1 min at 45°C or for 2 min at 35°C. The bacterial counts of all three pathogens in control samples remained the same throughout the incubation at all four temperatures. Results indicate that electrolyzed oxidizing water may be a useful disinfectant, but appropriate applications need to be validated.

Enterohemorrhagic *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes* are food-borne pathogens of major public health concern in the United States. A variety of foods, including poultry, eggs, meat, milk, fruits, and vegetables, have been implicated as vehicles of one or more of these pathogens in outbreaks of food-borne illness (2, 4, 5). The Pathogen Reduction program of the U.S. Department of Agriculture Food Safety and Inspection Service recommends antimicrobial treatments as a method for reducing or inactivating pathogenic bacteria in foods (13). Effective methods of reducing or eliminating pathogens in foods are important to the successful implementation of Hazard Analysis and Critical Control Point (HACCP) programs by the food industry and for the establishment of critical control points in restaurants, homes, and other food service units. Washing of raw agricultural produce with water is practiced in the industry; however, washing alone does not render the product completely free from pathogens. Although many chemicals generally recognized as safe (GRAS), including organic acids, possess antimicrobial activity against food-borne pathogens, none can eliminate high populations of pathogens when they are used individually at concentrations acceptable in foods. Treatments of fruits and vegetables with water containing sanitizers, including chlorine, may reduce but not eliminate pathogens on the surface of produce (2, 14). Hence, there is a need for, and interest in, developing practical and effective antimicrobial treatments for the inactivation of pathogenic microorganisms on foods.

Electrolyzed oxidizing water (EO water) is the product of a new concept developed in Japan. Research carried out in Japan revealed that electrolysis of deionized water containing a low concentration of sodium chloride (0.1%) in an electrolysis chamber where anode and cathode electrodes were separated by a diaphragm imparted strong bactericidal and virucidal properties to the water collected from the anode (EO water). Water from the anode normally has a pH of 2.7 or lower, an oxidation-reduction potential (ORP) greater than 1,100 mV, and a free-chlorine concentration of 10 to 80 ppm (10). EO water has been experimentally used in Japan by medical and dental professionals for treating wounds or disinfecting medical equipment. The objective of this study was to evaluate the efficacy of EO water for killing *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes* with a view to its potential application to foods and food contact surfaces as an antimicrobial treatment.

Bacterial culture and media. Five strains each of *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes* were used for the study. The five strains of *E. coli* O157:H7 (with origins in parentheses following strain designations) were E06 (milk), E08 (meat), E10 (meat), E16 (meat), and E22 (calf feces). The *S. enteritidis* isolates included SE180 (human), SE457 (egg), SE565 (salad), SE294 (egg), and SE1697 (human). The five strains of *L. monocytogenes* were LM ATCC 19117 (sheep), LM101 (salami), LM109 (pepperoni), LM116 (cheese), and LM201 (milk). The *E. coli* O157:H7 and *L. monocytogenes* strains, but not ATCC 19117, were isolated by one of the authors, whereas the *S. enteritidis* isolates were obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. The strains of each pathogen were cultured separately in 100 ml of sterile tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) in 250-ml Erlenmeyer flasks at 37°C for 24 h with agitation (150 rpm). Following incubation, 10 ml of each culture was sedimented by centrifugation (4,000 × *g* for 20 min), washed, and resuspended in 10 ml of 0.1% peptone water (pH 7.1). The optical density of the suspension was determined and adjusted with 0.1% peptone water to 0.5 at 640 nm (representing approximately 10^9 CFU/ml). The bacterial population in each culture was confirmed by plating 0.1-ml portions of appropriately di-

* Corresponding author. Mailing address: Center for Food Safety and Quality Enhancement, College of Agricultural and Environmental Sciences, University of Georgia, 1109 Experiment St., Griffin, GA 30223-1797. Phone: (770) 228-7284. Fax: (770) 229-3216. E-mail: mdoyle@cfsqe.griffin.peachnet.edu.

TABLE 1. Inactivation of *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes* by EO water at 4 or 23°C

Bacterial species	Temp (°C)	Surviving bacterial population (mean log CFU/ml) after exposure for:				EO water property		
		0 min	5 min	10 min	15 min	pH	ORP (mV)	Free chlorine (ppm)
E. coli O157:H7	4	7.98 ± 0.04	<1.0[a]	0[b]	0[b]	2.36 ± 0.03	1,153 ± 3	86.3 ± 5.4
Control		7.98 ± 0.04	7.99 ± 0.07	7.96 ± 0.06	7.99 ± 0.04			
S. enteritidis		7.74 ± 0.08	1.06 ± 0.15	0[b]	0[b]	2.48 ± 0.03	1,153 ± 2	83.5 ± 7.8
Control		7.74 ± 0.08	7.68 ± 0.09	7.61 ± 0.11	7.60 ± 0.12			
L. monocytogenes		7.91 ± 0.05	1.34 ± 0.37	0[b]	0[b]	2.63 ± 0.03	1,160 ± 4	43.0 ± 4.6
Control		7.91 ± 0.05	7.88 ± 0.06	7.87 ± 0.06	7.91 ± 0.03			
E. coli O157:H7	23	8.04 ± 0.07	<1.0[a]	0[b]	0[b]	2.37 ± 0.01	1,155 ± 1	82.3 ± 2.2
Control		8.04 ± 0.07	7.97 ± 0.03	7.99 ± 0.07	7.76 ± 0.42			
S. enteritidis		7.76 ± 0.08	<1.0[a]	0[b]	0[b]	2.45 ± 0.12	1,151 ± 1	82.0 ± 5.8
Control		7.76 ± 0.08	7.65 ± 0.09	7.73 ± 0.08	7.69 ± 0.10			
L. monocytogenes		7.89 ± 0.10	1.25 ± 0.33	0[b]	0[b]	2.63 ± 0.04	1,158 ± 5	48.5 ± 4.1
Control		7.89 ± 0.10	7.83 ± 0.06	7.85 ± 0.04	7.85 ± 0.07			

[a] Positive by enrichment.
[b] Negative by enrichment and no detectable survivors by a direct plating procedure.

luted culture on tryptic soy agar (TSA) (Difco Laboratories) plates and incubating the plates at 37°C for 48 h. For each pathogen, equal portions from each of the five strains were combined, and 1 ml of the suspension was used as the inoculum (10^9 CFU).

EO water. EO water was generated with a model ROX-20TA EO water generator (Hoshizaki Electric Company Ltd., Toyoake, Aichi, Japan). The current passing through the EO water generator and the voltage between the electrodes were set at 19.8 A and 10 V, respectively. A 12% solution of sodium chloride (Sigma Chemical Co., St. Louis, Mo.) and deionized water from the laboratory supply line were simultaneously pumped into the equipment. The display indicator was activated and observed until the machine stabilized at a reading of 19.8 A. The EO water was collected from the appropriate outlet in sterile containers and was used within 5 min for the microbial study. Samples for determination of the pH, ORP, and free-chlorine concentration also were collected simultaneously.

Sample inoculation and treatments. A volume of 9 ml of EO water (treatment) or sterile deionized water (control) was transferred to separate, sterile screw-cap tubes, and the caps were tightly closed. The tubes were placed in a water bath in order to prewarm the water samples to the desired temperature. To each tube containing 9 ml of EO water or deionized water, 1 ml (equivalent to 10^9 CFU) of the five-strain mixture of *E. coli* O157:H7, *S. enteritidis*, or *L. monocytogenes* was added, and the samples were incubated in a water bath (Pharmacia LKB, Piscataway, N.J.) at 4°C for 0, 5, 10, and 15 min; at 23°C for 0, 5, 10, and 15 min; at 35°C for 0, 2, 4, and 6 min; and at 45°C for 0, 1, 3, and 5 min. Following each incubation, the number of viable cells in each sample was determined by plating 0.1-ml portions directly or after serial (1:10) dilutions in 0.1% peptone water on duplicate TSA plates. Colonies of the inoculated pathogen were enumerated on TSA plates after incubation at 37°C for 48 h. A volume of 1 ml of the inoculated solution (treatment or control) after exposure to each temperature-time combination was also transferred to separate 250-ml Erlenmeyer flasks containing 100 ml of sterile TSB and incubated at 37°C for 24 h. Following enrichment in TSB, the culture was streaked on either sorbitol MacConkey agar no. 3 (Oxoid Division, Unipath Co., Ogdensburg, N.Y.) (for *E. coli* O157:H7), xylose lysine deoxycholate agar (Gene-Trak, Framingham, Mass.) (for *S. enteritidis*), or Oxford agar (Gene-Trak) (for *L. monocytogenes*), and the plates were incubated at 37°C for 24 h. Representative colonies of *E. coli* O157:H7 and *S. enteritidis* from the respective plates were confirmed by the *E. coli* O157:H7 latex agglutination assay (Remel Microbiology Products, Lenexa, Kans.) and the *Salmonella* latex test (Oxoid), respectively. The colonies of *L. monocytogenes* on Oxford agar were confirmed by the API-20E diagnostic test kit (Biomerieux, Hazelwood, Mo.). At least duplicate samples of treatments and controls were assayed at each sampling time, and the entire study with each pathogen was replicated three times.

The pH and ORP of the EO water were measured in duplicate samples immediately after sampling by using pH and ORP electrodes (model 50, ACCUMET meter; Denver Instrument Company, Denver, Colo.). The free-chlorine concentration was determined by an iodometric method using a digital titrator (model 16900; Hach Company, Loveland, Colo.). The assay was verified periodically by using a 100 ± 0.05 ppm chlorine standard solution (Orion Research Inc., Beverly, Mass.).

Statistical analysis. For each treatment, the data from the independent replicate trials were pooled and the mean value and standard deviation were determined (11).

The mean pH, ORP, and free-chlorine concentration of EO water at the different temperatures used for treatment are presented in Tables 1 through 3. The mean pH and ORP of sterile deionized water were 7.1 ± 0.15 and 355 ± 7.0 mV, respectively. No free chlorine was detected in deionized water.

TABLE 2. Inactivation of *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes* by EO water at 35°C

Bacterial species	Surviving bacterial population (mean log CFU/ml) after exposure for:				EO water property		
	0 min	2 min	4 min	6 min	pH	ORP (mV)	Free chlorine (ppm)
E. coli O157:H7	7.97 ± 0.03	0[b]	0[b]	0[b]	2.38 ± 0.00	1,154 ± 1	84.3 ± 4.6
Control	7.97 ± 0.03	7.94 ± 0.04	7.96 ± 0.03	7.94 ± 0.04			
S. enteritidis	7.68 ± 0.14	<1.0[a]	0[b]	0[b]	2.44 ± 0.04	1,153 ± 1	79.8 ± 3.3
Control	7.68 ± 0.14	7.63 ± 0.06	7.59 ± 0.11	7.64 ± 0.11			
L. monocytogenes	7.91 ± 0.10	0[b]	0[b]	0[b]	2.48 ± 0.05	1,159 ± 4	73.3 ± 1.8
Control	7.91 ± 0.10	7.88 ± 0.11	7.86 ± 0.08	7.81 ± 0.12			

[a] Positive by enrichment.
[b] Negative by enrichment and no detectable survivors by a direct plating procedure.

EO water had major antibacterial activity at 4 and 23°C on the five-strain mixtures of *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes* (Table 1). At time zero, both treatment and control samples for all three pathogens had approximate mean bacterial counts of 8.0 log CFU/ml. At 5 min of exposure at 4°C, the *E. coli* O157:H7 count in the treatment samples was reduced to less than 1.0 log CFU/ml (detected only by enrichment in TSB for 24 h), whereas the populations of *S. enteritidis* and *L. monocytogenes* were slightly greater than 1.0 log CFU/ml. All three pathogens decreased to undetectable levels (as determined by both plating and enrichment procedures) after 10 min of exposure to EO water at 4°C. However, no differences in bacterial counts were observed in the control samples throughout the study. At 5 min of exposure at 23°C, the populations of *E. coli* O157:H7 and *S. enteritidis* in the treatment samples decreased to less than 1.0 log CFU/ml, whereas the *L. monocytogenes* count was reduced to 1.25 log CFU/ml. In agreement with the results obtained at 4°C, all three pathogens were undetectable after 10 min of contact with EO water at 23°C.

E. coli O157:H7, *S. enteritidis*, and *L. monocytogenes* were more rapidly inactivated by EO water at 35 or 45°C (Tables 2 and 3) than at 4 or 23°C. At 35°C, the populations of *E. coli* O157:H7 and *L. monocytogenes* in the treated samples decreased to undetectable levels within 2 min of exposure to EO water, whereas *S. enteritidis* was detected only by enrichment of the treated sample in TSB. After 1 min of exposure to EO water at 45°C, *E. coli* O157:H7 was killed completely (a reduction of approximately 8.0 log CFU/ml), whereas the populations of *S. enteritidis* and *L. monocytogenes* were reduced by approximately 7.0 log CFU/ml. The bacterial counts of all three pathogens in control samples remained the same throughout the study at both 35 and 45°C.

The theoretical sequence of chemical reactions involved in the production of EO water can be summarized as follows (1). During electrolysis, sodium chloride dissolved in deionized water in the electrolysis chamber dissociates into negatively charged chloride (Cl^-) and hydroxy (OH^-) ions and positively charged sodium (Na^+) and hydrogen (H^+) ions. The chloride and hydroxy ions are adsorbed to the anode, with each ion releasing an electron (e^-) to become a radical. The chloric and hydroxy radicals combine, forming hypochlorous acid (HOCl), which separates from the anode. Two chloric radicals can also combine to produce chlorine gas. In the cathode section, each positively charged sodium ion receives an electron and becomes metallic sodium. The metallic sodium combines with water molecules, forming sodium hydroxide and hydrogen gas. A bipolar membrane separating the electrodes enhances the electrolysis of water to produce strong acidic and alkali waters from the anode and cathode, respectively.

The antagonistic effects of chlorine and low pH on microorganisms are well documented. Although organic acids (with low pH) and hypochlorite solution (with free chlorine) have been used widely in treatments for killing food-borne bacteria

TABLE 3. Inactivation of *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes* by EO water at 45°C

Bacterial species	Surviving bacterial population (mean log CFU/ml) after exposure for:				EO water property		
	0 min	1 min	3 min	5 min	pH	ORP (mV)	Free chlorine (ppm)
E. coli O157:H7	7.96 ± 0.03	0[b]	0[b]	0[b]	2.39 ± 0.02	1,153 ± 4	85.8 ± 2.7
Control	7.96 ± 0.03	7.89 ± 0.03	7.87 ± 0.03	7.86 ± 0.11			
S. enteritidis	7.70 ± 0.12	1.13 ± 0.33	0[b]	0[b]	2.44 ± 0.03	1,155 ± 1	79.33 ± 3.0
Control	7.70 ± 0.12	7.63 ± 0.12	7.67 ± 0.15	7.61 ± 0.14			
L. monocytogenes	7.91 ± 0.10	<1.0[a]	0[b]	0[b]	2.48 ± 0.05	1,159 ± 4	73.3 ± 1.8
Control	7.91 ± 0.10	7.88 ± 0.10	7.88 ± 0.08	7.83 ± 0.12			

[a] Positive by enrichment.
[b] Negative by enrichment and no detectable survivors by a direct plating procedure.

in the food industry, systems involving high ORP values, greater than 1,000 mV, have not normally been used. The ORP of a solution is an indicator of its ability to oxidize or reduce, with positive and higher ORP values correlated to greater oxidizing strength (6, 8, 9). An ORP of +200 to +800 mV is optimal for growth of aerobic microorganisms, whereas an optimum range of −200 to −400 mV is favored for growth of anaerobic microorganisms (6). Since the ORP of EO water in this study was greater than 1,100 mV, the ORP likely played an influential role, in combination with low pH and free chlorine, in killing microorganisms. A possible explanation for the high ORP of EO water is the oxygen released by the rupture of the weak and unstable bond between hydroxy and chloric radicals (1). It is hypothesized that the low pH in EO water sensitizes the outer membranes of bacterial cells, thereby enabling hypochlorous acid to enter the bacterial cells more efficiently. Acid-adapted cells of *Salmonella typhimurium* were reported to be more sensitive to inactivation by hypochlorous acid than nonadapted cells, due to increased outer membrane sensitivity to hypochlorous acid in acid-adapted cells (7). Experiments to identify the contributions of the different components of EO water to its antimicrobial activity are under way in our laboratory.

The effects of EO water on the three pathogens were evaluated at low and moderate temperatures in the interest of developing potential antibacterial dip treatments for unprocessed agricultural foods. No differences in the inactivation rates of the three pathogens were observed between treatment at 4°C and treatment at 23°C. However at 35 and 45°C, much higher rates of inactivation were observed for all three pathogens.

Since chlorine is one of the antimicrobial components of EO water, we evaluated the survival of *E. coli* O157:H7 and *L. monocytogenes* in sterile deionized water containing a free-chlorine concentration of 70 to 80 ppm, which was similar to that present in EO water. Results revealed reductions in the bacterial counts of both pathogens similar to those observed with EO water, indicating that the concentration of free chlorine present in EO water is sufficient to bring about the reductions in bacterial counts achieved by EO water. Although chlorine is highly effective in killing pathogenic microorganisms in simple aqueous systems, its antibacterial effect on microorganisms on foods is minimal, especially in the presence of organic materials which convert chlorine into inactive forms (3). For example, treatment of fresh produce with 200 ppm chlorine results in a reduction in the *L. monocytogenes* count of less than 2 log CFU/g (15). Studies comparing the efficacies of chlorinated water and EO water for inactivating *E. coli* O157:H7 on apples are in progress in our laboratory.

Results revealed that EO water is highly effective in killing *E. coli* O157:H7, *S. enteritidis*, and *L. monocytogenes*, indicating its potential application for decontamination of food and food contact surfaces. An advantage of EO water is that it can be produced with tap water, with no added chemicals other than sodium chloride.

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Biophysical Chemistry 107 (2004) 71–82

Biophysical Chemistry

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The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis

Kokichi Hanaoka[a,*], Dongxu Sun[c], Richard Lawrence[c], Yoshinori Kamitani[b], Gabriel Fernandes[c]

[a]*Bio-REDOX Laboratory Inc. 1187-4, Oaza-Ueda, Ueda-shi, Nagano-ken 386-0001, Japan*
[b]*Hoshizaki Electric Company, Limited 3-16 Minamiyakata, Toyoake, Aichi-ken 470-1194, Japan*
[c]*Divison of Clinical Immunology, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA*

Received 8 July 2003; received in revised form 22 August 2003; accepted 22 August 2003

Abstract

We reported that reduced water produced by electrolysis enhanced the antioxidant effects of proton donors such as ascorbic acid (AsA) in a previous paper. We also demonstrated that reduced water produced by electrolysis of 2 mM NaCl solutions did not show antioxidant effects by itself. We reasoned that the enhancement of antioxidant effects may be due to the increase of the ionic product of water as solvent. The ionic product of water (pK_w) was estimated by measurements of pH and by a neutralization titration method. As an indicator of oxidative damage, Reactive Oxygen Species- (ROS) mediated DNA strand breaks were measured by the conversion of supercoiled ϕX-174 RF I double-strand DNA to open and linear forms. Reduced water had a tendency to suppress single-strand breakage of DNA induced by reactive oxygen species produced by H_2O_2/Cu (II) and HQ/Cu (II) systems. The enhancement of superoxide anion radical dismutation activity can be explained by changes in the ionic product of water in the reduced water.

Keywords: Reduced water; Antioxidant; Ionic product of water; DNA damage

1. Introduction

Recently, a new technology involving electrolysis of water has been proposed for clinical improvement of various diseases. Reduced water produced by electrolysis of tap water has a higher pH (9.0–10.0), lower oxidation reduction potential (ORP), lower dissolved oxygen (DO) and higher dissolved hydrogen (DH) than non-electrolyzed water. Reduced water [1–3], which has such parameters, is used extensively as drinking water which in addition to its use as a pure filtered drinking water may also act as an antioxidant against oxidative stress. In contrast, the water collected from the anode compartment is oxidized water and has been used extensively as an antiseptic [4–6].

*Corresponding author. Tel.: +81-268-25-1736; fax: +81-268-24-0050.
E-mail address: hanak@rapid.ocn.ne.jp (K. Hanaoka).

0301-4622/04/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2003.08.007

Oxidative stress [7–12] in the human body is thought to be due to excess reactive oxygen species or free radicals including superoxide anion radicals (O_2^{-}•), hydroxyl radicals (•OH), hydroperoxyl radicals (•OOH), nitrogen monoxide radicals (NO•), singlet oxygen ($^1O_2$) and hydrogen peroxide molecules (H_2O_2). Among these, superoxide anion radicals are the best known. It is considered that the dismutation activity for superoxide anion radicals is the most important indicator of antioxidant effects. We have studied the antioxidant effects of reduced water produced by electrolysis [13]. The commonly reported parameters of reduced water which are pH, ORP, DO and DH, do not explain the mechanism of enhanced antioxidant effects but they are useful parameters for determining the energy of electrolysis, if measured immediately after electrolysis. They are the parameters of the solute in the reduced water. We have investigated the parameters of the solvent water. The parameter of the solvent, which is directly related to the enhancement of dismutation activity by reduced water is the ionic product of water. We could obtain the ionic product of water (pK_w) by using pH and the neutralization titration method. We defined the ionic product of water (pK_w) as pIP for electrolyzed water. Water passed through the electrolysis system with no current applied was used as a control. In this study, pIP and the increase of entropy have been estimated from the experimental results. We demonstrate that the reduced water protects DNA from damage by oxygen radicals based on the pIP of the reduced water. The mechanism of the observed antioxidant effects is discussed in relation to these parameters.

2. Materials and methods

2.1. Electrolysis cell

Two electrolysis half-cells, made of acrylonitrile-butadien-styrene mounted in polyvinyl chloride resin, were prepared as shown in Fig. 1. A non-charged membrane, (YUMICRON Y9201-T, YUASA CORPORATION) with an effective area of 100 cm^2 and 0.12 mm thickness was mounted between the two half-cells. Electrolysis was carried out across the non-charged membrane between these two half-cells equipped with platinum-coated titanium electrodes having an effective area of 100 cm^2 on the cathode.

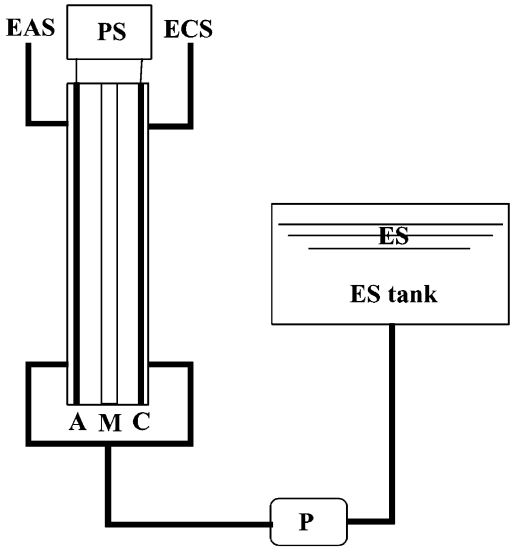

Fig. 1. Electrolysis cells made of an acrylonitrile–butadien–styrene mounted in polyvinyl chloride resin. (A) anode, (C) chathode, (M) non-charged membrane, (ES) electrolyte solutions, (ECS) electrolyzed cathode solutions (the reduced water), (EAS) electrolyzed anode solutions (the oxidized water), (PS) electric power source and (p) pump.

2.2. Chemicals and reagents

The reagents used for this study were special-grade NaCl, KCl, $MgCl_2$, $CaCl_2$, L-ascorbic acid (Wako Pure Chemical Industries Co., Ltd.), and as a solvent, water, which was distilled and deionized with an ion-exchange resin to below 0.3 μs cm^{-1}. To measure the superoxide dismutation activity, a sodium phosphate buffer containing 2 mM hypoxanthine (Sigma Chemical Company), a sodium phosphate buffer containing, 5,5-dimethyl-1-pyrroline-oxide (DMPO, Labotec Co., Ltd.), superoxide dismutase (Boehringer Manheim), 0.4 unit ml^{-1} xanthine oxidase in sodium phosphate buffer (Merck and Co., Inc.) and L-ascorbic acid (AsA) were used. ΦX-174RF1 plasmid DNA (New England Biolabs, Beverly, MA), H_2O_2, $CuSO_4$, hydroquinone, Tris base, sodium acetate, EDTA, and ethidium bromide (Sigma Chemical

Co., St. Louis, MO) and Agarose (Bio-Rad Laboratories, Hercules, CA) were used for the DNA damage measurements.

2.3. Electrolysis of 2 mM NaCl, KCl, 1 mM $MgCl_2$ and $CaCl_2$

20 L sample solutions, containing 2 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ or 1 mM $CaCl_2$ were prepared. These samples were pumped from the solution container through a pair of electrolysis half-cells with a non-charged membrane mounted between them. The sample solutions were subjected to electrolysis under set conditions of 0 to 0.8 A and 25 °C using a constant electric current source. Electrolysis was carried out with a constant flow rate of 1000 ± 50 ml min^{-1} in the cathode compartment and, 2000 ± 100 ml min^{-1} in the anode compartment. The cathode solution produced by electrolysis, which was called reduced water, was used for the experiments in this study.

2.4. Measurements of pH, oxidation and reduction potential (ORP), and dissolved oxygen (DO)

The parameters that were measured to characterize the reduced water produced by electrolysis of 2 mM NaCl and KCl, and 1 mM $MgCl_2$ and $CaCl_2$ were electrical potential (V) from the power source, electrical conductivity (EC) by an EC meter (TOA EC METER CM-14P), pH by a pH meter (TOA ION METER IM-4OS), oxidation reduction potential (ORP) by an ORP meter (TOA ION METER IM-4OS) and dissolved oxygen (DO) by a DO meter (HORIBA OM-12) at 25 °C.

2.5. Measurement of OH^- ions

The concentration of OH^- ions was obtained from the measurement of pH and by a neutralization titration method using an automatic titrator. In this study, pH was measured using a pH meter (TOA ION METER lM-40S, Toa Electronics Ltd.) in samples produced at each of the following levels of electric current, 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 A. Neutralization titrations with 20 mM HCl were carried out using a COMITE-550 titrator (Hiranuma Industry Co.) for the reduced water produced at each of the same levels of electric current as the pH measurements.

2.6. Measurement of superoxide dismutation activity

1 ml of 10 mM L-ascorbic acid was added to 50 ml samples of the reduced water and to 50 ml of 2 mM NaC1 and KCl, and 1 mM $MgCl_2$ and $CaCl_2$ solutions, respectively. Superoxide dismutation activity was measured using an electron spin resonance (ESR) spectrometer (ES-10, Nikkiso Co. Ltd.). Each reaction mixture contained 50 mm^3 of 2 mM hypoxanthine in sodium phosphate buffer, 50 mm^3 of sample, 16 mm^3 of DMPO and 50 mm^3 xanthine oxidase in a sodium phosphate buffer. These reaction mixtures were poured into a special flat cell to conduct ESR measurements. This ESR measurement was carried out using 3.7 mW microwave power, 339.1 ± 5.5 mT magnetic field, 100 kHz frequency, 0.1 mT modulation, 0.12 s response time and 1 min sweep time. As the unit for superoxide dismutation activity, we used the same unit as adopted by Friedovich et al. [14]. The superoxide dismutation activity of the samples was estimated by means of interpolation based on a standard curve of 0 to 30 units per ml of superoxide dismutase.

2.7. The ratio of concentration of cations to that of anions in the cathode compartment

When electrolysis of diluted electrolyte solutions is carried out the concentration of ions in both the cathode and the anode will be changed compared to the initial solutions. The concentration of transported ions in the cathode side was measured by an ion chromatography system (TOA ICA-5000 System) and the ratio of cations and anions for each sample solution was estimated.

2.8. Titration of the reduced water and the control by 10 mM AsA solutions

The amount of 10 mM AsA solutions consumed by the neutralization titration for the reduced water and the control solution was measured. Non-elec-

trolyzed water brought to the same pH as the electrolyzed water by addition of NaOH was used as the control. The pH of the reduced water was changed from 10.1 to 10.94 and that of the control was changed from 10.05 to 10.94.

2.9. The estimation of ionic product $p(IP)_{RW}$ of reduced water and the entropy of reduced water

When electrolysis is carried out with 2 mM NaCl and KCl solutions, and 1 mM $MgCl_2$ and $CaCl_2$ solutions, the following reactions will be observed in the cathode compartment,

$$2H_2O + 2e^- \leftrightharpoons 2OH^- + H_2 \quad (1)$$

and designating monovalent electrolytes as M_A and divalent electrolytes as M_B, for NaCl, or KCl, and $MgCl_2$ or $CaCl_2$, respectively,

$$M_A^+ + OH^- \rightarrow M_AOH \quad (2)$$

$$M_B^{2+} + 2OH^- \rightarrow M_B(OH)_2 \quad (3)$$

In general, even if pure water is used H_2CO_3 (beyond 1.1×10^{-2} mM at 25°) is present in an open system. Therefore, most solutes are dissolved as carbonates as shown in Eqs. (4)–(6).

$$H_2O + CO_2 \leftrightharpoons H_2CO_3 \leftrightharpoons H^+ + HCO_3^- \quad (4)$$

$$M_AOH + H_2CO_3 \rightarrow (M_A)_2CO_3 + 2H_2O \quad (5)$$

$$M_B(OH)_2 + H_2CO_3 \rightarrow M_BCO_3 + 2H_2O \quad (6)$$

The pH of the reduced water is shown as the result of hydrolysis of the carbonate salts. In general, hydrolysis of salts will be represented by the following equations,

$$HA \rightleftharpoons H^+ + A^- \quad (7)$$

Where *HA* is a weak acid.

The dissociation constant in this hydrolysis is given by Eq. (8),

$$Ka = C\alpha^2/(1-a) \quad (8)$$

Where *K*a, α and *C* are dissociation constant, degree of dissociation and initial concentration, respectively. If α is much smaller than 1, Eq. (9) can be obtained from Eq. (8). Eq. (10) can be obtained from Eq. (9).

$$\alpha \cong (Ka/C)^{1/2} \quad (9)$$

Eq. (10) can be obtained from Eq. (8).

$$[H^+] = C\alpha \cong (Ka/C)^{1/2} \quad (10)$$

Thus, pH will be described as follows:

$$pH = (1/2)(pKa - \log C) \quad (11)$$

In the case of $Kw = 10^{-14}$, the used pH will be shown as Eq. (12).

$$pH = 7 + (1/2)(pKa + \log C) \quad (12)$$

However, the dissociation of water will be shown as Eq. (13),

$$H_2O + H_2O \rightleftharpoons H_3O^+ + OH^- \quad (13)$$

Ionic product of water, K_w and IP will be shown as Eq. (14).

$$[H_3O^+][OH^-] = K_w = IP \quad (14)$$

where IP will be described as Eq. (15) under the conditions of 25 °C and 1 atm,

$$(IP)_w = 10^{-14}\ (mol/l)^2 \quad (15)$$

Therefore, $p(IP)_w$ will be shown as Eq. (16),

$$-\log(pK_w) = pK_w = p(IP)_w \quad (16)$$

The titration for 1-1 carbonates with HCl solutions will be carried out as follows,

$$M_2CO_3 + HCl \rightarrow MCl + MHCO_3 \quad (17)$$

$$MHCO_3 + HCl \rightarrow MCl + H_2O + CO_2 \quad (18)$$

$$M_2CO_3 + 2HCl \rightarrow 2MCl + H_2O + CO_2 \quad (19)$$

If the neutralization titration is carried out with 20 mM HCl, two peaks of differential values (dE/dV) are observed, where E and V are potential and volume. The concentration at each neutralization titration point is shown as Eqs. (20) and (21).

$$[OH^-]_{MOH} = [HCl]_{A\ point} - [HCl]_{B\ point} \quad (20)$$

$$[HCl]_{B\ point} = 2[M_2CO_3]_{dissociation} = [MHCO_3]_{total} \quad (21)$$

$$-\log[OH^-]_{M_2CO_3} = 14 - (pH)_{M_2CO_3} \quad (22)$$

$$[OH^-]_{total} = ([OH^-]_{MOH} + [OH^-]_{M_2CO_3} \quad (23)$$

$$p(IP)_{RW} = -\log K_{RW} = 14 + \log([OH^-]_{total}/[OH^-]_{pH}) \quad (24)$$

where $[OH^-]_{pH}$ is the concentration of $[OH^-]$ estimated from pH value of the reduced water.

The free energy change ΔG^0 for a reaction is related to the equilibrium constant K, enthalpy ΔH, and entropy ΔS at equilibrium as shown in following equations.

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT\ln\ K \quad (25)$$

$$\Delta S^0 = \Delta H^0/T + R\ \ln\ K \quad (26)$$

where R, T and K are the gas constant (1.987 cal mol^{-1} K^{-1}), the absolute temperature and the equilibrium constant which is the ionic product of water, K_{RW} in the reduced water produced by electrolysis. At 25 °C and 1 atm.

$$\Delta S^0 = -68.3/298 + 1.9872 \times \ln\ K_{RW} \quad (27)$$

From Eq. (27), the entropy difference, ΔS^0 of the solvent in the reduced water will be estimated using K_{RW} which is defined as $(IP)_{RW}$.

2.10. Measurement of DNA strand break under oxidative stress

The ROS-mediated DNA strand breaks were measured by the conversion of supercoiled ϕX-174 RF I double-strand DNA to open and linear forms, according to the method described by Li [15] and Win [16]. Briefly, 0.2 μg DNA was incubated with the indicated concentration of H_2O_2 and, 25 μM $CuSO_4$ in 20 μl of 20 mM sodium phosphate buffer (pH 7.5) containing 17 μl of reduced water or, NaOH solution of the same pH as the reduced water at 37 °C for 30 min. For the hydroquinone/copper II (HQ/Cu(II)) induced breaks, 0.2 μg of DNA was incubated with the indicated concentrations of HQ and Cu (II) in PBS at 37 °C at a final volume of 20 μl containing 17 μl of reduced water. Following incubation, the samples were immediately loaded in a 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, and subjected to electrophoresis in Tris/acetate gel buffer. After electrophoresis, the gels were stained with a 0.5 μg/ml solution of ethidium bromide for 30 min, followed by another 30 min destaining in water. The gels were then photographed under UV light.

3. Results

When electrolyte solutions are electrolyzed across the membrane, reduction occurs at the cathode and oxidation at the anode. Oxidation of water molecules produces H^+ and O_2 at the anode, and OH^- and H_2 at the cathode. Therefore, cathodic alkaline water (reduced water) is abundant in dissolved hydrogen (DH), whereas anodic acidic water (oxidized water) is abundant in DO. DH and DO produced by electrolysis have particular characteristics [2]. Results of the measurement of electric potential (V), electric conductivity (EC), pH, ORP and DO at different electric currents are shown in Figs. 2–6. The electrolysis potential varies linearly with electric current between 0.1 A and 0.8 A as shown in Fig. 2. The slopes, V/A increased in the order of KCl, $<CaCl_2$, $<MgCl_2$, $<$NaCl solutions. Fig. 3 shows results of electric conductivity measurements. The electrical conductivity for a fixed electric current increased in the order of $MgCl_2$, $<$NaCl, $<CaCl_2$, $<$KCl. The conductivity of KCl solutions was very high compared with the other electrolyte solutions. pH increased from 9.9 to 10.96 in NaCl solutions,

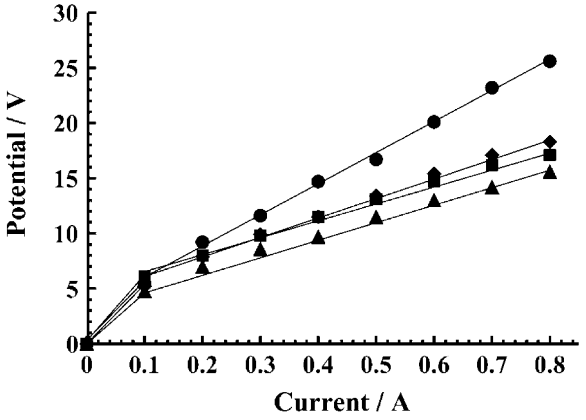

Fig. 2. The relationship between electric potential (*V*) and electric current (*A*) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

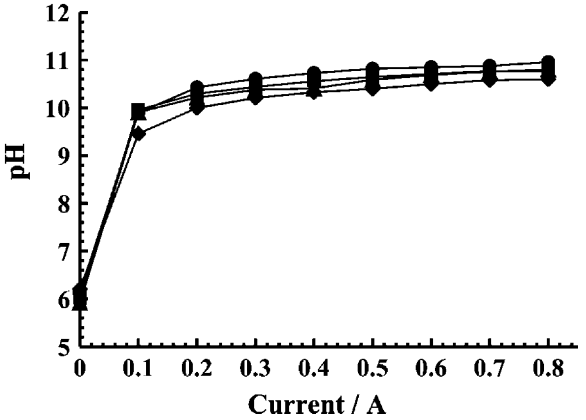

Fig. 4. The relationship between pH and electric current (*A*) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

9.91 to 10.77 in KCl solutions, 9.46 to 10.6 in $MgCl_2$ solutions and 9.96 to 10.77 in $CaCl_2$ solutions when the current was increased from 0.1 to 0.8 A. ORP decreased from −50 mV to −170 mV in NaCl solutions, −51 mV to −179 mV in KCl solutions, −70 mV to −176 mV in $MgCl_2$ solutions and −73 mV to −137 mV in $CaCl_2$ solutions when the current was increased from 0.1 to 0.8 A. DO decreased from 6.97 mg l^{-1} to 6.28 mg l^{-1} in NaCl solutions, 6.9 mg l^{-1} to 6.2 mg l^{-1} in KCl solutions, 6.63 mg l^{-1} to 6.03 mg l^{-1} in $MgCl_2$ solutions and 6.61 mg l^{-1} to 6.18 mg l^{-1} in $CaCl_2$ solutions, respectively.

Fig. 7 shows the superoxide dismutation activity of L-ascorbic acid in the reduced water of NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions, and the control solutions adjusted to the same pH and concentration, respectively. The results were obtained through ESR measurements conducted using 10 mM L-ascorbic acid as proton donor. Electrolysis of 2 mM NaCl and KCl, and 1 mM $MgCl_2$ and

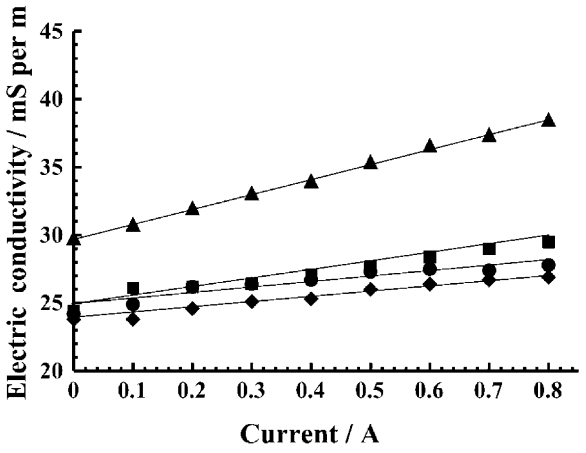

Fig. 3. The relationship between electric conductivity (mS per m) and electric current (*A*) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

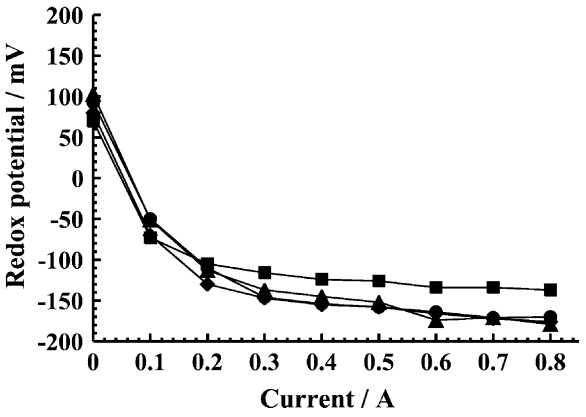

Fig. 5. The relationship between redox potential (mV) and electric current (*A*) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

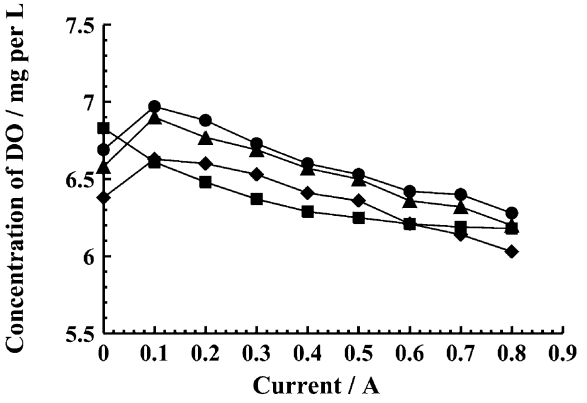

Fig. 6. The relationship between DO and electric current (*A*) using various electrolyte solutions. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

$CaCl_2$ solutions increased the superoxide dismutation activity from 119 to 173 units per 20 ml, 115 to 154.3 units per 20 ml, 105 to 140 units per 20 ml, and 128 to 165 units per 20 ml for L-ascorbic acid, respectively, compared to the same solutions without electrolysis adjusted to the same pH as the reduced water,. In the previous paper, we demonstrated that the reduced water did not show any dismutation activity without added ascorbic acid. These results show the enhancement of superoxide dismutation activity by electrolysis compared the control solutions.

Fig. 8 shows the results of the ratio of concentration of cations to that of anions, $[\text{cations}]_{\text{cathode}}/[\text{anions}]_{\text{cathode}}$. The ratio of $[\text{cations}]_{\text{cathode}}/[\text{anions}]_{\text{cathode}}$ increased in the order of $CaCl_2$, $<MgCl_2$, $<$KCl, $<$NaCl. The value of $CaCl_2$ was negative between 0.1 and 0.8 A of electric current. For KCl and $MgCl_2$ the ratio was nearly 1. The NaCl solution, showed the highest value (between 3.848 and 4.151).

In order to confirm the difference in consumed amount for neutralization titration between the reduced water and the sample solution, the solutions were titrated using a 10 mM AsA solution from pH 10.05 to 10.95. As the result, the consumed amount of 10 mM AsA solution for the reduced water was lower than that of sample solutions at all pH levels as shown in Fig. 9.

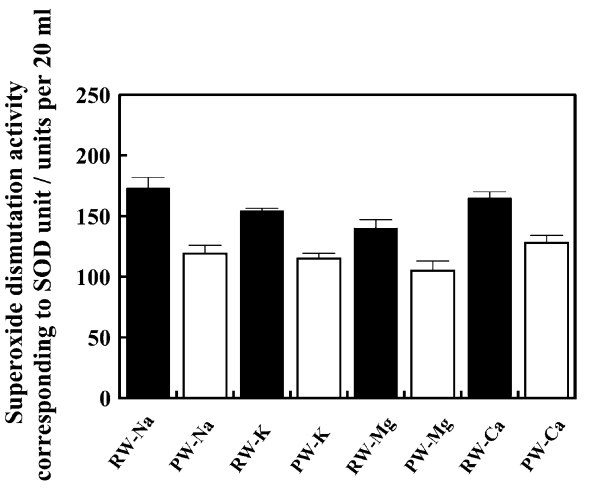

Fig. 7. Superoxide dismutation activity of L-ascorbic acid for the reduced water and control solutions adjusted to the same pH and initial concentration of electrolyte solutions (2 mM NaCl and KCl, and 1 mM $MgCl_2$ and $CaCl_2$), (RW–Na) the reduced water from 2 mM NaCl solutions, (RW–K) the reduced water from 2 mM KCl solutions, (RW–Mg) the reduced water from 1 mM $MgCl_2$ solutions and (RW–Ca) reduced water from 1 mM $CaCl_2$, (PW–Na) the control 2 mM NaCl solutions and (PW–K) the control 2 mM KCl solutions, and (PW–Mg) the control 1 mM $MgCl_2$ solutions and (PW–Ca) the control 1 mM $CaCl_2$ solution.

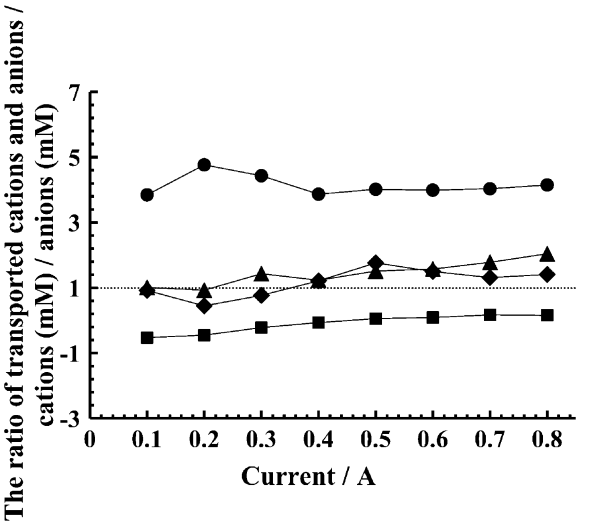

Fig. 8. The ratio of the concentration of cations and anions in the cathode compartment corresponding to electric current (*A*). (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$.

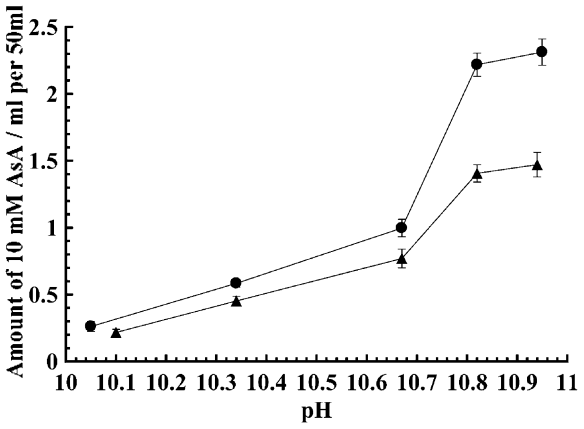

Fig. 9. The amount of consumed AsA for the reduced water and the control solutions by neutralization titration corresponding to pH. (●) the control solutions of NaOH and (▲) the reduced water.

Fig. 10 shows the results of $p(IP)_{RW}$ values for the reduced water of NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions and control solutions of NaCl and KCl. $p(IP)_{RW}$ decreased in reduced NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions in proportion to the increase in pH, but control solutions of NaCl and KCl showed no change of $p(IP)_{RW}$ between pH:9.8 and pH:10.96. The $p(IP)_{RW}$ values of the reduced water of the electrolyte solutions decreased from 14.058 to 13.474. However, there was almost no change in $p(IP)_{CS}$ between pH:9.8 and pH:10.96 in the control solutions.

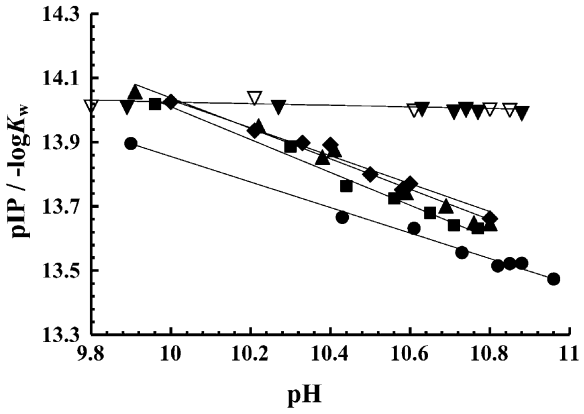

Fig. 10. The relationship between pIP ($-\log K_W$) and pH for the reduced water made from NaCl, KCl, $MgCl_2$ and $CaCl_2$, and control solutions of NaCl and KCl. (●) NaCl, (▲) KCl, (♦) $MgCl_2$ and (■) $CaCl_2$ for the reduced water, and (▼) NaCl and (▽) KCl for the control solutions.

Table 1
The increase, $(\Delta S^{\circ})_{A=0.8}-(\Delta S^{\circ})_{A=0}$, of entropy between 0 and 0.8 A of electric current for the reduced water made from NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions

Current A	NaCl 2 mM	KCl 2 mM	$CaCl_2$ 1 mM	$MgCl_2$ 1 mM
0	0	0	0	0
0.8	2.4	1.6	1.6	1.1

Eq. (27) shows the entropy of $p(IP)_{RW}$. We can estimate the increase in entropy by using Eq. (27). Table 1 shows the estimated entropy increase in the reduced water from the sample solutions. The reduced water with 2 mM NaCl solution showed the largest entropy increase (2.4 Kcal/mol). Conversely, the reduced water of $MgCl_2$ showed the lowest entropy increase (1.1 Kcal/mol).

The effects of reduced water on oxidative DNA damage are presented in Figs. 11 and 12. In the present study, oxidative DNA damage is clearly

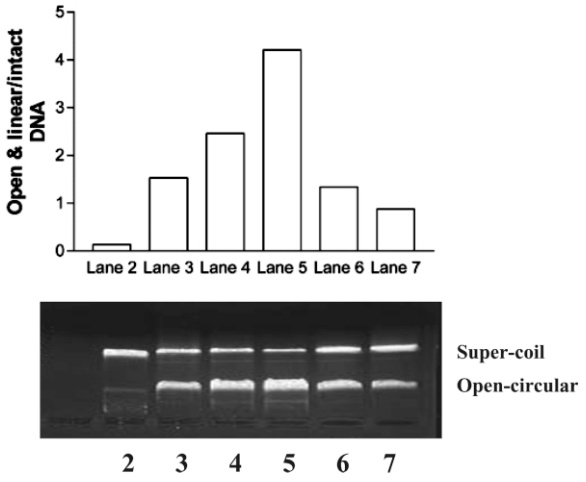

Fig. 11. Effect of electrolyzed water on hydrogen peroxide induced DNA damage. Lane 1: Negative control; Lane 2: Control; Lane 3: $H_2O_2+Cu(II)$; Lane 4: $H_2O_2+Cu(II)+KOH$ solution; Lane 5: $H_2O_2+Cu(II)+2$ mM KCl solution (0 A), pH 6.30; Lane 6: $H_2O_2+Cu(II)+$electrolyzed reduced water (0.4 A), pH 10.47; and Lane 7: $H_2O_2+Cu(II)+$electrolyzed reduced water (0.8 A), pH 10.74.

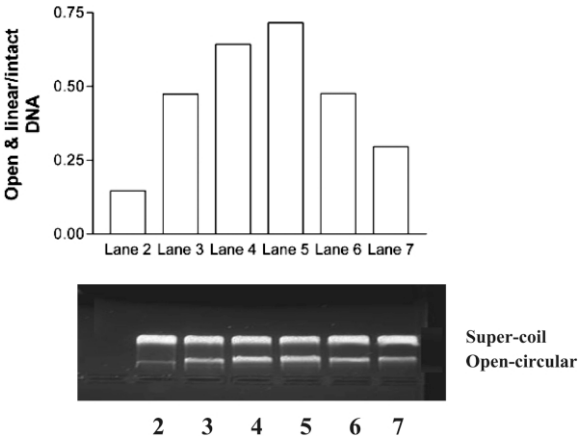

Fig. 12. Effect of electrolyzed water on hydroquinone (HQ) induced DNA damage. Lane 1: Negative control; Lane 2: Control; Lane 3: HQ + Cu(II); Lane 4: HQ + Cu(II) + KOH solution; Lane 5: HQ + Cu(II) + 2 mM KCl solution (0 A), pH 6.30; Lane 6: HQ + Cu(II) + electrolyzed reduced water (0.4 A), pH 10.47; and Lane 7: HQ + Cu(II) + electrolyzed reduced water (0.8 A), pH 10.74.

evident in the H_2O_2 /Cu (II) and the HQ/Cu (II) systems (lane 3). Densitometric results, expressed as the fraction of DNA converted to open circular and linear forms, clearly showed that reduced water significantly inhibited oxidative stress induced DNA damage in both H_2O_2/Cu (II) and HQ/Cu (II) systems (lanes 6 and 7) compared to the control solution passed through the apparatus with no voltage applied (lane 5).

4. Discussion

4.1. The characterization of solutes in the reduced water

The parameters related to solutes in water reduced by electrolysis are pH, ORP, DO, DH and EC. pH is the indicator which shows the hydrogen ion concentration. When electrolysis is carried out in electrolyte solutions such as NaCl, KCl, $MgCl_2$ or $CaCl_2$ the pH of the reduced water produced in the cathode compartment will be measured as hydrolysis of carbonates of those electrolytes. Therefore, the pH value depends on the initial concentration of the carbonates and the parameters of hydrolysis. For example, Na_2CO_3 shows pH:9.2 to 10.6 between 10^{-6} mol l^{-1} and 10^{-3} mol l^{-1} and in $NaHCO_3$, pH:7.2 to 8.7 between 10^{-6} mol l^{-1} and 10^{-3} mol l^{-1}, respectively, [17]. At 25 °C, 1.15×10^{-2} mM carbon dioxide is dissolved in pure water. As mentioned above, when sufficiently dilute electrolyte solutions are used for the reduced water, carbonates cannot be neglected. ORP is the oxidation reduction potential and it can be estimated from the Nernst equation as follows,

$$E_V = E_V^0 + 2.303RT/nF\log(\mathrm{ox})/(\mathrm{red}) \quad (28)$$

Where, E_V, E_V^0, R, T, n, F, (ox) and (red) are the oxidation reduction potential, standard potential, gas constant, absolute temperature, number of electrons transferred in the reaction, Faraday constant, product of the activities of the oxidized species and product of activities of reduced species, respectively. Thus, in the case of reduced water, as oxidation and reduction are included for oxygen in the original water and hydrogen in the reduced water, the ORP equation will be shown as Eq. (29)

$$E_V = E_V^0 + (n)\mathrm{pH} + (m)_\mathrm{O}\log[\mathrm{O_2}] + (m)_\mathrm{H}\log[\mathrm{H_2}] \quad (29)$$

Where, (n), $(m)_\mathrm{O}$ and $(m)_\mathrm{H}$ are coefficients relative to pH, O_2 and H_2 [18]. Therefore, ORP is the parameter indicating the concentrations of oxygen and hydrogen dissolved in the reduced water. As DO and DH are the concentrations of oxygen and hydrogen in the reduced water, they are parameters relative to solutes in it. Those solutes will be generated by the process of electrolysis.

The electric potential at each level of electric current was higher in NaCl solution than that in KCl, and $MgCl_2$ solutions, which was higher than in $CaCl_2$ solution as shown in Fig. 2. It is assumed that the difference in ionic mobility between cations and anions will be proportional to the electric potential. For example, the ratio of Cl^- ions to Na^+ ions is 0.767, that of Cl^- ions to K^+ ions is 0.987, and that of Cl ions to Mg^{2+} ions is 0.713 and Cl^- ions to Ca^{2+} is 0.799. It is strongly suggested that the higher the ratio of anions to cations, the lower the electric potential gradient.

Results for electrical conductivity were contrary to those for electrical potential as mentioned above. This is a reasonable result. Furthermore, pH, ORP and DO also changed as expected in proportion to the magnitude of the electrical field energy.

4.2. The enhancement of superoxide dismutation activity in reduced water

Reduced water prepared from all of the electrolyte solutions (NaCl, KCl, $MgCl_2$ and $CaCl_2$) had higher superoxide dismutation activity, when measured using AsA as an antioxidant indicator, than the corresponding control solutions adjusted to the same pH (Fig. 7). AsA has the structure of a 2,3-enediol which is a kind of saccharic acid, and it plays a role in the protection system against oxidative stress in animals and plants [19,20]. The OH at the 2nd functional group has p*K*a = 11.79 and that at the 3rd functional group has p*K*a = 4.25. If AsA is added to reduced water and control solutions which are adjusted to the same pH as the reduced water, the proton on the OH of the 3rd functional group will react with the OH ions of the reduced water and the OH ions in control solutions, and will be neutralized because the OH of the 3rd functional group of AsA is dissociated as shown in Eq. (30),

$$-\mathrm{OH} \rightarrow \mathrm{O}^- + \mathrm{H}^+ \tag{30}$$

Furthermore, it is considered that proton in OH of the third functional group of AsA will be used for the neutralization of the alkaline reduced water and proton in that of the 2nd functional group of AsA which has very low dissociation activity at a p*K*a = 11.79 will be used for the superoxide radical dismutation reaction [21,22]. If AsA is mixed with reduced water, the dissociation activity will increase because of the higher dissociation activity of the reduced water. The higher dissociation activity of the reduced water increases the dissociation activity of substances with lower activity. As shown in Fig. 9, when the neutralization titration was carried out at several different pH levels in reduced water and the control solutions of 2 mM NaOH, there were significant differences at each pH. It is considered that the increase in dissociation of protons at the 3rd functional group of AsA, dissolved in the reduced water accounts for the significant difference between the reduced water and the control solutions. This property is due to water molecules as solvent and also a very stable molecular structure. As described in a previous paper, the increase in dissociation activity of the reduced water is due to the process of electrolysis [10]. When a sufficiently large electric field is applied to the boundary between the electrode and the electrolyte bulk solutions, a very high electric potential will be generated (above 10^7 V cm^{-1}). If a sufficient amount of reduced water is taken into the body, it will increase the dissociation activity for the water-soluble antioxidant substances of relatively lower dissociation activity. As a result, we speculate that their antioxidant capacity will be enhanced. It is quite possible that many useful phenomena related to reduced water will be found to be the result of the increase of dissociation activity of water as solvent by electrolysis. The enhancement of the reduced water will depend on the ionic properties of the solutes such as species and membrane or ionic mobility [23,24]. When a non-charged membrane is used for the production of reduced water a greater difference in ionic mobility between cations and anions will produce higher dissociation activity as shown in Fig. 8. In general, the dissociation activity of water depends on the temperature and pressure. The ionic product of water p(K_w) has been calculated in a wide range of temperatures (0–600) and densities by H. Sato et al. [25].

The difference between $p(IP)_{RW}$ for reduced water and $p(IP)_{CS}$ for control solutions is shown in Eq. (31).

$$\Delta p(IP) = p(IP)_{CS} - p(IP)_{RW} \tag{31}$$

It is considered that the enhancement of antioxidant effects for superoxide radicals will increase in proportion to the increase of $\Delta p(IP)$. Therefore, $\Delta p(IP)$ for reduced water as solvent is the essential parameter.

4.3. The estimation of pIP and entropy produced by electrolysis in NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions

Fig. 10 shows estimates of $p(IP)_{RW}$ for reduced water made from NaCl, KCl, $MgCl_2$, $CaCl_2$, and control solutions using NaCl and KCl. These results showed significant differences between reduced water and control solutions adjusted to the same pH and initial concentration as the reduced water before electrolysis. In general, ΔG^0 is related to the dissociation constant K and the electric potential E° at the equilibrium state as shown in Eqs. (25) and (27), can be shown as Eq. (32)

$$\Delta G^0 = -nFE^\circ \tag{32}$$

E° and hydrogen ionic concentration can be shown as Eq. (33).

$$E^\circ = (RT/mnF)\ln[H^+] \tag{33}$$

Where m is the coefficient of the correction of the relationship between E° and pH at $K_W = 10^{-14}$.

$$\log IP = 2.303(RT/mnF)\log[H^+] + C \tag{34}$$

Thus, pIP is described as Eq. (35).

$$p(IP)_{RW} = (0.0591/m)pH + C \tag{35}$$

The plot of $p(IP)_{RW}$ vs pH is a straight line with a slope of (0.0591/m) and a constant. The constant C will depend on the properties of electrolytes and m will depend on the properties of the membrane and the interaction between the membrane and the ions or solvent. The $p(IP)_{CS}$ of the control solutions did not change with a change of pH from 9.8 to 10.88 but that of all sample solutions changed linearly with a change of pH from 9.9 to 10.9. The $p(IP)_{RW}$ of the reduced water decreased in proportion to the increase in pH. This change will depend on the magnitude of the applied electric current. Therefore, the dissociation energy depends on the electric current used during electrolysis.

Entropy is estimated by Eq. (31) using IP instead of K_W. Table 1 shows the entropy difference of each electrolyte solution at 0.8 A $(\Delta S^\circ)_{A=0.8} - (\Delta S^\circ)_{A=0}$. The entropy increased in proportion to the increase of $\Delta p(IP)_w$. The increase of entropy means that the reduced water became more active and offered a higher reaction field for dissolved substances. Thus, if substances of lower dissociation activity are dissolved in the reduced water the dissociation activity will increase compared to non-electrolyzed water.

4.4. The inhibitive effect of reduced water on the single-strand breakage of DNA by using H_2O_2/Cu (II) and HQ/Cu system

In the present study, the effects of reduced water on oxidative DNA damage were investigated by using H_2O_2/Cu (II) and HQ/Cu (II) systems. Induction of single-strand breaks in the supercoiled double-stranded ΦX174 plasmid DNA leads to formation of open circular DNA, while the formation of a linear form of DNA is indicative of double-strand breaks [15]. Cu (II), H_2O_2 is able to cause strand breaks in isolated DNA. As such, H_2O_2/Cu (II) has been widely used as a model system to induce oxidative DNA damage [16,26]. Although the exact reactive species remain to be chemically defined, a bound hydroxyl radical or its equivalent derived from the reaction of H_2O_2 and copper has been suggested to participate in the oxidative DNA damage [15,26]. The addition of reduced water to H_2O_2/Cu (II) resulted in marked inhibition of conversion of supercoiled DNA to open circular forms, suggesting that reduced water is capable of protecting against the H_2O_2/Cu (II)-mediated DNA strand breaks. To further determine the inhibitory effects of reduced water on oxidative DNA damage, HQ+Cu (II) was used in the present study. It has been previously shown that the HQ/Cu (II) system is able to induce DNA breaks, with both Cu (II)/Cu (I) redox cycle and H_2O_2 being critically involved. Similar to what was observed with the H_2O_2/Cu (II), the presence of reduced water also markedly protected against DNA strand breaks induced by the HQ/Cu (II) system. Therefore, the results suggest that reduced water can prevent oxidative DNA damage possibly by enhanced antioxidant effects against superoxide anion radicals as shown

originally by S. Shirahata et al. [27]. Thus, it appears that consumption of electrolyzed reduced drinking water may potentially serve to prevent DNA damage induced by oxygen free radicals produced by the mitochondria due to the rise in oxidative stress.

Acknowledgments

We would like to thank Dr Yoshiaki Matsuo and Dr Dick Wullaert for stimulating discussion and encouragement.

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 234, 269–274 (1997)
ARTICLE NO. RC976622

Electrolyzed–Reduced Water Scavenges Active Oxygen Species and Protects DNA from Oxidative Damage

Sanetaka Shirahata,[1] Shigeru Kabayama, Mariko Nakano, Takumi Miura, Kenichi Kusumoto, Miho Gotoh, Hidemitsu Hayashi,* Kazumichi Otsubo,** Shinkatsu Morisawa,** and Yoshinori Katakura

*Institute of Cellular Regulation Technology, Graduate School of Genetic Resources Technology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-81, Japan; *Water Institute, Nisshin Building 9F, 2-5-10 Shinjuku, Tokyo 160, Japan; and **Nihon Trim Co. Ltd., Meiji Seimei Jusou Building 6F, 1-2-13 Shinkitano, Yodogawa-ku, Osaka 532, Japan*

Received March 21, 1997

Active oxygen species or free radicals are considered to cause extensive oxidative damage to biological macromolecules, which brings about a variety of diseases as well as aging. The ideal scavenger for active oxygen should be 'active hydrogen'. 'Active hydrogen' can be produced in reduced water near the cathode during electrolysis of water. Reduced water exhibits high pH, low dissolved oxygen (DO), extremely high dissolved molecular hydrogen (DH), and extremely negative redox potential (RP) values. Strongly electrolyzed–reduced water, as well as ascorbic acid, (+)-catechin and tannic acid, completely scavenged $O_2^{\cdot-}$ produced by the hypoxanthine-xanthine oxidase (HX-XOD) system in sodium phosphate buffer (pH 7.0). The superoxide dismutase (SOD)-like activity of reduced water is stable at 4°C for over a month and was not lost even after neutralization, repeated freezing and melting, deflation with sonication, vigorous mixing, boiling, repeated filtration, or closed autoclaving, but was lost by opened autoclaving or by closed autoclaving in the presence of tungsten trioxide which efficiently adsorbs active atomic hydrogen. Water bubbled with hydrogen gas exhibited low DO, extremely high DH and extremely low RP values, as does reduced water, but it has no SOD-like activity. These results suggest that the SOD-like activity of reduced water is not due to the dissolved molecular hydrogen but due to the dissolved atomic hydrogen (active hydrogen). Although SOD accumulated H_2O_2 when added to the HX-XOD system, reduced water decreased the amount of H_2O_2 produced by XOD. Reduced water, as well as catalase and ascorbic acid, could directly scavenge H_2O_2. Reduced water suppresses single-strand breakage of DNA by active oxygen species produced by the Cu(II)-catalyzed oxidation of ascorbic acid in a dose-dependent manner, suggesting that reduced water can scavenge not only $O_2^{\cdot-}$ and H_2O_2, but also $^1O_2$ and $^{\cdot}OH$.

Active oxygen species or free radicals, such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) are considered to cause extensive oxidative damage to biological macromolecules (DNA, membrane polyunsaturated fatty acid chains, enzymes and so on), which bring about a variety of diseases, as well as aging (1, 2). We believe that the ideal countermeasure against active oxygen is 'active hydrogen'. Electrolysis of water produces reduced and oxidized water near the cathode and anode, respectively. Reduced water exhibits high pH, low dissolved oxygen (DO), high dissolved hydrogen (DH) and significant negative redox potential (RP) values. Soft water in Japan made it possible to develop domestic devices to reform water by electrolysis about half a century ago.

So far, the characteristics of neither reduced water nor oxidized water have been well clarified. Based upon the interesting clinical improvement of a variety of diseases by intake of reduced water since 1985, Hayashi proposed the hypothesis 'Water Regulating Theory' (3). Here, based on his theory we first demonstrate that reduced water scavenges active oxygen species and protects DNA from damage by oxygen radicals.

MATERIALS AND METHODS

Electrolysis of water. Ultrapure water produced by an ultrapure system (ULTRAPUR LV-10T, TORAY, Tokyo) was added 0.1 g/l NaCl

[1] To whom correspondence should be addressed. Fax: 81-92-642-3052. E-mail: sirahata@grt.kyushu-u.ac.jp.

Abbreviations: AET, 2-(aminoethyl)isothiuronium; AsA, ascorbic acid; CL, chemiluminescence; CLA, *Cypridina* luciferin analog; DO, dissolved oxygen; DH, dissolved hydrogen, EC, electrical conductance; HX, hypoxanthine; RP, redox potential; SOD, superoxide dismutase; XOD, xanthine oxidase.

0006-291X/97 $25.00

to elevate electrical conductance (EC) to about 20 ms/m. The water was then electrolyzed with various voltages by an electrolyzing device (Type TI-7000S and TI-7000SL, Nihon Trim Co., Osaka) equipped with a platinum-coated titanium electrode to produce reduced water which exhibited various RP. RP, EC, DO and DH were measured using a RP meter (type, HM-14P), a EC meter (CM-14P), a DO meter (DO-14P) and a DH meter (DHDI-1) from Toa Electronics Ltd. (Tokyo) at 25°C. pH was measured using a pH meter (Beckman, Type pHI32) at 25°C.

Assay of the SOD-like activity of reduced water, ascorbic acid (AsA), (+)-catechin, and tannic acid. The reaction mixture (1 ml) for measuring chemiluminiescence (CL) intensity specific to $O_2^{\bullet-}$ contained 500 mM hypoxanthine (HX), 200 mM EDTA, 40 mM sodium phosphate buffer (pH 7.0), 0.6 ml of the reduced water or NaOH solution of the same pH as reduced water, 2.5 mM 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a] pyrazin-3-one (*Cypridina* luciferin analog (CLA), Tokyo Kasei Industrial Co., Tokyo) and 0.5 U/l of xanthine oxidase (XOD) (Wako Purechemical Industries, Tokyo). The CL intensity of the reaction mixture (0.85 ml) except for XOD solution was measured in a glass-tube in a CL reader (Aloka, type BLR-301) at 26 °C. At 18 seconds time point, 0.15 ml of XOD solution was injected into the tube and the CL intensity was continuously measured for 120 sec. The change of the CL intensity in the presence of reduced water, SOD derived from bovine erythrocytes (Sigma), AsA (Wako), (+)-catechin (Wako) or tannic acid (Wako) was measured with the HX-XOD system to evaluate the scavenging activity of $O_2^{\bullet-}$. The strongly reduced water (RP, −820 mV; pH 11.0) was diluted with a NaOH solution (pH 11.0) to assure the same final pH of the reaction mixture (pH 7.3) among the test samples. The extinguishing rate of $O_2^{\bullet-}$ (%) was calculated by dividing the total CL intensity of the test sample by that of the control.

Analysis of catalase-like activity of reduced water and AsA. The reaction mixture (100 μl) of the HX-XOD system described above in the presence or absence of SOD or reduced water was put into a 96-well microplate and incubated at 37 °C. Time course of the change in the H_2O_2 concentration was measured by the addition of 200 μl of substrate containing peroxidase (0.3 mg/ml 2,2′-azino-di-(3-ethyl benzthiazolin sulfonic acid), 0.1 M citrate buffer, pH 4.0, streptavidine-horseradish peroxidase conjugate (Amersham) diluted to 1000 times). Absorbency of the reaction product was measured at 405 nm by an ELISA reader. In order to examine the catalase-like activity, the H_2O_2 solution was directly incubated with control NaOH solution (pH 11.0), reduced water (pH 11.0), AsA or catalase (Wako) in 40 mM sodium phosphate buffer (pH 7.0) at 37 °C and the H_2O_2 concentrations were determined.

Analysis of single-strand breakage of DNA caused by active oxygen species produced by the Cu(II)-catalyzed oxidation of AsA. Time-course of the single-strand DNA breaking reaction caused by Cu(II)-catalyzed oxidation of AsA was examined by using super-coil plasmid DNA. One μg of pBluescript II plasmid DNA (Stratagene, La Jolla, CA) was incubated with the mixture of 25 μM AsA and 25 μM $CuSO_4$ in 20 μl of 20 mM sodium phosphate buffer (pH 7.5) containing 17 μl of reduced water, NaOH solution of the same pH as the reduced water, SOD, catalase, 2-(Aminoethyl)isothiuronium (AET)(Wako), KI (Wako), or NaN_3 (Wako) at 37°C for various periods of time. The reaction was stopped by adding 4 μl of 15 mM EDTA (pH 7.0). Super-coil DNA, open-circular DNA and linear DNA were separated by electrophoresis with 1% agarose gel and any changes of the amount of super-coil DNA were determined from the photo images by a NIHimage computer software.

RESULTS AND DISCUSSION

Characteristics of electrolyzed–reduced water. The principle of electrolysis was founded by Michael Faraday (1791-1867). In this process, reduction occurs at the cathode and oxidation at the anode. Dissociation of H_2O produces H^+ and OH^- ions. At the cathode, H^+ ions gain electrons to change into active atomic hydrogen (H). Active atomic hydrogen exhibits high reducing potential. It is then changed to hydrogen molecules (H_2) which are chemically inert at room temperature. At the anode, OH^- ions lose electrons to form OH, which results in the production of O_2 and H_2O. Cathodic alkaline water (reduced water) is abundant in DH, whereas anodic acidic water (oxidized water) is abundant in DO. The relationships of RP with pH, DO, and DH in reduced water were shown in FIG. 1. Marked changes in these values occur in water after electrolysis. It should

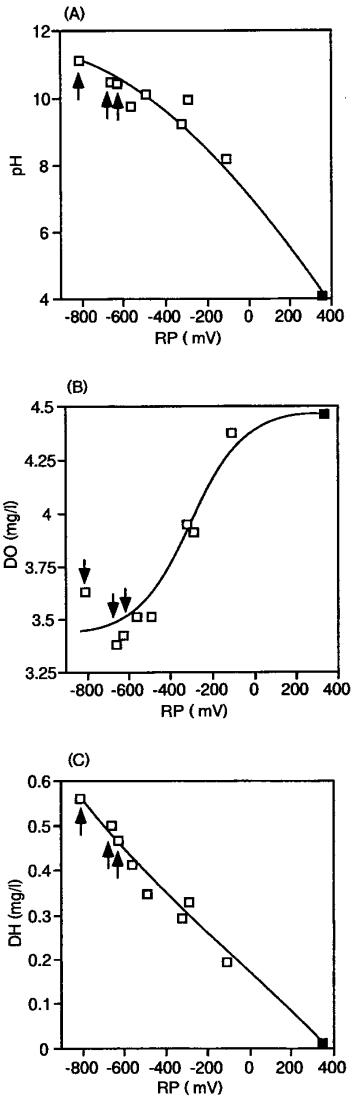

FIG. 1. Relationships of RP with pH (A), DO (B) and DH (C) in electrolyzed-reduced water. Ultrapure water was electrolyzed by an electrolyzing device and RP, pH, DO and DH values were immediately measured. The data of water before electrolysis was shown by dark squares. Arrows show the reduced water which exhibited the strong SOD-like activity.

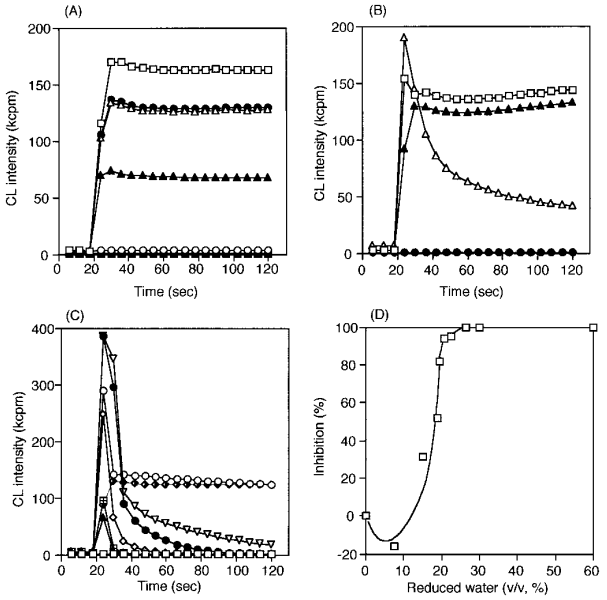

FIG. 2. Analysis of the SOD-like activity of reduced water. (A) Effects of reduced water, SOD, H_2-water and N_2-water on the accumulation of $O_2^{\bullet-}$ generated by the HX-XOD system. The CL intensity was determined in phosphate buffer (pH 7.0) in the presence of reduced water (RP −820 mV, pH 11.0; IC_{50}SO(18%)), a NaOH solution of the same pH as reduced water, SOD, H_2-water, or N_2-water. □, control NaOH solution; ■, reduced water; ▲, SOD (0.13 U/ml); ○, SOD (66.7 U/ml); ●, H_2-water; △, N_2-water. (B) Stability of the SOD-like activity of reduced water. □, control NaOH solution. ●, reduced water and reduced water treated with repeated freezing and melting, deflation with sonication, vigorous mixing for 10 minutes, boiling for 10 minutes, repeated filtration with 0.22μm filter, or closed autoclaving at 121°C for 20 minutes. ▲, reduced water treated with opened autoclaving at 121°C for 20 minutes. △, reduced water treated with closed autoclaving in the presence of tungsten trioxide (0.5 g/70ml reduced water) at 121°C for 20 minutes. (C) The SOD-like activity of diluted reduced water. Amount of reduced water (%): □, 60%; +, 30%; *, 26%; ▲, 23%; □, 21%; ◇, 20%; ●, 19%; ▽, 15%; ○, 7.5%; ◆, 0% (control). (D) Inhibition of the accumulation of $O_2^{\bullet-}$ by variously diluted reduced water. All experiments were triplicated and the average values were shown in the figures. The SD errors were within 5%.

be noticed that the DH value is higher in reduced water than in the original water by two orders of magnitude.

The SOD-like activity of reduced water. XOD oxidizes HX to xanthine, coupling to generation of $O_2^{\bullet-}$ from O_2. CLA specifically reacts with $O_2^{\bullet-}$ and $^1O_2$ and emits a CL (4). As shown in FIG. 2A, reduced water completely inhibited the CL, demonstrating the SOD-like activity of reduced water. Since the CL was completely inhibited by SOD, the CL was specific to $O_2^{\bullet-}$.

Bubbling of hydrogen gas into a NaOH solution (pH 10.5) for 5 minutes resulted in remarkable changes of RP from +116 mV to −842 mV; DO from 7.66 mg/l to 1.98 mg/l; DH from 0.0002 mg/l to 0.938 mg/l; but no change of pH. Bubbling of nitrogen gas into a NaOH solution (pH 10.5) resulted in changes of RP from +116 mV to +83 mV; DO from 7.66 mg/l to 1.82 mg/l; DH from 0.0002 mg/l to 0.0001 mg/l; but no change of pH. As shown in FIG. 2A, water bubbled with hydrogen gas (H_2-water) and nitrogen gas (N_2-water) showed decreased CL intensity by about 20% as compared to that of the control in the steady state, suggesting that the decreased CL intensity in N_2-water and H_2-water was due to low DO and H_2-water had no SOD-like activity. The SOD-like activity of reduced water is retained during storage, although the RP and DH values exhibit decay. These results clearly indicate that dissolved molecular hydrogen gas in reduced water was responsible for the negative RP value, but not for the SOD-like activity.

Stability of the SOD-like activity of reduced water. The SOD-like activity of reduced water was very stable in a closed glass bottle at 4°C for over a month. The activity was not lost even after neutralization, repeated freezing and melting, deflation with sonication for 10 minutes, repeated filtration with a 0.22 μm filter, boiling for 10 minutes, or autoclaving in a closed glass bottle at 121°C for 20 minutes (FIG. 2B). However, 90% the SOD-like activity was lost by autoclaving in an opened glass bottle, suggesting that the active substance in the reduced water is volatile. Atomic hydrogen is volatile and can reduce metallic oxide such as tungsten trioxide, though molecular hydrogen cannot easily do this (5). A sensitive detection method of atomic hydrogen is based on the color change of tungsten trioxide by reduction (6). The closed autoclaving of reduced water with tungsten trioxide at 121°C for 20 minutes resulted in the loss of 52% of the SOD-like activity (FIG. 2B). These results strongly suggest that the substance responsible for the SOD-like activity of reduced water is active atomic hydrogen. Active hydrogen in gas phase is rather stable taking into consideration of the collision rate (7). Since two atoms of hydrogen in the ground state have larger energy than molecular hydrogen in any stable state, the extra energy is required to be eliminated by a third substance to produce a hydrogen molecule from two atoms of hydrogen by collision (8). Atomic hydrogen can stably exist avoiding the attack by oxygen in solution and crystals at room temperature for a period longer than a year (9). Water vapor is known to prevent the recombination of atomic hydrogen on a tungsten surface (8). The characteristics in aqueous solution of active atomic hydrogen produced by electronic discharge is under investigation.

When XOD solution was injected into the reaction mixture containing the diluted reduced water, strong CL was emitted immediately after injection (FIG. 2C), but the intensity of CL rapidly dropped below the control value. The initial strong transient emission of CL in diluted reduced water could be inhibited by SOD, indicating that a large amount of $O_2^{\bullet-}$ was transiently generated just after the addition of XOD. AsA, (+)-

catechin and tannic acid, as well as SOD, did not show such a strong initial transient emission of CL (data not shown). Injection of the solvent without enzyme did not result in an emission of CL. More detailed experiments will be needed to clarify the mechanism of this phenomenon. The inhibitory effect of reduced water on the accumulation of $O_2^{\bullet-}$ was increased in a dose-dependent manner as shown in FIG. 2D, suggesting the stoichiometric action of active substance in reduced water.

Definition of the SOD-like activity of reduced water. Since this paper first reports the $O_2^{\bullet-}$ scavenging activity of electrolyzed-reduced water, the standardization of this activity is needed to compare the reducing potency of the reduced water prepared each time. In order to standardize the reducing potency of the reduced water, we defined a $IC_{50}SO$ unit as a reducing potency of which reduced water can scavenge 50% of $O_2^{\bullet-}$ generated by the HX-XOD system under the conditions described in the MATERIALS AND METHODS section and a $IC_{50}SO$ (%) as the concentration (%) of reduced water in which the 50% of $O_2^{\bullet-}$ generated by the HX-XOD system is scavenged. The $IC_{50}SO$ values of SOD, AsA, (+)-catechin, and tannic acid were 0.05 U/ml, 3 μM (0.53 μg/ml), 130 μM (38 μg/ml), and 33 μg/ml in the HX-XOD system used here.

Catalase-like activity of reduced water. Although reduced water could scavenge $O_2^{\bullet-}$, there was a possibility that reduced water inhibited the enzyme activity of XOD or inhibited the reaction between $O_2^{\bullet-}$ and the luciferin analog reagent. To eliminate this possibility, the production of H_2O_2 was determined in the HX-XOD system. XOD can produce not only $O_2^{\bullet-}$ but also H_2O_2 in this HX-XOD system. As shown in FIG. 3A, XOD produced H_2O_2 even in the presence of reduced water, demonstrating no inhibition of the enzyme activity by reduced water. As expected, the addition of SOD to the HX-XOD system resulted in the accumulation of H_2O_2, indicating that $O_2^{\bullet-}$ produced by XOD was changed to H_2O_2 by SOD. Reduced water first accumulated H_2O_2 and then gradually lowered the concentration of H_2O_2, suggesting that reduced water exhibits not only SOD-like activity but also catalase-like activity. The fact that reduced water stimulated the accumulation of H_2O_2 in the HX-XOD system in the first 5 minutes indicated that the decreased CL intensity in the presence of reduced water is not due to the inhibition of the reaction between $O_2^{\bullet-}$ and luciferin analog by reduced water, but due to the conversion of $O_2^{\bullet-}$ into H_2O_2 by reduced water. To demonstrate the catalase-like activity of reduced water, H_2O_2 was directly incubated with reduced water. As shown in FIG. 3B, reduced water scavenged H_2O_2 as well as AsA and catalase.

Suppressive effect of reduced water on the single-strand breakage of DNA caused by the Cu(II)-catalyzed oxidation of AsA. The DNA strand breakage is caused by the mixture of AsA and Cu(II) (10, 11). The Cu(II)-catalyzed oxidation of AsA is known to produce $O_2^{\bullet-}$ and H_2O_2 which react to produce $^{\bullet}OH$ (12). In order to demonstrate that reduced water can scavenge not only $O_2^{\bullet-}$ and H_2O_2 but also other active oxygen species, we examined the effect of reduced water on DNA breakage caused by the mixture of AsA and Cu(II). Super-coil plasmid DNA (Form I) changes to open-circular DNA (Form II) by a single-strand breakage. Open-circular DNA changes to linear DNA (Form III) by a double-strand DNA breakage. When plasmid DNA was incu-

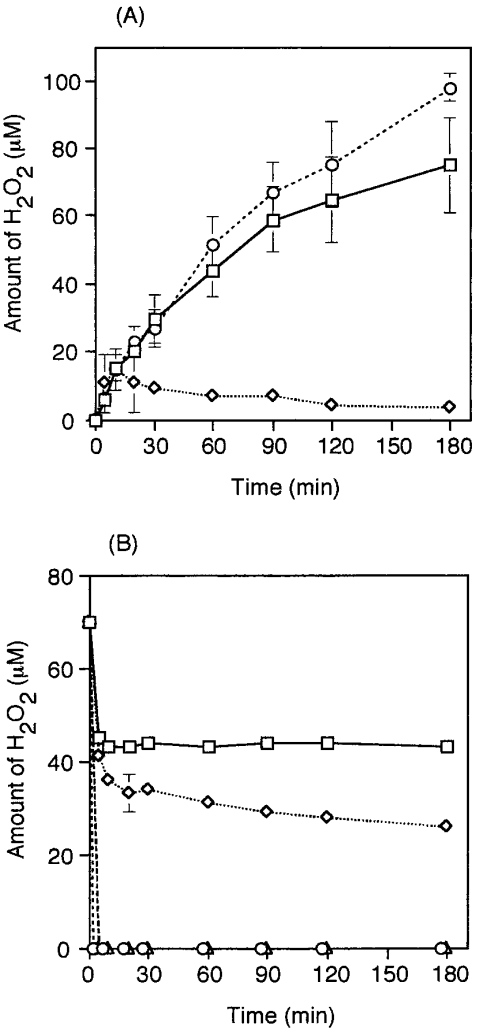

FIG. 3. The SOD-like and catalase-like activity of reduced water and AsA. (A) Change of the amount of H_2O_2 in the HX-XOD system in the presence of SOD or reduced water. SOD (70 U/ml) or reduced water (12 $IC_{50}SO$ units) were incubated in the HX-XOD system at 37 °C. □, control NaOH; ◇, reduced water; ○, SOD. (B) Degradation of H_2O_2 by reduced water, AsA and catalase. Reduced water (12 $IC_{50}SO$ units), AsA (100 μM) or catalase (20 U/ml) was incubated with H_2O_2 (70 μM) in 20 mM sodium phosphate buffer (pH 7.5) at 37 °C. □, control; ◇, reduced water; △, AsA; ○, catalase. All experiments were triplicated and the average values were shown. The standard errors were shown by vertical bars. In (B) most of the SD error bars are embedded in the symbols.

bated with the mixture of AsA and Cu(II), the amount of super-coil DNA gradually decreased (FIG. 4). However, reduced water significantly inhibited the single-strand breakage by the mixture of AsA and Cu(II). As shown in Table 1, reduced water inhibited the DNA breaking reaction in a dose-dependent manner. Catalase also inhibited the breaking reaction but SOD did not, indicating that H_2O_2 participated in the breaking reaction, but $O_2^{\bullet -}$ did not. Both radical scavengers specific to $^{\bullet}OH$ or $^1O_2$ inhibited the breaking reaction. These results indicated that reduced water can prevent DNA damage caused by active oxygen species such as H_2O_2, $^{\bullet}OH$, and $^1O_2$ produced by the Cu(II)-catalyzed oxidation of AsA. Significant synergistic effect between reduced water and the radical scavengers used here was not observed (data not shown). It is noteworthy that reduced water can prevent DNA damage even in the presence of metallic ions which catalyze the autooxidation of most established antioxidants and reverse their protective effect against active oxygen species. However, the water must have a higher reducing ability for this purpose than scavenging $O_2^{\bullet -}$.

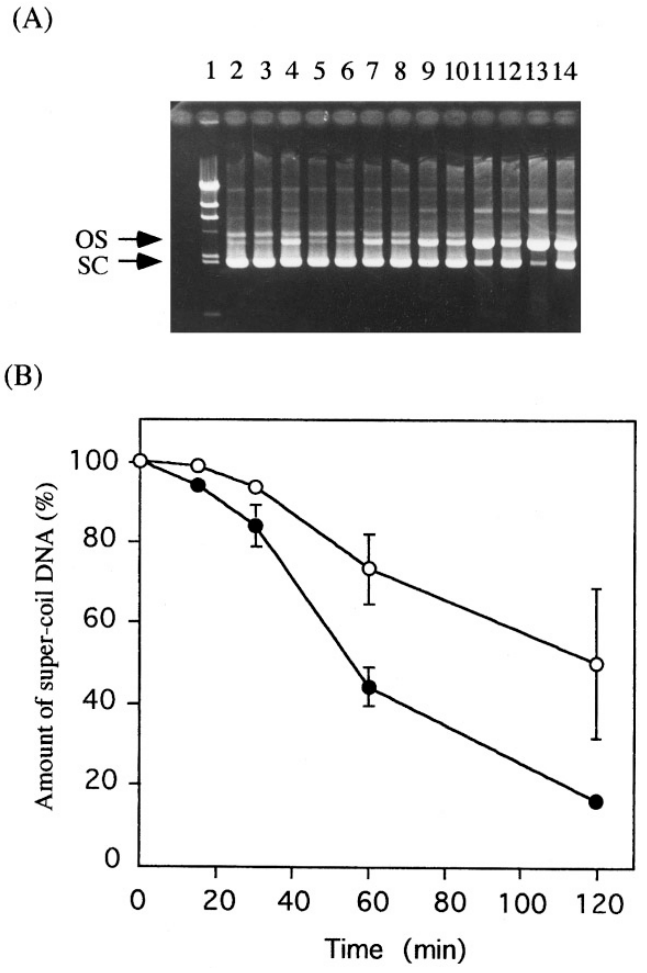

FIG. 4. Inhibitory effect of reduced water on single-strand breakage of DNA caused by oxygen radicals produced by the Cu(II)-catalyzed oxidation of AsA. (A) Electrophoresis of plasmid DNA treated with the mixture of AsA and Cu(II) in the presence of 4.1 $IC_{50}SO$ units of reduced water (RP, −659 mV; pH 10.5; $IC_{50}SO(18\%)$) or NaOH solution (pH 10.5). SC, super-coil DNA. OC, open-circular DNA. Lane 1, marker (λ DNA-HindIII); lane 2, control DNA (2 hours); lane 3, DNA + Cu(II) (2 hours); lane 4, DNA + AsA (2 hours). Lanes 5, 7, 9, 11 and 13 show the DNA breaking reaction by the mixture of AsA and Cu(II) in the presence of NaOH solution for 0, 15, 30, 60 and 120 minutes, respectively. Lanes 6, 8, 10, 12 and 14 show the DNA breaking reaction by the mixture of AsA and Cu(II) in the presence of reduced water for 0, 15, 30, 60 and 120 minutes, respectively. (B) Decrease of the relative amount (%) of super-coil DNA during the incubation of plasmid DNA with the mixture of AsA and Cu(II) in the presence of reduced water (○) or NaOH solution (●). Average values of two independent experiments (n=2, each) were shown. The vertical bars show the SD errors.

TABLE 1

Effect of Reduced Water, SOD, Catalase, and Various Radical Scavengers on Single-Strand Breakage of DNA by the Cu(II)-Catalyzed Oxidation of AsA

Addition	Concn	Specificity	Inhibin, %
Reduced water	3.7 $IC_{50}SO$ units		19
	7.5 $IC_{50}SO$ units		38
	15 $IC_{50}SO$ units		49
SOD	150 U/ml	$O_2^{\bullet -}$	2
Catalase	0.8 U/ml	H_2O_2	6
	4 U/ml	H_2O_2	36
	20 U/ml	H_2O_2	90
AET[a]	4×10^{-5} M	gereral	44
KI	1×10^{-2} M	$^{\bullet}OH$	18
	5×10^{-2} M	$^{\bullet}OH$	53
NaN_3	1×10^{-2} M	$^1O_2$	14
	5×10^{-2} M	$^1O_2$	43

[a] AET = 2-(aminoethyl)isothiuroium.

Although aerobic organisms have evolved by acquisition of the ability to utilize oxygen, oxygen is principally a toxic substance. Recently biological activation of hydrogen by hydrogenases was reported (13). Hydrogenases, which are among in the oldest enzymes (3.8 billion years old), can reversibly split molecular hydrogen to produce active atomic hydrogen. Active atomic hydrogen may have participated in the redox regulation of cellular functions. Water can permeate everywhere in the body and penetrates every membrane including the blood-brain barrier. To neutralize the toxic action of active oxygen species, electrolyzed-reduced water may be an ideal and very powerful antioxidant. Further intensive investigation on the effect of reduced water on cell biology, immunology and oncology should be promoted.

ACKNOWLEDGMENTS

We are grateful to Dr. David Barnes, Oregon State University, and Mr. Mark Selby for their critical reading of the manuscript.

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Preservative Effect of Electrolyzed Reduced Water on Pancreatic β-Cell Mass in Diabetic *db*/*db* Mice

Mi-Ja KIM,[a,b] Kyung Hee JUNG,[c] Yoon Kyung UHM,[c] Kang-Hyun LEEM,[d] and Hye Kyung KIM*,[e]

[a] *Department of Obesity Management, Graduate School of Obesity Science, Dongduk Women's University; Seoul 136–714, South Korea:* [b] *Imagine Obesity Institute, 117 Purynsol Mun Wa Gyun, Kyung Hee University; 1 Hoegi-Dong, Dongdaemun-Gu, Seoul 136–701, South Korea:* [c] *Department of Pharmacology, College of Medicine, Kyung Hee University; 1 Hoegi-Dong, Dongdaemun-Gu, Seoul 136–701, South Korea:* [d] *College of Korean Medicine, Semyung University; Jaechon, 370–711, South Korea: and* [e] *Department of Food and Biotechnology, Hanseo University; Seosan 356–706, South Korea.* Received July 27, 2006; accepted November 10, 2006

Oxidative stress is produced under diabetic conditions and involved in progression of pancreatic β-cell dysfunction. Both an increase in reactive oxygen free radical species (ROS) and a decrease in the antioxidant defense mechanism lead to the increase in oxidative stress in diabetes. Electrolyzed reduced water (ERW) with ROS scavenging ability may have a potential effect on diabetic animals, a model for high oxidative stress. Therefore, the present study examined the possible anti-diabetic effect of ERW in genetically diabetic mouse strain C57BL/6J-*db*/*db* (*db*/*db*). ERW with ROS scavenging ability reduced the blood glucose concentration, increased blood insulin level, improved glucose tolerance and preserved β-cell mass in *db*/*db* mice. The present data suggest that ERW may protects β-cell damage and would be useful for antidiabetic agent.

Key words electrolyzed reduced water; diabetic mice; blood glucose; insulin; glucose tolerance; pancreatic β-cell mass

All oxidative reactions are a continuous source of potentially cytotoxic reactive oxygen species (ROS), which play an important role in living systems both through their beneficial and detrimental effects.[1)] Under physiological conditions, ROS are fully inactivated by an elaborated cellular and extracellular antioxidant defense system.[2)] However, under certain conditions increased generation of ROS and/or reduction of the antioxidant capacity leads to enhanced ROS activity and oxidative stress.

Hyperglycemia, the primary clinical manifestation of diabetes, is the most responsible factor for the development of various chronic diabetic complications.[1)] The chronic presence of high glucose levels enhances the production of ROS from protein glycation and glucose autoxidation.[3)] Diabetes also disturbs natural antioxidant defense systems.[4)] Previous studies have shown that antioxidants such as quercetin, ascorbate and β-carotene resulted in an improvement of the antioxidant status in diabetic rats.[5,6)]

During the progression of type 2 diabetes, the development of both insulin resistance and α- and β-cell dysfunctions appear to be the basic metabolic abnormalities leading to the long term disease.[7)] Once hyperglycemia becomes apparent, β-cell function progressively deteriorates: glucose-induced insulin secretion becomes further impaired and degranulation of β-cells becomes evident, often accompanied by a decrease in the number of β-cells.[8)] The maintenance of β-cell mass is a dynamic process, undergoing both increases and decreases to maintain glycemia within a narrow physiological range.[9)] The majority of patients with obesity causing insulin resistance are not diabetic, as their capacity for β-cell compensation is maintained but, 15—20% of these individuals become diabetic, when the β-cells lose their compensatory ability.[9)] Therefore, one approach to preventing and treating diabetes could be through the enhancement of β-cell mass.

Electrolysis of aqueous NaCl or KCl solutions by diaphragm-type electrolyzing devices produces oxidized and reduced water at the anodic and the cathodic side, respectively. Electrolyzed reduced water (ERW) exhibits high pH (pH 10—12), low dissolved oxygen (1.3—3.5 mg/l), high dissolved hydrogen (0.3—0.6 mg/l), and significant negative redox potential (−400—−900 mV). The ideal scavenger for ROS is "reactive hydrogen". Since oxidative damage has been implicated in the etiology of diabetic complication, ERW, a potent ROS scavenger, may have a therapeutic role in diabetic mellitus. Therefore, the present study examined the beneficial effect of ERW on β-cell damage and glycemic control in diabetic mouse strain of C57BL/6J-*db*/*db* (*db*/*db*) which exhibits many of the metabolic disturbances of human type 2 diabetes including hyperglycemia, obesity, and insulin resistance.[10)]

MATERIALS AND METHODS

Materials The ERW was produced by AK-3000 (Nexus, Korea). This water was produced by the electrolysis of water from a municipal water system. The ERW used in this study has the following physical properties: pH 10.24±0.03 and an oxidative-reduction potential of −400.2±17.3 mV.

Animals Genetically diabetic male *db*/*db* (C57BL/6J *db*/*db*) and their non-diabetic heterozygous littermates (*db*/−) at 3 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A.). Mice were housed under temperature (20—26 °C) and light (12-h light/dark cycle) controlled conditions. All animals had free access to standard rodent pellet food (NIH #31M, Samtako, Korea), except when fasted before experiments. The study has been carried out along the "Principles of laboratory animal care" (WHO, 1985), and the "guidelines of the Animal Care and Use Committee" of the Kyunghee University. The *db*/*db* (D) and non-diabetic control mice (N) were each randomly divided into 2 groups (n=8); tap water (DC, NC) or ERW (DE, NE) group.

Blood Glucose and Insulin Measurements At the end of 4 weeks of experimental period, mice were fasted overnight and injected with glucose (1 g/kg, i.p.). Blood samples were collected from the tail vein at various time points

* To whom correspondence should be addressed. e-mail: hkkim111@hanseo.ac.kr; mijakim@dongduck.ac.jp

(0—120 min) after glucose loading, and blood glucose levels were measured by One touch Basic glucose measurement system (Life Scan Inc., U.S.A.). Mice were killed by decapitation immediately after 120 min blood sample was taken and blood samples were taken from the cervical wound. Plasma glucose and insulin concentrations were determined with commercially available kits (Sigma Co., U.S.A.).

Immunohistochemical Staining of Pancreatic Islet Cell Pancreatic islet cell mass is a major determinant of the insulin secretory capacity. The four groups were used for histological studies. Pancreases were removed, and then fixed with 10% formalin. Immunohistochemical staining of β-cells using an anti-insulin antibody (Dako, Santa Barbara, CA, U.S.A.) was performed[11] with 5 μm sections of formalin fixed and paraffin embedded pancreas.

Statistical Analysis Results were presented as mean±S.E.M., and the data were analyzed by ANOVA followed by Tukey HSD's *post-hoc* test.

RESULTS AND DISCUSSION

No differences in food intake, water intake, and body weight were observed by ERW consumption in *db*/*db* and non-diabetic control mice (data not shown). Blood glucose levels of diabetic mice (DC) were significantly higher than non-diabetic control mice (NC), and ERW consumption significantly lowered (41%) the blood glucose levels in hyperglycemic *db*/*db* mice (DE) without any effect in non-diabetic control mice (NE, Table 1). Plasma insulin levels of diabetic mice were more than 2-fold higher than control mice indicating insulin resistance in type 2 diabetes. Interestingly, ERW administration also increased insulin level in diabetic mice without any effect in control mice, suggesting elevated insulin release in diabetic mice. This increased insulin levels in diabetic mice resulted from increased pancreatic β-cell mass (Fig. 2).

Intraperitoneal glucose tolerance test revealed that glucose tolerance in diabetic *db*/*db* mice exhibited significantly higher glucose level during all time points determined (Fig. 1). After glucose loading, the increase in serum glucose concentrations in diabetic mice was very slow, while normal mice exhibited sharp increase in glucose level with peak concentration at 15 min, indicating delayed glucose homeostasis in *db*/*db* mice. ERW administration ameliorated glucose tolerance in diabetic *db*/*db* mice without any affect in control mice.

Significant histological differences were noted between *db*/*db* mice and their non-diabetic littermates. When β-cells were stained with anti-insulin antibodies, weak staining of β-cells were observed in *db*/*db* mice (Fig. 2c) compared with control mice (Fig. 2a). ERW supplemented *db*/*db* mice exhibited strong staining (Fig. 2d), as seen in the lean littermates (Fig. 2b).

This study clearly demonstrated that treatment with ERW ameliorates hyperglycemia and improves glucose handling capacity in obese diabetic *db*/*db* mice. In addition, immunohistochemical examination revealed that treatment with ERW can prevent loss of β-cell mass resulting in increase of insulin secretory capacity.

Recently, pancreatic β-cells emerged as a target of oxidative stress-mediated tissue damage. Because of the relatively low expression of antioxidant enzymes,[12] pancreatic β-cells may be rather sensitive to ROS attack when they are exposed to oxidative stress. Chronic hyperglycemia is not only a marker of poor glycemic control in diabetes but is itself a cause of impairment of both insulin secretion and biosynthesis: prolonged exposure of pancreatic β-cells to high glucose levels is known to cause β-cell dysfunction, called glucose toxicity.[13] Such damaged β-cells often display extensive degranulation, and are clinically associated with the development of diabetes in some model animals for type 2 dia-

Table 1. Fasting Blood Glucose and Insulin Levels

	NC	NE	DC	DE
Blood glucose (mg/dl)	129.0±6.8[a]	125.4±3.9[a]	490.1±32.4[b]	287.9±34.5[c]
Blood insulin (ng/ml)	0.71±0.02[a]	0.68±0.01[a]	1.55±0.09[b]	5.72±0.49[c]

Values are means±S.E.M. (n=8); means in same raw with different superscripts are significantly different (p<0.05).

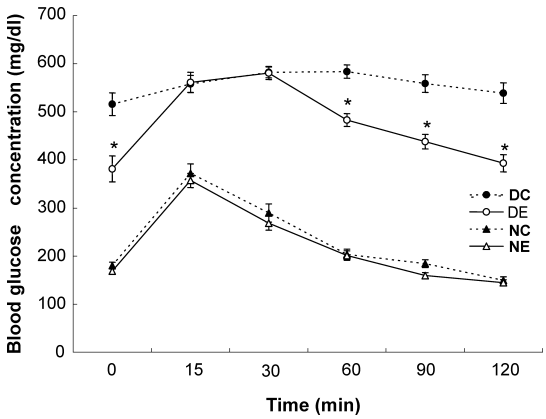

Fig. 1. Effect of Electrolyzed Reduced Water (ERW) on Glucose Tolerance in Genetically Diabetic *db*/*db* Mice

Normal *db*/− mice fed tap water (NC); normal *db*/− mice fed ERW (NE); *db*/*db* mice fed tap water (DC); *db*/*db* mice fed ERW (DE). Data are expressed as means±S.E.M.

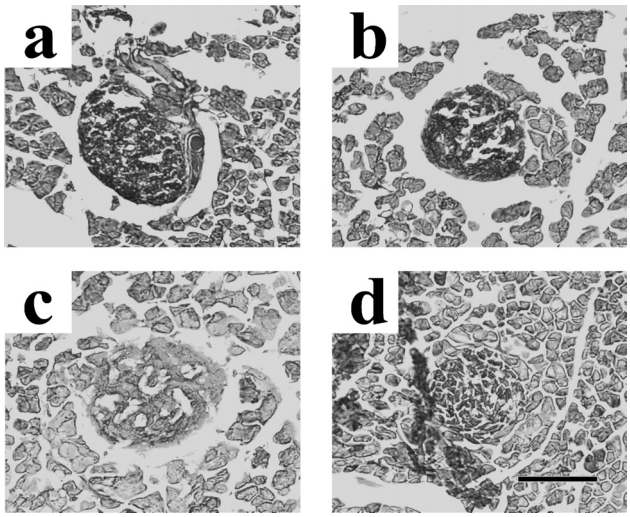

Fig. 2. Images of Pancreases Immunohistochemical Staining for Insulin

Pancreases from non-diabetic control (a), non-diabetic treated with ERW (b), diabetic *db*/*db* control (c), or ERW treated *db*/*db* mice (d) were immunostained with anti-insulin antibody. (c) shows weak staining of β-cells with anti-insulin antibodies in *db*/*db* mice compared with non-diabetic control (a). *db*/*db* mice consumed ERW exhibited strong staining (d), as seen in control (a). The bar indicates 100 μm.

betes.[14] Thus, it is likely that oxidative stress plays a major role in β-cell deterioration in type 2 diabetes. The restoration of glucose induced secretory capacity after ERW consumption is thought to be due to elimination of glucose toxicity resulting in protection from the toxic effects of ROS. Indeed, ERW consumption increased β-cell mass (Fig. 2) and insulin level (Table 1). Tanaka *et al.*[15] reported that chronic hyperglycemia impairs β-cell function at the level of insulin synthesis as well as insulin secretion, and all of these adverse consequences can be prevented by antioxidants. ERW, containing active atomic hydrogen with high reducing ability which can contribute to ROS scavenging activity,[16] could reduce oxidative stress in pancreatic islets and loss of β-cell mass was eventually prevented. Therefore, ERW administration improved islet β-cell function resulting in increased release of circulating insulin and improved insulin sensitivity, and thus, ameliorate hyperglycemia and delay the development of diabetes in these diabetic mice model.

To our knowledge, this is the first report showing protective effect of ERW on β-cell mass in diabetic animal models.

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Kidney International, Vol. 58 (2000), pp. 1859–1869

Citrate therapy for polycystic kidney disease in rats

GEORGE A. TANNER and JUDITH A. TANNER

Department of Physiology and Biophysics, Indiana University School of Medicine, Indianapolis, Indiana, USA

Citrate therapy for polycystic kidney disease in rats.

Background. Few treatments are available to slow the progression to renal failure in autosomal dominant polycystic kidney disease (PKD). In an animal model of PKD, the male heterozygous Han:SPRD rat, intake of a solution of potassium citrate plus citric acid (KCitr) from age one to three months prevented a decline in glomerular filtration rate (GFR). The present study tested whether this beneficial effect is sustained and explored handling of citrate and ammonia in normal and cystic kidneys.

Methods. Rats were provided with tap water or citrate solutions to drink, and clearance and survival studies were performed.

Results. The GFRs of rats with PKD that consumed KCitr from one month of age were normal at six months of age, while those of their counterparts on water were about one third of normal. Long-term KCitr treatment extended the average life span of rats with PKD from 10 to 17 months. Compared with normal rats, water-drinking rats with PKD had higher plasma [citrate], renal cortical [citrate], and fractional excretion of citrate, and lower rates of renal citrate consumption, ammonia synthesis, and ammonia excretion. Cortical P_{NH3} was not elevated in cystic kidneys. Intake of Na_3 citrate/citric acid solution or K_3 citrate solution, but not ammonium citrate/citric acid solution, prevented a decline in GFR in three-month-old rats with PKD.

Conclusions. Rats with PKD show abnormal renal handling of citrate and ammonia. Citrate salts that have an alkalinizing effect preserve GFR and extend survival.

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited disorder that afflicts more than 500,000 people in the United States alone and millions more worldwide. This disease is accompanied by the formation and enlargement of numerous fluid-filled epithelial sacs (cysts) in both kidneys and eventual development of renal failure. There is currently no specific treatment to halt or slow the progression of renal failure in patients with PKD [1].

In 1991, Kaspareit-Rittinghausen, Deerberg, and Wcislo discovered a mutant strain of Sprague-Dawley rats with autosomal dominant PKD, the Han:SPRD strain, in a laboratory animal-breeding facility in Hanover, Germany [2]. Homozygous animals with altered PKD genes develop massively enlarged cystic kidneys and die at about three to four weeks of age. Heterozygous males and females develop a slowly progressive cystic disease, which is much more severe in males than in females. Death from renal failure in male heterozygotes occurs at a median age of about 6 [2] or 17 months [3] in different colonies. The heterozygous Han:SPRD rat with PKD may be a useful model for human autosomal dominant PKD [2–5], even though the abnormal gene in the rat [6] may not correspond to the *PKD-1* or *PKD-2* genes that are defective in almost all patients with ADPKD.

We recently demonstrated that if heterozygous male Han:SPRD rats with PKD were provided with a solution of potassium citrate/citric acid (abbreviated "KCitr") to drink, starting at one month of age, then the glomerular filtration rate (GFR) was sustained at a normal level and the kidneys showed less cystic disease when the rats were three months old [7]. By contrast, littermate rats with PKD that drank tap water had a GFR one half of normal, large kidney cysts, and extensive renal interstitial disease. The present study had two purposes: (*1*) to ascertain whether the benefits of KCitr therapy would be sustained beyond three months, and (*2*) to examine the mechanism of this beneficial effect.

To assess the effectiveness of KCitr administration beyond three months of age, we treated Han:SPRD rats with KCitr for longer times in two sets of experiments. First, we treated rats, starting at the age of one month, and measured renal function at six months of age. Second, we treated rats from the age of one month until their deaths to see whether this treatment prolonged survival.

To gain some insight into the mechanism of KCitr therapy, we examined several parameters. First, we measured blood and urine pH values to determine whether KCitr intake affected these variables. Second, we hypothesized that renal citrate handling might be abnormal in rats with PKD, based on a previous report that citrate excretion is abnormally elevated in these rats [8]. We therefore measured plasma and renal tissue citrate levels and renal reabsorption and consumption of citrate; the

Key words: autosomal dominant polycystic kidney disease, potassium citrate, sodium citrate, citric acid, renal ammonia, Han:SPRD rat.

Received for publication October 19, 1999
and in revised form May 12, 2000
Accepted for publication May 19, 2000

effects of KCitr treatment on these parameters were also studied. Third, since Torres et al had suggested that increased ammonia levels contribute to damage in cystic kidneys, we determined renal cortical tissue P_{NH3} and ammonia production and excretion [9, 10]. Fourth, we tested the idea that KCitr's beneficial effect is dependent on the well-known interaction between citrate and calcium ions in the body [11]. Finally, we substituted ammonium or sodium ions for potassium ions in the citrate solution and also gave K_3 citrate (tripotassium citrate without citric acid) to determine whether the beneficial effect of the KCitr solution was related to its potassium content or to its alkalinizing effect.

METHODS

Animals and solutions

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on heterozygous male Han:SPRD rats with PKD and their normal littermates. The breeding stock was obtained from the Polycystic Kidney Program at the University of Kansas. All animals were allowed free access to a diet containing 24% protein and 6% fat (Teklad 6% mouse/rat diet 7002; Harlan, Madison, WI, USA). In most experiments, rats were provided with either a solution of 55 mmol/L tripotassium citrate/67 mmol/L citric acid (KCitr) or tap water to drink beginning at one month of age, and they were studied at six months of age or long-term survival was followed. In other experiments, normal rats or rats with PKD were provided with solutions of either (*1*) 82.5 mmol/L diammonium citrate/39.5 mmol/L citric acid, (*2*) 55 mmol/L trisodium sodium citrate/67 mmol/L citric acid, or (*3*) 55 mmol/L K_3 citrate to drink from one month of age, and they were studied at three months of age. The milliequivalents of citrate in all citrate solutions were the same. Drinking solutions were made up in tap water.

Clearance studies

Before experiments, six-month-old rats were deprived overnight of food but had free access to water or citrate solution. They were intraperitoneally anesthetized with the thiobarbiturate Inactin (130 mg/kg body weight; Byk Gulden, Konstanz, Germany). Each animal was placed on a heated table, and rectal temperature (monitored with a probe) was kept at 37°C. The trachea was cannulated, and a slow flow of moistened 35% O_2/65% N_2 was passed over the opening of the cannula. A femoral vein was cannulated for infusions. One milliliter of 6 g/100 mL fraction V bovine serum albumin in 0.9% NaCl was administered intravenously during the surgical preparation. This was followed by a priming dose (0.2 mL/100 g body weight) and constant intravenous infusion at 3 mL/hour of a solution containing 3% polyfructosan (Laevosan Co., Linz, Austria), 5.5 to 11 mg/mL sodium *p*-aminohippurate (PAH), 2 g/100 mL bovine serum albumin, 24 mmol/L $NaHCO_3$, and 125 mmol/L NaCl. A femoral artery was cannulated for blood sampling and for measuring blood pressure with a transducer (Gould-Statham, Hato Rey, Puerto Rico). The abdomen was opened via a midline incision. The left ureter was cannulated, and a cannula was inserted into the bladder to drain urine from the right kidney. A number 25 needle, connected to a short length of Silastic tubing, was inserted into the left renal vein for blood sampling.

Renal clearance measurements were made as follows: About one hour after the left ureter had been cannulated, we collected two 30-minute urine samples under water-equilibrated light mineral oil. Urine volume was determined by weighing, assuming a density of one. Urine pH was measured immediately at room temperature using a 9802BN micro-pH combination electrode (Orion Research, Beverly, MA, USA). Arterial and renal venous blood samples (0.60 to 0.85 mL) were collected at the beginning and end of the urine collections. When determinations of plasma ammonia levels were made, blood samples were collected in iced syringes. Plasma and blood cells were separated immediately. Plasma proteins were precipitated with iced 10% trichloroacetic acid. Supernatants were frozen, and analyses were completed within a few hours. An arterial blood sample (0.25 mL) was also collected anaerobically for measurement of blood gases and pH using an IRMA series 2000 blood system (Diametrics Medical, St. Paul, MN, USA) or model 1400 blood gas electrolyte analyzer (Instrumentation Laboratories, Lexington, MA, USA). In some experiments, a terminal blood sample (0.25 mL) was collected for plasma calcium and phosphate determinations.

The clearance studies in three-month-old rats were done in exactly the same way as in our previous study on rats of this age [7].

Long-term survival

Survival studies were performed on 18 male heterozygous rats with PKD that drank water and on six male heterozygous rats that drank KCitr solution starting at one month of age. Three normal rats on KCitr intake since one month of age were sacrificed when 21 months old.

Tissue collection

At the end of most clearance experiments, one kidney (usually the left) was rapidly removed. Cortex and medulla were separated, and wet and dry weights (samples heated in an oven at 120°C for 16 hours) were determined. In other experiments, samples for tissue calcium were obtained after separating kidney cortex and medulla. Samples for tissue citrate were obtained by rapidly slicing off a piece of cortex, freeze-clamping it in liquid nitrogen, and then homogenizing the sample in iced 10%

perchloric acid. The samples were centrifuged, and the supernatants were then filtered through a 10,000 molecular weight cutoff UltraFuge filter (Micron Separations, Westboro, MA, USA).

Histology

The remaining kidney was fixed by retrograde aortic perfusion with a solution of 3% paraformaldehyde, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4, 4 mmol/L Na_2HPO_4, and 2 mmol/L picric acid at a perfusion pressure of 150 to 170 mm Hg. The kidney was kept in the same fixative solution for several days in the refrigerator and then weighed, sliced with a razor blade, immersed in 0.1 mol/L cacodylate solution (pH 7.25), and embedded in paraffin. Sections were stained with hematoxylin and eosin. The degree of cystic disease (size of cysts, interstitial widening and fibrosis, presence of inflammatory cells) was evaluated blindly using an arbitrary scale of 0 to 4, where 0 represents the normal condition and 4 represents severe changes.

Chemical analyses

Polyfructosan (a synthetic inulin) in plasma and urine was determined by an anthrone method [12]. PAH was determined by Bratton and Marshall's method [13]. Citrate in plasma, urine, and tissue samples was determined spectrophotometrically using a citrate lyase method (Boehringer Mannheim, Indianapolis, IN, USA). Recovery of citrate added to plasma averaged 99 ± 6% (N = 11). When kidney tissue was analyzed for citrate, we observed a 100 ± 5% (N = 25) recovery of an internal citrate standard at five minutes after adding the citrate lyase, indicating completion of the reaction. The absorbance readings, however, continued to drift appreciably with time, probably because of the presence of nicotinamide adenine dinucleotide (NADH) oxidase activity in kidney cortex extracts. To correct for this "creep," we measured absorbance every 5 minutes for a total of 20 minutes after adding the citrate lyase and extrapolated the absorbance back to time zero [14]. Ammonia was determined using a glutamate dehydrogenase-based kit (Boehringer Mannheim). Recovery of ammonia added to plasma averaged 100 ± 1.4% (N = 4). Phosphate in plasma was determined by the Fiske-SubbaRow method.

Potassium in urine and plasma and calcium in plasma, urine, and kidney tissue were determined using an atomic absorption spectrophotometer (model 951; Instrumentation Laboratory, Wilmington, MA, USA). Tissue samples were homogenized in a solution containing 4% (vol/vol) butanol, 0.2 mol/L HCl, and 36 mmol/L $LaCl_3$ [15] using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA). Recovery of calcium added to tissue samples averaged 103 ± 3% (N = 9). Samples and standards were always prepared in the same matrix solutions.

Calculations

The GFR was calculated from the rate of excretion of polyfructosan divided by the plasma polyfructosan concentration. Renal plasma flow (RPF) was calculated from the PAH clearance divided by the PAH extraction ratio ((arterial minus renal venous plasma [PAH]) ÷ arterial plasma [PAH]). Renal blood flow (RBF) was calculated from this formula: RBF = RPF/(1 − hematocrit). Renal citrate consumption was calculated from RPF × (arterial plasma [citrate] − renal vein plasma [citrate]) minus urinary citrate excretion rate. Tubular reabsorption was calculated from the filtered load minus the excretion rate. Peritubular uptake of citrate was calculated from the renal citrate consumption minus the rate of tubular citrate reabsorption. Fractional excretion was calculated from the excretion rate divided by the filtered load. Renal ammonia production was calculated from the RBF × (renal venous blood [ammonia] − arterial blood [ammonia]) plus the urinary excretion rate. The partial pressure of cortical tissue ammonia (P_{NH3}) was estimated from the renal vein total NH_4^+ concentration and arterial blood pH [16, 17] and this formula:

$$P_{NH3} = (\text{total } NH_4^+ \times 22.09)/(10^{pK - pH} \times \alpha)$$

where the pK for NH_4^+ is 9.02. The pH of renal venous and arterial blood are assumed to be the same, and the solubility coefficient (α) for ammonia is 0.91 [18].

Statistical methods

Data presented are means ± SD. They were analyzed by two-way analysis of variance (ANOVA), after a preliminary test for homogeneity of variances. Individual groups were compared with the Bonferroni method. If variances were heterogeneous, the Kruskal–Wallis test and Dunn's test were used to compare means. A P value of less than 0.05 was considered significant.

RESULTS

Overall function in six-month-old rats

The effects of KCitr consumption in six-month-old normal rats and rats with PKD are summarized in Table 1. The most remarkable finding is that GFR was completely normal in rats with PKD that had been treated with KCitr, whereas GFR was only 37% of normal in rats with PKD that had consumed tap water. RBF also was normal in the rats with PKD that had been treated with KCitr and was about half of normal in untreated rats with PKD. KCitr treatment had no effect on body weight or blood pressure, in agreement with our earlier findings at three months of age [7].

A comparison of water-drinking normal rats and water-drinking rats with PKD (Table 1) shows that the animals with cystic disease had heavier kidneys, higher

Table 1. Effects of KCitr consumption on function in six-month-old normal rats and rats with PKD

	Normal rats		Rats with PKD	
	Tap water	KCitr	Tap water	KCitr
Body weight *g*	464 ± 28 (14)	446 ± 25 (13)	462 ± 29 (13)	464 ± 10 (12)
Kidney weight *g*	1.55 ± 0.14 (14)	1.59 ± 0.15 (13)	2.64 ± 0.40 (13)[c]	2.83 ± 0.28 (12)[e]
Kidney cortex % H_2O	78.9 ± 0.4 (10)	80.2 ± 0.6 (9)[c]	87.5 ± 0.6 (9)[c]	84.4 ± 0.5 (7)[e,g]
Kidney medulla % H_2O	83.0 ± 0.6 (10)	84.0 ± 0.6 (9)[b]	85.0 ± 0.7 (9)[c]	83.9 ± 0.5 (7)[f]
MABP *mm Hg*	107 ± 6 (14)	106 ± 7 (13)	116 ± 10 (13)[a]	119 ± 6 (12)[e]
Hematocrit % *cells*	47 ± 1 (14)	44 ± 1 (13)[a]	39 ± 4 (13)[b]	43 ± 1 (12)
GFR *μL/min per 100 g body weight*	392 ± 31 (14)	448 ± 43 (13)[a]	146 ± 67 (13)[c]	418 ± 37 (12)[g]
$\dot{V}$ *μL/min per 100 g body weight*	6.8 ± 1.7 (14)	6.7 ± 1.7 (13)	8.4 ± 3.6 (13)	9.3 ± 2.4 (12)[d]
PAH extraction	0.88 ± 0.02 (14)	0.84 ± 0.04 (13)	0.37 ± 0.17 (13)[b]	0.72 ± 0.05 (12)
Renal blood flow *mL/min*	18.5 ± 2.5 (14)	18.5 ± 2.3 (13)	9.7 ± 2.3 (13)[c]	17.0 ± 1.4 (12)[g]

Values are means ± SD (number of rats). Kidney data are for the left kidney. Abbreviations are: PKD, polycystic kidney disease; KCitr, potassium citrate/citric acid solution; MABP, mean arterial BP; $\dot{V}$, urine flow rate.

[a] $P < 0.05$, [b] $P < 0.01$, and [c] $P < 0.001$ compared with normal rats on tap water

[d] $P < 0.05$ and [e] $P < 0.001$ compared with normal rats on KCitr

[f] $P < 0.01$ and [g] $P < 0.001$ compared with rats with PKD on tap water

kidney water contents, higher blood pressure, lower hematocrit, and lower PAH extraction. The increased kidney size and water content reflect cyst fluid accumulation.

KCitr treatment did not prevent overall renal enlargement in rats with PKD (Table 1). The histology of cystic kidneys in untreated and treated rats was, however, quite different (Fig. 1). In tap water-drinking rats with PKD, there was severe cystic disease, with numerous large cysts, interstitial widening and fibrosis, and numerous inflammatory cells; the disease score averaged 4 ± 0 ($N = 7$). In KCitr-treated rats with PKD, the disease score averaged 2.6 ± 0.4 ($N = 7$), and in no case was cystic disease judged to be severe (a score of 4).

In the normal rats, KCitr treatment had no significant effect on any of the variables measured (Table 1) or on renal histology, except for about a 10% increase in GFR, a small increase in cortex and medulla water contents, and a fall in hematocrit. In rats with PKD, KCitr treatment overall tended to produce more normal renal function and structure (Table 1 and Fig. 1).

Long-term survival

KCitr treatment significantly prolonged the life of rats with PKD (Fig. 2). The average survival time of rats that drank tap water was 288 ± 39 days ($N = 18$). The average survival time of rats with PKD on KCitr was 506 ± 33 days ($N = 6$). The KCitr-treated animals lived well beyond the time all of the untreated rats with PKD had died (Fig. 2). Histologic examination of kidneys from old KCitr-treated rats with PKD demonstrated marked cystic changes; hence, this treatment does not stop progression of the disease. Normal plasma chemistry values (urea, creatinine, and potassium) were found in three normal rats that had consumed KCitr solution for 20 months (data not shown).

Renal citrate, ammonia, and electrolyte handling in six-month-old rats

Acid-base measurements are presented in Table 2. Rats with PKD that drank tap water showed evidence of a metabolic acidosis (average plasma pH of 7.30 and bicarbonate concentration of 19 mEq/L). KCitr treatment increased blood pH and plasma bicarbonate concentration in the rats with PKD, but did not significantly affect these variables in normal rats. KCitr treatment significantly increased urine pH, by about 0.5 pH unit, in both normal rats and rats with PKD.

Data on renal citrate handling in normal rats and in rats with PKD that had consumed either tap water or KCitr solution are presented in Table 3. It is notable that in the normal rats, the arterial plasma citrate concentration and renal cortical citrate concentration were not affected by increased citrate intake. In fact, KCitr intake in normal rats did not affect any of the parameters measured except for expected increases in urine citrate concentration, excreted citrate, and the fraction of filtered citrate excreted in the urine.

Rats with PKD that were on tap water had an elevated arterial plasma citrate concentration (about twice normal), diminished citrate extraction and consumption, elevated urinary citrate excretion (about 10 times normal), and elevated renal cortical citrate concentration when compared with normal rats on tap water (Table 3). The higher rate of citrate excretion in rats with PKD is not due to a difference in urine pH [19, 20], since both groups that drank tap water had urine pH values about 5.9 (Table 2). A novel finding is that cortical tissue citrate concentration is also elevated in rats with PKD, although to a variable extent. Figure 3 illustrates that this variability is related to the degree of renal impairment in untreated rats with PKD; tissue citrate concentration was inversely related to the GFR.

Increased citrate intake paradoxically led to a lower

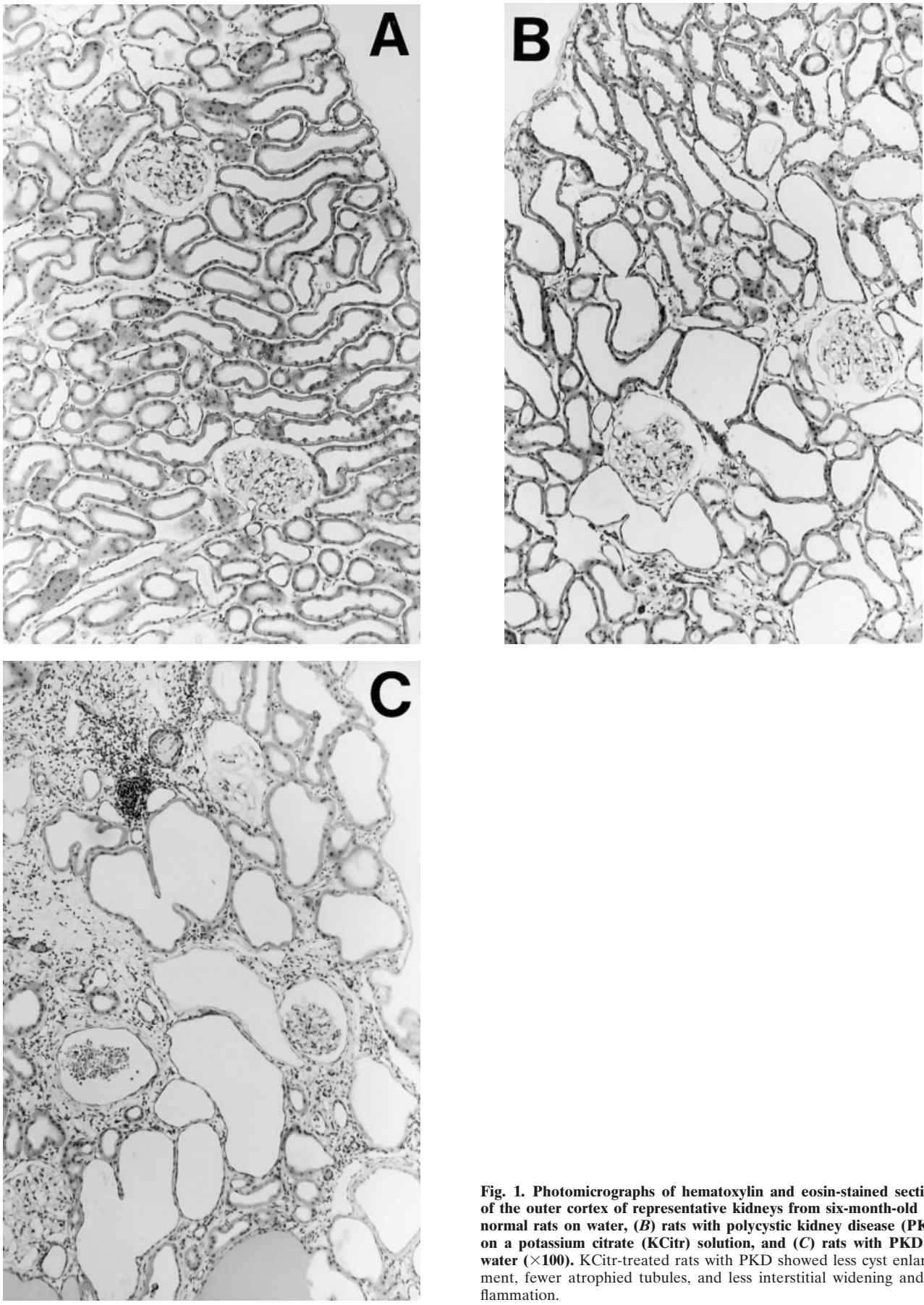

Fig. 1. Photomicrographs of hematoxylin and eosin-stained sections of the outer cortex of representative kidneys from six-month-old (*A*) normal rats on water, (*B*) rats with polycystic kidney disease (PKD) on a potassium citrate (KCitr) solution, and (*C*) rats with PKD on water (×100). KCitr-treated rats with PKD showed less cyst enlargement, fewer atrophied tubules, and less interstitial widening and inflammation.

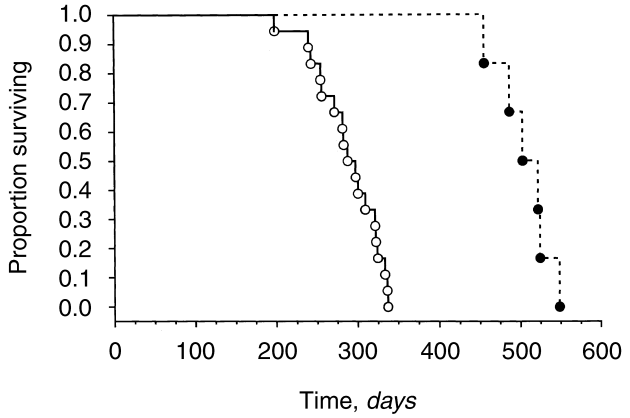

Fig. 2. Survival of male heterozygous rats with PKD drinking tap water (○) or on a KCitr solution (●). The age of death is plotted as a function of time. Treatment with KCitr starting at the age of one month led to an increase in survival time from 288 ± 39 days ($N = 18$) to 506 ± 33 days ($N = 6$).

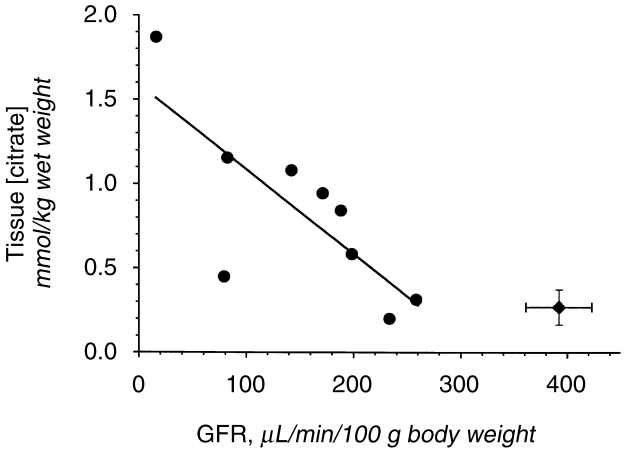

Fig. 3. Inverse relationship between cortical [citrate] and glomerular filtration rate (GFR) in untreated rats with PKD (●). The equation of the least squares line is tissue [citrate] = $-0.00503 \times$ GFR + 1.59 ($r = -0.77$, $P < 0.05$). Average values for eight normal rats consuming water are shown on the right (◆).

arterial plasma citrate concentration and a lower concentration of citrate in cortical tissue of rats with PKD compared with rats with PKD that drank tap water (Table 3). Overall, KCitr treatment of rats with PKD led to a more normal pattern of renal handling of citrate.

Measurements of renal handling of ammonia are summarized in Table 4. KCitr treatment in normal rats led to significant decreases in renal ammonia production and excretion. This may be due to two effects: (*1*) oxidation of citrate to bicarbonate and its consequent alkalinizing effect, and (*2*) inhibition of ammoniagenesis by citrate [21]. Rats with PKD on tap water had lower rates of production and excretion of ammonia than did normal rats on tap water. KCitr-treated rats with PKD had the same rates of production and excretion of ammonia as normal KCitr-treated rats.

Arterial ammonia concentration was lower in the rats with PKD than in normal rats when both drank tap water. Otherwise, there were no statistically significant differences in arterial ammonia concentration, renal venous ammonia concentration, or cortical P_{NH3} among the four groups (Table 4).

Data on electrolyte handling in normal rats and in rats with PKD that drank tap water or KCitr solution are shown in Table 5. Surprisingly, the plasma potassium concentration was significantly decreased in both normal rats and in rats with PKD that consumed KCitr when compared with water-drinking rats. Urinary potassium excretion was elevated with KCitr intake, as expected. The fractional excretion of potassium (FE_K) was increased with potassium intake in normal rats; in the rats with PKD treated with KCitr, FE_K was below that observed in untreated rats, most likely because of the higher GFR and filtered potassium load in the KCitr-treated rats.

Plasma calcium was significantly decreased by KCitr consumption in the normal rats, but was not affected by this treatment in the rats with PKD (Table 5). No significant differences in calcium excretion were seen in the four groups. In untreated rats, cortical tissue calcium levels were higher with PKD than in normals. Potassium citrate treatment actually raised the tissue calcium levels in the cortex and medulla of normal rats and in the medulla of rats with PKD. Plasma phosphate concentration was elevated in the rats with PKD on water, but was brought closer to normal in the rats with PKD that had been treated with KCitr. These changes in plasma phosphate level most likely reflect retention of phosphate in animals with severe renal disease (untreated rats with PKD) and adequate phosphate disposal in animals with a normal GFR (KCitr-treated rats with PKD).

Effects of varying drinking fluid composition

Effects of varying the composition of the drinking solutions were determined in clearance studies on three-month-old rats. Figure 4 shows the measurements of left kidney GFR from these new experiments along with results from our previous study on the effects of intake of tap water or KCitr solution [7]. The data are normalized per 100 g body weight. None of the treatments had a significant effect on body weight, and none prevented renal enlargement in rats with PKD (data not shown).

The intake of an ammonium citrate/citric acid solution had no beneficial effect on GFR in rats with PKD; GFR averaged 261 ± 22 μL/min-100 g body weight, about half of normal, and was the same as in water-drinking rats of the same age (Fig. 4). Urine pH in rats consuming ammonium citrate/citric acid solution was 5.77 ± 0.04 ($N = 4$) in normal rats and 5.60 ± 0.09 ($N = 4$) in rats

Table 2. Acid-base measurements in six-month-old normal rats and rats with PKD

	Normal rats		Rats with PKD	
	Tap water ($N = 14$)	KCitr ($N = 13$)	Tap water ($N = 13$)	KCitr ($N = 12$)
Arterial pH	7.38 ± 0.03	7.39 ± 0.06	7.30 ± 0.05[b]	7.39 ± 0.04[d]
Arterial P_{CO_2} *mm Hg*	37 ± 4	40 ± 6	39 ± 5	37 ± 4
Arterial plasma [HCO_3^-] *mEq/L*	22 ± 2.5	24 ± 2.1	19 ± 1.9[a]	22 ± 2.5[c]
Urine pH	5.96 ± 0.22	6.43 ± 0.25[b]	5.89 ± 0.22	6.39 ± 0.24[d]

Values are means ± SD.
[a] $P < 0.01$ and [b] $P < 0.001$ compared with normal rats on tap water
[c] $P < 0.01$ and [d] $P < 0.001$ compared with rats with PKD on tap water

Table 3. Renal citrate handling in six-month-old normal rats and rats with PKD

	Normal rats		Rats with PKD	
	Tap water ($N = 9$)	KCitr ($N = 8$)	Tap water ($N = 8$)	KCitr ($N = 7$)
Arterial [citrate] *mmol/L*	0.086 ± 0.018	0.081 ± 0.013	0.154 ± 0.038[c]	0.077 ± 0.015[g]
Citrate extraction	0.34 ± 0.08	0.33 ± 0.10	0.21 ± 0.08[b]	0.33 ± 0.09
Urine [citrate] *mmol/L*	0.08 ± 0.06	0.70 ± 0.54[c]	0.65 ± 0.33[c]	1.04 ± 0.57
Filtered citrate *nmol/min*	149 ± 31	158 ± 37	107 ± 18[b]	161 ± 39[f]
Excreted citrate *nmol/min*	2.3 ± 2.1	13.4 ± 8.6[c]	25.4 ± 16.9[c]	43.6 ± 20.3[d]
Reabsorbed citrate *nmol/min*	146 ± 30	145 ± 37	82 ± 17[c]	117 ± 24
$FE_{citrate}$ %	1.4 ± 1.0	8.8 ± 5.7[c]	23.0 ± 12.8[c]	26.3 ± 6.7[e]
Citrate consumption *nmol/min*	257 ± 66	267 ± 137	141 ± 57[b]	202 ± 68
Peritubular citrate uptake *nmol/min*	110 ± 62	122 ± 108	59 ± 41	85 ± 73
Cortical [citrate] *mmol/kg wet weight*[a]	0.269 ± 0.104	0.264 ± 0.069	0.825 ± 0.516[c]	0.271 ± 0.071[g]

Values are means ± SD. Kidney data are for the left kidney.
[a] Measurements in the four groups were done in 8, 7, 9, and 7 rats, respectively
[b] $P < 0.05$ and [c] $P < 0.001$ compared with normal rats on tap water
[d] $P < 0.01$ and [e] $P < 0.001$ compared with normal rats on KCitr
[f] $P < 0.05$ and [g] $P < 0.001$ compared with rats with PKD on tap water

with PKD; intake of this solution was associated with an acidic urine pH.

By contrast, substitution of sodium for potassium in the drinking solution yielded results similar to KCitr. GFR in the rats with PKD, 532 ± 96 μL/min-100 g body weight, was normal. Urine pH averaged 6.57 ± 0.23 ($N = 4$) in normal rats and 6.51 ± 0.33 ($N = 4$) in rats with PKD. The renal histology of rats with PKD that had consumed the sodium citrate salt (data not shown) was identical to that seen in rats of the same age that had consumed KCitr [7]. These results clearly indicate that it is the intake of citrate or citric acid, not the provision of extra potassium in the diet, that is responsible for the beneficial effect of KCitr.

With the administration of K_3 citrate (potassium citrate but no citric acid), GFR was maintained as with KCitr. Urine pH in the rats on K_3 citrate solution was 6.08 ± 0.25 ($N = 2$) in normal rats and 5.83 ± 0.35 ($N = 5$) in rats with PKD. Overall, the results demonstrate that alkalinizing citrate salts prevent a decline in GFR in rats with PKD.

DISCUSSION

This study demonstrates that chronic treatment of rats which have PKD with a KCitr solution, starting at one month of age, results in normal GFR and renal blood flow in six-month-old animals (Table 1). These results extend our previous study in which a beneficial effect of KCitr treatment was seen in rats treated from one to three months of age [7]. Long-term KCitr therapy, started at the age of one month, prolongs the survival time of rats with PKD (Fig. 2). We do not know whether initiation of treatment at an older age, when some degree of renal impairment is already present, would also be effective in slowing the progression of PKD.

Renal handling of citrate

We studied renal handling of citrate in an attempt to understand why it was effective. Some of our results were not expected (*1*) citrate concentrations in plasma, renal cortical tissue, and urine were all higher than normal in untreated rats with PKD; and (*2*) KCitr treatment of rats with PKD led to decreases in plasma and tissue citrate levels, probably as a consequence of more normal renal function.

Plasma citrate. Two factors may explain the elevated arterial plasma citrate concentration in untreated rats with PKD (Table 3). First, renal consumption of citrate was decreased. The kidneys are the major site of citrate utilization in the body [22]. Second, rats or patients with

Table 4. Ammonia handling in six-month-old normal rats and rats with PKD

	Normal rats		Rats with PKD	
	Tap water ($N = 5$)	KCitr ($N = 5$)	Tap water ($N = 5$)	KCitr ($N = 5$)
Arterial ammonia *μmol/L*	40 ± 6	37 ± 3	32 ± 4[a]	35 ± 4
Renal vein ammonia *μmol/L*	84 ± 11	77 ± 6	79 ± 19	84 ± 17
Cortical P_{NH_3} *mm Hg* × 10^{-6}	47 ± 6	53 ± 15	33 ± 9	48 ± 12
Ammonia excretion *μmol/min*	0.74 ± 0.12	0.40 ± 0.09[b]	0.28 ± 0.19[c]	0.32 ± 0.07
Ammonia production *μmol/min*	1.67 ± 0.23	1.10 ± 0.26[a]	0.77 ± 0.41[c]	1.14 ± 0.32

Values are means ± SD.
[a] $P < 0.05$, [b] $P < 0.01$, and [c] $P < 0.001$ compared with normal rats on tap water

Table 5. Electrolyte handling in six-month-old normal rats and rats with PKD

	Normal rats		Rats with PKD	
	Tap water	KCitr	Tap water	KCitr
Plasma [K^+] *mEq/L*	3.70 ± 0.30 (9)	2.91 ± 0.45 (8)[b]	4.04 ± 0.56 (8)	3.34 ± 0.17 (7)[d]
Excreted K^+ *μEq/min*	1.59 ± 0.16 (9)	2.95 ± 0.48 (8)[c]	1.78 ± 0.28 (8)	3.16 ± 0.29 (7)[f]
FE_K %	26 ± 4 (9)	51 ± 6 (8)[c]	64 ± 16 (8)[c]	47 ± 8 (7)[d]
Plasma [calcium] *mg/100 mL*	8.60 ± 0.24 (6)	8.01 ± 0.37 (4)[a]	8.64 ± 0.24 (3)	8.47 ± 0.33 (4)
Excreted calcium *μg/min*	0.62 ± 0.32 (9)	0.36 ± 0.12 (6)	1.10 ± 0.59 (6)	0.74 ± 0.26 (8)
Cortex [calcium] *mg/kg wet weight*	95 ± 4 (6)	176 ± 89 (3)[a]	153 ± 8 (3)[a]	181 ± 28 (3)
Medulla [calcium] *mg/kg wet weight*	156 ± 21 (6)	855 ± 677 (3)[c]	222 ± 37 (3)	1001 ± 462 (3)[e]
Plasma [phosphate] *mg/100 mL*	4.97 ± 0.34 (10)	5.37 ± 0.67 (9)	8.70 ± 1.45 (9)[c]	6.04 ± 0.61 (7)[f]

Values are means ± SD (number of animals). Kidney data are for the left kidney.
[a] $P < 0.05$, [b] $P < 0.01$, and [c] $P < 0.001$ compared with normal rats on tap water
[d] $P < 0.05$, [e] $P < 0.01$ and [f] $P < 0.001$ compared with rats with PKD on tap water

renal failure develop secondary hyperparathyroidism, and parathyroid hormone promotes release of citrate from bone and elevates the plasma citrate concentration [11].

In normal rats, plasma citrate was not affected by the level of chronic intake (Table 3). Likewise, patients on long-term intake of potassium citrate do not show an increase in serum citrate concentration [23].

Tissue citrate. The elevated renal cortical tissue citrate levels in untreated rats with PKD (Table 3 and Fig. 3) could be due to both an increase in intracellular citrate concentration and/or an increase in the tubular fluid or urine citrate level. It appears to be mainly due to an increase in intracellular level, since urine concentrations of citrate were elevated in the KCitr-treated rats and yet tissue citrate levels were not increased in these animals (Table 3).

Many factors can influence renal tissue citrate levels, including intracellular pH, citrate production or consumption, or altered citrate transport by luminal and peritubular cell membrane carriers [20]. Although we cannot say why the tissue citrate level was increased in rats with PKD, the finding of Ogborn et al that tissue succinate levels are low suggests impaired mitochondrial metabolism of citrate [8].

Citrate reabsorption and excretion. High urine citrate concentration and elevated citrate excretion in rats with PKD was first reported by Ogborn et al [8]. Our finding that renal cortical tissue citrate is elevated in rats with PKD may provide an explanation for increased citrate excretion. A high level of citrate in proximal tubule cells would diminish the driving force for reabsorption of filtered citrate across the luminal cell membrane and in this way could lead to increased citrate excretion [20].

Although the tenfold higher rate of citrate excretion in rats with PKD compared with normal rats is most impressive (Table 3), it is important to note that only 1% of the filtered load is excreted by the normal rat kidney. Fractional excretion of citrate in the untreated rat with PKD, 23% (Table 3), is similar to fractional excretion of citrate in the normal human kidney, about 10 to 35% [20]. Excessive urinary excretion of citrate in the rat with PKD may contribute to the development of metabolic acidosis, since loss of citrate represents a loss of potential bicarbonate.

Citrate consumption. Citrate is an important metabolic substrate in the kidneys, accounting for about 10% of their energy production [22, 24, 25]. In untreated rats with PKD, renal citrate consumption was lower than normal (Table 3). Decreased GFR and consequent decreased tubular sodium reabsorption in cystic kidneys probably contributes to the decreased citrate consumption, although we cannot rule out an intrinsic defect in citrate utilization.

Citrate and PKD. Citrate handling by cystic kidneys is clearly abnormal. It is still not obvious, however, why citrate treatment is beneficial. Had we known before

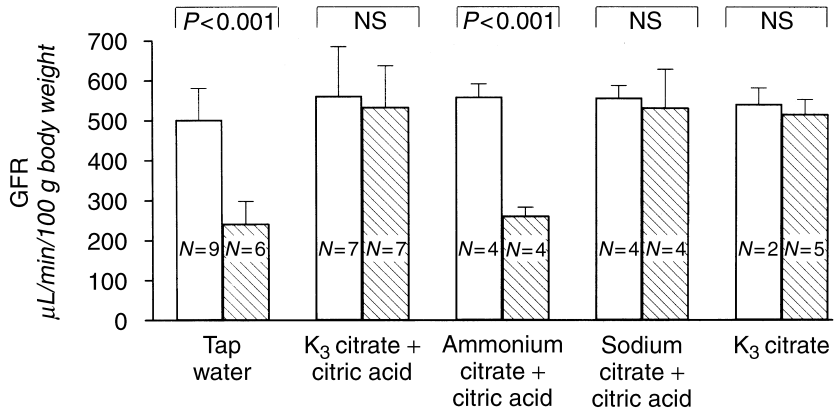

Fig. 4. Summary of effects of various treatments on GFR in three-month-old normal rats (□) and rats with PKD (▨). The data from the rats treated with KCitr or tap water are from our previous study [7]. GFR was preserved in the rats with PKD, which consumed solutions of KCitr (K_3 citrate + citric acid), Na_3 citrate + citric acid, or K_3 citrate alone, but was about half of normal in the rats that drank tap water or the $(NH_4)_2$ citrate + citric acid solution.

undertaking these experiments that plasma and kidney cortex citrate concentrations are elevated in untreated rats with PKD, we might have been dissuaded from administering additional citrate. These surprising results bolster our conclusion (discussed later in this article) that the alkalinizing effect of citrate, rather than some other attribute of citrate, underlies the success of citrate therapy.

Data on citrate handling in patients with PKD are limited. In one study of PKD patients with nephrolithiasis, an abnormally low rate of citrate excretion was seen in 67% of the patients, but this could be due an abnormally low urine pH [26]. Renal citrate handling by PKD patients, as far as we know, has not been systematically studied.

Renal handling of ammonia

The suggestion has been made that increased intrarenal levels of ammonia might contribute to tubulointerstitial injury in chronic renal diseases [27, 28], including PKD [9, 10, 29]. Torres et al reported an elevated ammonia level in human cyst fluid, but whether the fluid was derived from proximal or distal cysts is not clear [10]. Also, cyst fluid total ammonia levels may not reflect interstitial ammonia levels, since fluid within tubules or cysts is generally more acidic and ammonia is trapped as NH_4^+. In the present study, we detected no increase in arterial or renal vein total ammonia concentrations or in cortical tissue P_{NH3} in rats with PKD (Table 4). Therefore, our data do not support the hypothesis that elevated intrarenal ammonia levels are responsible for kidney damage in this disease.

Urinary ammonia production and excretion were below normal in cystic rat kidneys. Preuss et al demonstrated earlier that ammonia excretion was reduced in patients with PKD and postulated that this was secondary to decreased renal ammonia production, as we found in the rat with PKD (Table 4) [30]. Impaired urinary excretion of ammonia could contribute to the metabolic acidosis in untreated rats with PKD (Table 2).

Calcium and citrate

We hypothesized that citrate administration might exert its beneficial effect by preventing precipitation of calcium salts and lowering tissue calcium levels. We found, however, that KCitr treatment for five months led to higher levels of calcium in the kidney, even in normal rats (Table 5). Accumulation of calcium in the rat kidney with citrate intake has been observed before [31] and appears to be a consequence of the elevated urine pH. Deposition of calcium salts in kidney tissue occurs in rats [2] and patients [26] with PKD, and can lead to tissue damage and ischemia. In our study, treatment with KCitr produced only a modest nephrocalcinosis that did not appear to progress; normal rats on KCitr for 20 months had tissue calcium levels the same as in six-month-old rats (data not shown). Nevertheless, the modest elevation in tissue calcium suggests that KCitr does not ameliorate PKD by lowering tissue calcium levels.

Citrate complexes calcium in the urine and inhibits the formation of renal stones [23]. Torres et al found that giving high concentrations of another alkalinizing agent, potassium bicarbonate (200 to 300 mmol/L), caused extensive precipitation of calcium phosphate in medullary collecting ducts [9]. Citrate may be a safer compound to administer than other agents that alkalinize the urine.

Alkalinizing effect of citrate

Data in the present study strongly suggest that the beneficial effect of treatment with KCitr is a consequence of the citrate ion and its oxidation in the body to bicarbonate (alkalinizing effect). Several observations confirm an alkalinizing effect of citrate. In rats with PKD, KCitr administration resulted in an arterial blood pH of 7.39, compared with 7.30 in water-drinking rats (Table 2). Urine pH was significantly more alkaline after the administration of KCitr in both normal rats and rats with PKD (Table 2). Renal ammonia synthesis, which is

inhibited by an alkaline pH, was decreased by KCitr intake in normal rats (Table 4).

The ion-substitution experiments demonstrated that the alkalinizing effect was the factor responsible for the benefits of KCitr treatment. Intake of extra potassium is not the explanation for preservation of GFR, because sodium citrate is just as effective as the potassium salt (Fig. 4). If unmetabolized citrate or citric acid were key, then one would have expected ammonium citrate plus citric acid to have slowed the progression of PKD. Ammonium citrate, when metabolized, does not produce net addition of base to the body; formation of urea from ammonia in the liver, a process that consumes bicarbonate, negates the effect of production of bicarbonate from citrate. Omitting citric acid (K_3 citrate) yielded results similar to KCitr, showing that it is the alkalinizing form of citrate that is effective.

Conclusion

Dietary intake of sodium or potassium citrate in rats with PKD dramatically slows the progression of renal disease. Citrate's beneficial action appears to be due to its alkalinizing effect.

Exogenous citrate is selectively taken up by the kidneys [22], so citrate administration may be a good way to target base to kidney cells. Fortuitously, citrate reduces the risk of urinary calcium stone formation concomitant with administration of other bases. Therefore, it may be an ideal alkalinizing agent in PKD.

There has been extensive clinical experience with citrate, but not, as far as we know, as an agent to slow the progression of inherited PKD. Igarashi et al found that alkali therapy with citrate was of benefit in stabilizing the size and number of renal cysts in one patient with distal renal tubular acidosis [32]. It did not help in three other patients with this disease [33]; this might be explained by lack of patient compliance or by diets too acidifying to have been adequately alkalinized by the amount of alkali prescribed. Citrate therapy is commonly used nowadays in patients for the treatment of a variety of stone-forming disorders [34]. In the early part of the 20th century, it was recommended for treating patients with chronic nephritis [35], but fell into disuse, probably because excessive intake of sodium or potassium salts may be dangerous in patients with impaired renal function. Whether an alkalinizing diet in conjunction with judicious citrate therapy will slow the progression of renal disease in patients with PKD is a question that deserves to be studied.

ACKNOWLEDGMENTS

We are indebted to the Polycystic Kidney Research Foundation for grant support. Portions of this work were presented at the American Society of Nephrology meeting, Miami Beach, FL, USA, November 1999. We thank Dr. James A. McAteer for preparing the histologic slides, Dr. Kodi Vijayalakshmi and Xianghong Ren, R.N., for watering the rats, Dr. Lynn R. Willis for use of his atomic absorption spectrophotometer, and Dr. Wiltz Wagner and Joseph Bailey for use of their blood gas analyzers.

Reprint requests to George A. Tanner, Ph.D., Department of Physiology and Biophysics, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, Indiana, USA.
E-mail: gtanner@iupui.edu

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Sterilization effect of electrolyzed water on rice food

Seiichiro Isobe, Chang-yong Lee, and Kyoichiro Yoshida

Recently, a lot of instant foods have been made from rice. The main categories are aseptic packaged rice and rice cookies. In this food process, the control of microorganisms, especially heat-resistant spores from raw materials to products, is the most important point to confirm safety in product flow. If the heat-resistant spores can be controlled by pretreatment before cooking, excess heat should be omitted to make long shelf-life rice products, thus improving the quality of the products. Several papers have demonstrated the sterilization effects of electrolyzed water on food ingredients (Koseki et al 2004a,b).

In this paper, we try to confirm the sterilization effect on rice using electrolyzed water and check the quality of rice during pretreatment.

Materials and methods

Electrolyzed water is produced by electrolyzing tap water with the addition of a small quantity of NaCl. Acidic electrolyzed water (AcEW) created at the anode has been observed to have sterilization effects on microorganisms, and alkaline electrolyzed water (AlEW) created at the cathode has been observed to have a rinsing effect on organic compounds. In Japan, AcEW already was approved as an indirect food additive in 2002. The principle of electrolyzed water is shown in Figure 1.

We used a flow-type electrolyzed water producer (Rox-20TA: Hoshizaki Electric Co., Japan). This apparatus generates electrolyzed water by the electrolysis of a dilute (0.1%) saline solution in an electrolytic cell separated into an anode and cathode region with a diaphragm. The current passing

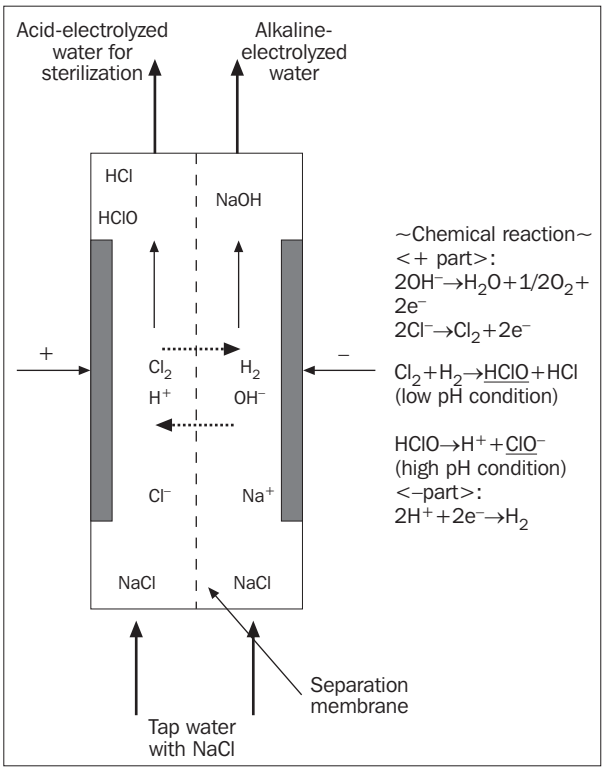

Fig. 1. Principle of acidic electrolyzed water. Left: process flow of apparatus, right: chemical reaction during electrolyzing process.

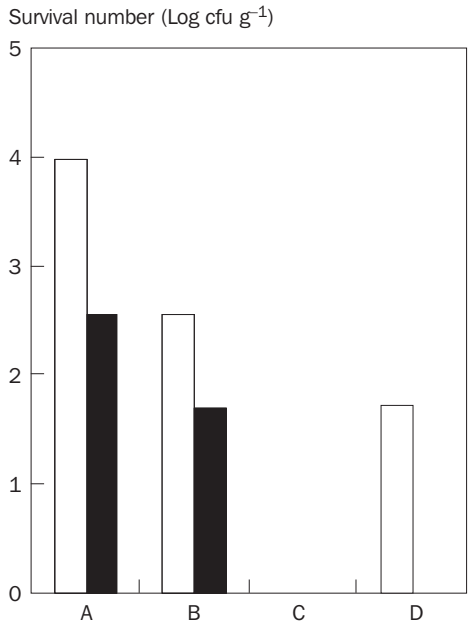

Fig. 2. Sterilization effects of combination of electrolyzed water on rice preparation. (A) Control rice, (B) washing with AlEW (5 min), (C) soaking with AcEW (30 min) after B treatment, (D) soaking with D/W (30 min) after C treatment.

through the electrolysis apparatus and voltage between the electrodes were set at 14A and 18V, respectively. AcEW was prepared within the anode region of the electrolytic cell, and AlEW was prepared within the cathode region. The physicochemical properties of electrolyzed water are as follows:

- AcEW: pH 2.7, oxidation reduction potential (ORP) 1,481 mV, available chlorine concentration 51.5 ppm.
- AlEW: pH 11.6, ORP –576 mV.

We used distilled water as a control processing solution.

Polished rice (Kinuhikari) was purchased and used as a sample. Prepared *Bacillus subtillis* PCI219 was used as a heat-resistant spore sample.

Some 1 mL of spore solution (10^9 cfu mL) was added to 9 mL of AcEW and mixed. The sample was collected from the mixed solution every 1 min and the survival number counted in each sample after cultivation.

Some 100 g of polished rice were prepared for each preparation test. We devised a preparation process with 3 stages (washing: 5 min, soaking 1 ½ min, soaking 2 ½ min). We used AcEW, AlEW, and distilled water, respectively, as each stage solution. pH and color of rice were measured by a conventional pH meter and color meter.

To confirm the microorganism control effect, we added the *B. subtillis* spore into the rice and we counted the survival number in each sample of the preparation test.

Results and discussion

We examined several tests to prove the influence on the microorganism and the change in the raw rice.

AcEW and AlEW changed the color and pH of rice rapidly. But, when the rice was treated with a combination of electrolyzed water (washing with AlEW, soaking with AcEW, and soaking with distilled water), pH and color of the treated rice were no different from conventional washing and soaking with distilled water.

AcEW showed a strong effect on microorganism control, even on heat-resistant spores in vitro. However, in the rice preparation test, AcEW could not kill *B. subtillis* spores on the rice completely. But, after washing by AlEW, AcEW could kill completely. So, finally, we set the optimum method of combination of electrolyzed water as follows. To remove rice bran powder and other foreign materials that coat the surface of the raw rice, at first we washed the rice with AlEW for 5 min and soaked it with AcEW to sterilize microorganisms for 30 min. Then, to remove the odor of chlorine and to revert the pH level, we resoaked the rice with distilled water for 30 min. Figure 2 shows the effects against microorganisms using the combination process of each electrolyzed water.

In this experiment, we could confirm the effect of the combination of electrolyzed water on microorganism control. This effect can be considered the result of available chlorine concentration and the rapid change in pH and it raises the sen-

sibility on the microorganism. From this experiment we can see the importance of the contact condition of the microorganism with the electrolyzed water, which increased the effect of sterilization.

We concluded that the combination of electrolyzed water for rice preparation before cooking (washing and soaking) is efficient in reducing heat-resistant microorganisms; therefore, this process will be able to help make rice products with a long shelf-life without excess heat treatment to keep the quality of these products.

In this experiment, we did not evaluate the quality (texture, flavor, chemical components, and so on) of the cooked rice. We must consider this quality before introducing this pretreatment.

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Notes

Authors' addresses: Seiichiro Isobe, National Food Research Institute, 2-1-12 Kannodai, Tsukuba, Ibaraki 305-8642, Japan, e-mail: seiichi@nfri.affrc.go.jp; Chang-yong Lee, Cheiljedang Corporation Korea; Kyoichiro Yoshida, Hoshizaki Electric Co., Japan.

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Food Control 19 (2008) 329–345
FOOD CONTROL
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Review

Application of electrolyzed water in the food industry

Yu-Ru Huang [a], Yen-Con Hung [b], Shun-Yao Hsu [c], Yao-Wen Huang [d,e], Deng-Fwu Hwang [d,*]

[a] *Department of Food Science, National Penghu University, Penghu, Taiwan, ROC*
[b] *Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of Georgia, Griffin, GA 30223-1797, USA*
[c] *Graduate Institute of Food Science and Technology, National Taiwan University, P.O. Box 23-14, Taipei 106, Taiwan, ROC*
[d] *Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, ROC*
[e] *Department of Food Science and Technology, Center for Food Safety, University of Georgia, Athens, GA 30602-7610, USA*

Received 12 February 2007; received in revised form 6 August 2007; accepted 10 August 2007

Abstract

Electrolyzed oxidizing (EO) water has been regarded as a new sanitizer in recent years. Production of EO water needs only water and salt (sodium chloride). EO water have the following advantages over other traditional cleaning agents: effective disinfection, easy operation, relatively inexpensive, and environmentally friendly. The main advantage of EO water is its safety. EO water which is also a strong acid, is different to hydrochloric acid or sulfuric acid in that it is not corrosive to skin, mucous membrane, or organic material. Electrolyzed water has been tested and used as a disinfectant in the food industry and other applications. Combination of EO water and other measures are also possible. This review includes a brief overview of issues related to the electrolyzed water and its effective cleaning of food surfaces in food processing plants and the cleaning of animal products and fresh produce.
Published by Elsevier Ltd.

Keywords: Electrolyzed water; Disinfectant; Food industry

Contents

* Corresponding author. Tel.: +886 2 24622192x5103; fax: +886 2 24626602.
E-mail address: dfhwang@mail.ntou.edu.tw (D.-F. Hwang).

0956-7135/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.foodcont.2007.08.012

1. Introduction

Food-borne illnesses are prevalent all over the world. The toll of that in terms of human life and suffering is enormous. Acute food-borne disease infections and intoxications are much more of a concern to governments and the food industry today than a few decades ago. From January 1988 through December 1997, a total of 5170 outbreaks of food-borne disease were reported to the Centers for Disease Control and Prevention. These outbreaks caused 163,000 persons to become ill (Bean, Goulding, Lao, & Angulo, 1996; Olsen, Mackinnon, Goulding, Bean, & Slutsker, 2000). Food-borne infections are estimated to cause 76 million illnesses, 300,000 hospitalizations and 5000 deaths annually in the USA (Mead et al., 1999). When excluding multi-ingredient foods, seafood ranked third on the list of products which caused food-borne disease between 1983 and 1992 in the USA (Lipp & Rose, 1997). Moreover, the top five food categories linked to food poisoning outbreaks in the USA from 1990 to 2003 were seafood, dairy products, eggs, beef, and poultry products which were responsible for 61% of all outbreaks according to the Center for Science in the Public Interest (CSPI)'s database (CSPI, 2006). Globally, the search for effective and safe protocols and agents for rendering food safety has been continued to engage the attention of researchers, food manufacturers and retailers as well as policy makers, in countries such as the USA, Japan, UK and Taiwan. In fact, recent outbreaks of food-borne illnesses in Taiwan, USA and Japan, have raised vast international concern.

The best way to reduce incidences of food-borne diseases is to secure safe food supply. Although Hazard Analysis Critical Control Point (HACCP) system has been implemented in many food processing establishments, most outbreaks of food-borne illnesses still occurred in foodservice sectors including institutions, fast food restaurants, and food stores, where food products had undergone various treatments and should have been rendered as safe (Chang, 2003). This situation indicates that hazards might still exist in the food supply systems. Today, food chains are becoming complicated in handling, processing, transportation, and storage ensuring a safe food supply becomes a challenge task.

Electrolyzed oxidizing (EO) water, also known as strongly acidic electrolyzed water (SAEW) or electrolyzed strong acid aqueous solution (ESAAS), is a novel antimicrobial agent which has been used in Japan for several years. It has been reported to possess antimicrobial activity against a variety of microorganisms (Fabrizio & Cutter, 2003; Horiba et al., 1999; Iwasawa & Nakamura, 1993; Kim, Hung, & Brachett, 2000a, 2000b; Kim, Hung, Brachett, & Frank, 2001; Kimura et al., 2006; Kiura et al., 2002; Park & Beuchat, 1999; Park, Hung, & Brackett, 2002a; Venkitanarayanan, Ezeike, Hung, & Doyle, 1999b; Vorobjeva, Vorobjeva, & Khodjaev, 2003). In recent years, EO water has gained interest as a disinfectant used in agriculture, dentistry, medicine and food industry. It has been shown as an effective antimicrobial agent for cutting boards (Venkitanarayanan, Ezeike, Hung, & Doyle, 1999a), poultry carcasses (Fabrizio, Sharma, Demirci, & Cutter, 2002; Park et al., 2002a), eggs (Russell, 2003), lettuce (Izumi, 1999; Koseki & Itoh, 2001; Koseki, Yoshida, Isobe, & Itoh, 2001; Koseki, Fujiwara, & Itoh, 2002; Koseki, Isobe, & Itoh, 2004a; Koseki, Yoshida, Kamitani, Isobe, & Itoh, 2004c; Park, Hung, Doyle, Ezeike, & Kim, 2001; Yang, Swem, & Li, 2003), alfalfa seeds, sprouts (Kim, Hung, Brackett, & Lin, 2003; Sharma & Demirci, 2003), pears (Al-Haq, Seo, Oshita, & Kawagoe, 2002), apples (Okull & Laborde, 2004), peaches (Al-Haq, Seo, Oshita, & Kawagoe, 2001), tomatoes (Bari, Sabina, Isobe, Uemura, & Isshiki, 2003; Deza, Araujo, & Garrido, 2003), strawberry (Koseki, Yoshida, Isobe, & Itoh, 2004b) and food processing equipments (Ayebah & Hung, 2005; Ayebah, Hung, & Frank, 2005; Kim et al., 2001; Park, Hung, & Kim, 2002b; Venkitanarayanan et al., 1999a; Walker, Demirci, Graves, Spencer, & Roberts, 2005a, 2005b). EO water also has the potential to be more effective and inexpensive than traditional cleaning agents. The greatest advantage of EO water for the inactivation of pathogenic microorganisms relies on its less adverse impact on the environment as well as users' health because of no hazard chemicals added in its production. Moreover, it has been clarified that EO water does no harm to the human body (Mori, Komatsu, & Hata, 1997). It is more effective, less dangerous and less expensive than most traditional preservation methods such as glutaraldehyde (Sakurai, Nakatsu, Sato, & Sato, 2003; Sakurai, Ogoshi, Kaku, & Kobayashi, 2002), sodium hypochlorite and acetic acid (Ayebah et al., 2005). Many aspects of EO water are elucidated in this review, including its chemical and physical properties, generation, antimicrobial properties and its applications in food industries, such as fresh vegetables, fruits, eggs, poultry and seafood.

2. Principles and characteristics of electrolyzed water

EO water was initially developed in Japan (Shimizu & Hurusawa, 1992). It has been reported to have strong bactericidal effects on most pathogenic bacteria that are important to food safety. EO water is produced by passing a diluted salt solution through an electrolytic cell, within which the anode and cathode are separated by a membrane. By subjecting the electrodes to direct current voltages, negatively charged ions such as chloride and

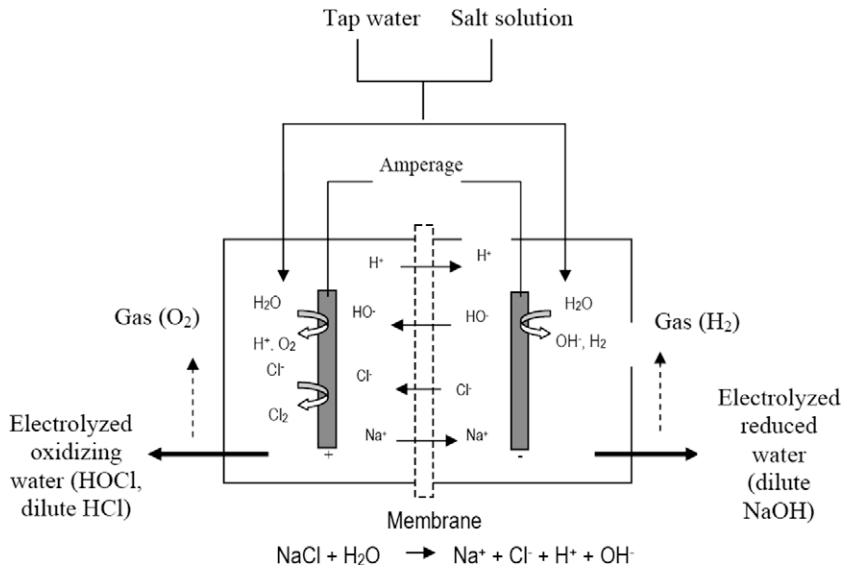

Fig. 1. Schematics of electrolyzed water generator and produced compounds.

hydroxide in the diluted salt solution move to the anode to give up electrons and become oxygen gas, chlorine gas, hypochlorite ion, hypochlorous acid and hydrochloric acid, while positively charged ions such as hydrogen and sodium move to the cathode to take up electrons and become hydrogen gas and sodium hydroxide (Hsu, 2005). Two types of water are produced simultaneously. EO water, with low pH (2.3–2.7), high oxidation–reduction potential (ORP, >1000 mV), high dissolved oxygen and contains free chlorine (concentration depends on the EO water machine setting), is produced from anode side. However, electrolyzed reduced (ER) water, with high pH (10.0–11.5), high dissolved hydrogen, and low ORP (−800 to −900 mV), is produced from the cathode side. ER water with strong reducing potential can be used to remove dirt and grease from items such as cutting boards and other kitchen utensils (Hsu, 2005).

The principle of producing electrolyzed water is shown in the Fig. 1 with the following:

$$\begin{aligned}\text{Positivepole}: & \quad 2H_2O \rightarrow 4H^+ + O_2 \uparrow + 4e^- \\ & \quad 2NaCl \rightarrow Cl_2 \uparrow + 2e^- + 2Na^+ \\ & \quad Cl_2 + H_2O \rightarrow HCl + HOCl \\ \text{Negativepole}: & \quad 2H_2O + 2e^- \rightarrow 2OH^- + H_2 \uparrow \\ & \quad 2NaCl + 2OH^- \rightarrow 2NaOH + Cl^-\end{aligned}$$

3. Systems for generation of electrolyzed water

Commercial EO water generators can be divided into three major types based on their automatic control systems. The first type of EO water generators, made by the ARV® and the Amano® companies, allows the users to select brine flow rate while the machines adjust voltages and/or amperages automatically. The second type of EO water generators, made by the Hoshizaki® Company, allows the users to select amperages and/or voltages, while the machines change brine flow rate accordingly. The third type of EO water generators, made by the Toyo® and the Nippon Intek® companies, allows the users to select a preset chlorine concentration level of EO water from a display panel and the machines change brine flow rate and amperages and/or voltages automatically (Hsu, 2003).

Hsu (2003) investigated relationship among water flow rate, water temperature and salt concentration on electrolysis efficiency, and separation efficiency of an EO water generator. He made following conclusions: (1) electric potential (7.9–15.7 V) and power consumption (16–120 W) of electrolysis cell were not affected by water flow rate, water temperature or salt concentration in the feed solution; (2) electric current changed with water temperature and water flow rate; and (3) electrolysis efficiency of the electrolysis cell and separation efficiency of the ion exchange membrane were significantly decreased by the increases in water flow rate and salt concentration in the feed solution. Later, Hsu (2005) also reported that ORP decreased with increases in water follow rate and free chlorine increased with increases of salt concentration and decrease of water flow rate.

4. The advantages and disadvantages of EO water

The main advantage of EO water is its safety. EO water which is also a strong acid, is different to hydrochloric acid or sulfuric acid in that it is not corrosive to skin, mucous membrane, or organic material. On the other hand, sodium hypochlorite was proved to have a strong toxicity, such as skin irritation, membrane irritation, acute toxicity, and so on (Mori et al., 1997; Sekiya, Ohmori, & Harii, 1997; Shigeto et al., 2000). Currently used hatchery sanitizers

(formaldehyde gas and glutaraldehyde) are noxious to humans and chicks, and may pose a serious health risk (Russell, 2003). Furthermore, the use of formaldehyde gas and glutaraldehyde are gradually being limited because of the adverse effects this chemical has on the environment. Sakurai et al. (2003) also stated that EO water provides a useful means of cleaning and disinfecting digestive endoscopes between patients. It is safe for the human body and for the environment. In addition, the cost of using EO water is much less expensive (5.3 yen/L) compared with glutaraldehyde (1200 yen/L) (Sakurai et al., 2003).

When EO water comes into contact with organic matter, or is diluted by tap water or reverse osmosis (RO) water, it becomes ordinary water again. Thus, it's less adverse impact on the environment as well as users' health. Moreover, compared with other conventional disinfecting techniques, EO water reduces cleaning times, is easy to handle, has very few side effects, and is relative cheap (Tanaka et al., 1999). Chemicals used for cleaning and disinfection are expensive and represent an operating expense for the dairy producer. Once the initial capital investment is made to purchase an EO water generator, the only operating expenses are water, salts and electricity to run the unit (Walker et al., 2005b).

The main disadvantage of EO water is that the solution rapidly loses its antimicrobial activity if EO water is not continuously supplied with H^+, HOCl and Cl_2 by electrolysis (Kiura et al., 2002). EO water is gaining a reputation in various fields as a more capable disinfectant than conventional chemical disinfectants. However, problems, such as chlorine gas emission, metal corrosion, and synthetic resin degradation, due to its strong acidity and free chlorine content have been a matter of concern. Although metal corrosion and synthetic resin degradation occurred, they were not serious on hemodialysis equipment (Tanaka et al., 1999). Ayebah and Hung (2005) also indicated that EO water did not have any adverse effect on stainless steel, it can still be safely used as a sanitizer to inactivate bacteria on food contact surfaces made from stainless steel in food processing. After disinfection, washing food equipment with sterile water can completely avoid metal corrosion. During the EO water generation process, chlorine ions are generated, and thus chlorine gas is emitted. This necessitates the use of standard-type extractor fan.

5. Inactivation of microbes using EO water

As shown in Table 1, many studies have been conducted in evaluating the bactericidal activity of EO water. EO water possess antimicrobial activity on a variety of microorganisms including *Pseudomonas aeruginosa* (Kiura et al., 2002; Vorobjeva et al., 2003), *Staphylococcus aureus* (Park et al., 2002b; Vorobjeva et al., 2003), *S. epidermidis*, *E. coli* O157:H7 (Kim et al., 2000a, 2000b; Park, Hung, & Chung, 2004; Venkitanarayanan et al., 1999b), *Salmonella* Enteritidis (Venkitanarayanan et al., 1999b), *Salmonella* Typhimurium (Fabrizio & Cutter, 2003), *Bacillus cereus* (Len, Hung, Erickson, & Kim, 2000; Sakashita, Iwasawa, & Nakamura, 2002; Vorobjeva et al., 2003), *Listeria monocytogenes* (Fabrizio & Cutter, 2003; Park et al., 2004; Vorobjeva et al., 2003), *Mycobacterium tuberculosis* (Iwasawa & Nakamura, 1993), *Campylobacter jejuni* (Park et al., 2002a), *Enterobacter aerogenes* (Park et al., 2002b) and *Vibrio parahaemolyticus* (Huang et al., 2006a; Kimura et al., 2006). EO water can also reduce germination of many fungal species, such as *Alternaria* spp., *Bortrytis* spp., *Cladosporium* spp., *Colletotrichum* spp., *Curvularia lunata*, *Didymella bryonaie*, *Epicoccum nigrum*, *Fusarium* spp., *Helminthosporium* spp., *Pestalotia* spp., *Phomopsis longicolla*, *Rhodosporidium toruloides*, *Stagonospora nodorum*, *Thielaviopsis basicola*, *Trichoderma spirale*, *Acidovorax avenae* subsp., *Erwinia chrysanthemi*, *Pantoea ananatis*, *Pseudomonas syringae* (Buck, Iersel, Oetting, & Hung, 2002), *Aspergillus* spp. (Buck et al., 2002; Suzuki et al., 2002b), *Botryosphaeria berengeriana* (Al-Haq et al., 2002), *Monilinia fructicola* (Al-Haq et al., 2001; Buck et al., 2002), *Penicillium expansum* (Okull & Laborde, 2004) and *Tilletia indica* (Bonde et al., 1999).

In general, bacteria generally grow in a pH range of 4–9. Aerobic bacteria grow mostly at ORP range +200 to 800 mV, while anaerobic bacteria grow well at −700 to +200 mV. The high ORP in the EO water could cause the modification of metabolic fluxes and ATP production, probably due to the change in the electron flow in cells. Low pH may sensitize the outer membrane of bacterial cells to the entry of HOCl into bacterial cells (McPherson, 1993). HOCl, the most active of the chlorine compounds, appears to kill the microbial cell through inhibiting glucose oxidation by chlorine-oxidizing sulfhydryl groups of certain enzymes important in carbohydrate metabolism. Other modes of chlorine action that have been proposed are: (1) disruption of protein synthesis; (2) oxidative decarboxylation of amino acids to nitrites and aldehydes; (3) reactions with nucleic acids, purines, and pyrimidines; (4) unbalanced metabolism after the destruction of key enzymes; (5) induction of deoxyribonucleic acid (DNA) lesions with the accompanying loss of DNA-transforming ability; (6) inhibition of oxygen uptake and oxidative phosphorylation, coupled with leakage of some macromolecules; (7) formation of toxic *N*-chlorine derivatives of cytosine; and (8) creation of chromosomal aberrations (Marriott & Gravani, 2006).

A theory for inactivation of bacteria based on the high oxidation potential of EO water causing damage of cell membranes was reported by Liao, Chen, and Xiao (2007). The chemical process of oxidation occurs when oxygen contacts with other compounds causing them to lose electrons and further causing the compounds to break down and change functions. In the case of microbes, oxidation could damage cell membranes, create disruption in cell metabolic processes and essentially kill the cell. The bactericidal effects of EO water on *Staphylococcus saprophyticus*, *Micrococcus luteus* and *Bacillus sphaericus* can be seen by

Table 1
A comparison of bactericidal effects on bacterial strains treated with electrolyzed oxidizing water

Bacterial species	Surviving bacterial population after exposing time (mean log CFU/mL)						EO water property				Ref.
	0 s	30 s	1 min	5 min	10 min	15 min	pH	ORP (mV)	Free chlorine (mg/L)	Temperature (°C)	
Gram-negative											
Escherichia coli O157:H7	7.98 ± 0.04	–	–	<1.0	0	0	2.36	1153	86.3	4	Venkitanarayanan et al. (1999b)
Escherichia coli O157:H7	8.04 ± 0.07	–	–	<1.0	0	0	2.37	1155	82.3	23	Venkitanarayanan et al. (1999b)
Salmonella Enteritidis	7.74 ± 0.08	–	–	1.06 ± 0.15	0	0	2.48	1153	83.5	4	Venkitanarayanan et al. (1999b)
Salmonella Enteritidis	7.76 ± 0.08	–	–	<1.0	0	0	2.45	1151	82.0	23	Venkitanarayanan et al. (1999b)
Salmonella Typhimurium	5.20 ± 1.0	–	–	5.13 ± 1.20	3.37 ± 0.70	3.32 ± 0.50	2.30	1155	50	4	Fabrizio and Cutter (2003)
Salmonella Typhimurium	5.11 ± 1.60	–	–	3.46 ± 1.40	0	0	2.60	1150	50	25	Fabrizio and Cutter (2003)
Pseudomonas aeruginosa	8.04 ± 0.07	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Escherichia coli	8.21 ± 0.04	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Citrobacter freundii	7.63 ± 0.06	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Flavobacter sp.	8.12 ± 0.02	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Proteus vulgaris	8.01 ± 0.04	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Alcaligenes faecalis	7.80 ± 0.03	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Aeromonas liquefaciens	7.90 ± 0.04	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Campylobacter jejuni	7.42 ± 0.26	<1	–	–	–	–	2.95	1072	25.7	4	Park et al. (2002a)
Campylobacter jejuni	7.47 ± 0.13	<1	–	–	–	–	2.67	1092	53.9	23	Park et al. (2002a)
Campylobacter jejuni	7.42 ± 0.26	0	–	–	–	–	2.67	1092	53.3	4	Park et al. (2002a)
Campylobacter jejuni	7.47 ± 0.13	0	–	–	–	–	2.57	1082	51.6	23	Park et al. (2002a)
Enterococcus faecalis	8.23 ± 0.03	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Gram-positive											
Listeria monocytogenes	7.91 ± 0.05	–	–	1.34 ± 0.37	0	0	2.63	1160	43.0	4	Venkitanarayanan et al. (1999b)
Listeria monocytogenes	7.89 ± 0.10	–	–	1.23 ± 0.33	0	0	2.63	1158	48.5	23	Venkitanarayanan et al. (1999b)
Listeria monocytogenes	5.89 ± 0.40	–	–	5.36 ± 0.80	5.12 ± 0.80	4.60 ± 1.10	2.60	1150	50	4	Fabrizio and Cutter (2003)
Listeria monocytogenes	5.10 ± 1.40	–	–	2.66 ± 1.10	0	0	2.60	1150	50	25	Fabrizio and Cutter (2003)
Staphylococcus aureus	8.36 ± 0.08	0	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Staphylococcus aureus	8.03 ± 0.03	0	–	–	–	–	2.53	1178	53.1	23	Park et al. (2002b)
Staphylococcus aureus	8.03 ± 0.03	0	–	–	–	–	2.79	1163	26.9	23	Park et al. (2002b)
Staphylococcus aureus	8.03 ± 0.03	3.92 ± 0.11	–	–	–	–	3.18	1116	11.3	23	Park et al. (2002b)
Bacillus cereus	6.72 ± 0.02	3.76 ± 0.02	–	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Bacillus cereus (spores)	7.98 ± 0.06	–	0	–	–	–	2.84	1125	43	23	Vorobjeva et al. (2003)
Enterobacter aerogenes	7.98 ± 0.04	0	–	–	–	–	2.53	1178	53.1	23	Park et al. (2002b)
Enterobacter aerogenes	7.98 ± 0.04	0	–	–	–	–	2.79	1163	26.9	23	Park et al. (2002b)
Enterobacter aerogenes	7.98 ± 0.04	0	–	–	–	–	3.18	1116	11.3	23	Park et al. (2002b)

0, Complete inactivation of bacterial culture; –, not measured.

using a scanning electron microscope. The cells treated with electrolyzed acidic water had wrinkled cell wall with round pores in which the cytoplasmic structures were flushed out (Osafune, Ehara, & Ito, 2006).

Little reports on the effects of chlorine, pH and ORP values of the EO water in inactivation of pathogens are available. Kim et al. (2000b) have developed chemically modified water from deionized water with the same properties (i.e., pH, chlorine and ORP) as EO water without using electrolysis. Their results suggested that ORP of EO water might be the primary factor responsible for the bactericidal effect. However, Koseki et al. (2001) noted that the ORP is not the main factor of antimicrobial activity because the higher ORP of ozonated water did not show higher disinfectant effect than lower ORP of EO water. They further defined that free chlorine of EO water, mainly hypochlorous acid (HOCl), produces hydroxyl radical (·OH) that acts on microorganisms. Ozone solution produces ·OH, too. The higher ·OH produced by higher HOCl concentration in EO water means the better the disinfectant efficacy than ozone solution. Len et al. (2000) reported that the relative concentrations of aqueous molecular chlorine, HOCl, hypochlorite ion (OCl^-) and chlorine gas (Cl_2) were also the factors that accounted for the bactericidal potency. At pH 4, EO water with the maximum concentration of HOCl had the maximum microbicidal activity.

Park et al. (2004) investigated the effects of chlorine and pH on efficacy of EO water for inactivating *E. coli* O157:H7 and *L. monocytogenes*. It was demonstrated that EO water is very effective for inactivating *E. coli* O157:H7 and *L. monocytogenes* in a wide pH range (between 2.6 and 7.0), if sufficient free chlorine (>2 mg/L) is present. For each chlorine content, bactericidal activity and ORP increased with decreasing pH. Based on fluorescent and spectroscopic measurements, Liao et al. (2007) reported that the ORP of EO water could damage the outer and inner membranes of *E. coli* O157:H7. The redox state of the glutathione disulfide–glutathione couple (GSSG/2GSH) can serve as an important indicator of redox environment. There are many redox couples in a cell that work together to maintain the redox environment. The inactivation mechanism hypothesized was that ORP could damage the redox state of GSSG/2GSH and then penetrate the outer and inner membranes of cell, giving rise to the release of intracellular components and finally cause the necrosis of *E. coli* O157:H7. Thus, the antimicrobial effect of EO water derives from the combined action of the hydrogen ion concentration, oxidation–reduction potential and free chlorine.

Storage conditions can affect chemical and physical properties of EO water. When stored under an open, agitated and diffused light condition the EO water had the highest chlorine loss rate. Under open condition, chlorine loss through evaporation followed first-order kinetics. The rate of chlorine loss was increased abound 5-fold with agitation, but it was not significantly affected by diffused light (Len, Hung, & Chung, 2002). EO water exposed to the atmosphere could reduce more chlorine and oxygen than that kept to a closed systems for a longer time (Hsu & Kao, 2004). Fabrizio and Cutter (2003) reported that EO water stored at 4 °C was more stable than stored at 25 °C.

The effectiveness of chlorine as a bactericidal agent is reduced in the presence of organic matter due to the formation of combined available chlorines. At an identical chlorine concentration, the combined available chlorines had much lower bactericidal activity than the free form (Oomori, Oka, Inuta, & Arata, 2000). For practical application, EO water usually must be used in the presence of amino acids or proteins containing materials produce a combined form. Although the electrolyzed solution is not a newly discovered disinfectant, it is important to examine its bactericidal effect on different bacteria (Table 1).

6. Inactivation of blood-virus using EO water

Researchers also indicated that EO water has antiviral potency on blood borne pathogenic viruses including hepatitis B virus (HBV), hepatitis C virus (HCV) (Morita et al., 2000; Sakurai et al., 2003; Tagawa et al., 2000) and human immunodeficiency virus (HIV) (Kakimoto et al., 1997; Kitano et al., 2003; Morita et al., 2000). EO water contained only 4.2 mg/L of free chlorine (pH 2.34, ORP 1053 mV) had a greater efficacy against hepatitis B virus surface antigen (HBsAg) and HIV-1 than sodium hypochlorite (Morita et al., 2000). The possible mechanisms underlying the EO water disinfection against blood-borne viruses might include (1) inactivation of surface protein; (2) destruction of virus envelope; (3) inactivation of viral nucleic acids encoding for enzymes; and (4) destruction of viral RNA (Morita et al., 2000). Hanson, Gor, Jeffries, and Collins, 1989 demonstrated that dried HIV is relatively resistant against disinfectants compared with wet HIV. In an insightful work, Kitano et al. (2003) stated that EO water has an inactivation potential against the infectivity of dried HIV-1. They found that the viral reverse transcript (RT) and the viral RNA in HIV-1 are targets of EO water. Sakurai et al. (2003) reported experiments with HBC and HCV-contaminated endoscopes, and concluded that neither HBV nor HCV was detected after the endoscopes were cleaned manually with a brush and disinfected with EO water. Viral DNA was not detected from any endoscope experimentally contaminated with viral-positive mixed sera (Lee et al., 2004; Tagawa et al., 2000). Thus, EO water directly inactivates viruses and its clinical application is recommended. Effectiveness of EO water in preventing viral infection in the food field needs to be further studied.

7. Inactivation of toxins using EO water

Staphylococcal food poisoning results from the consumption of a food in which enterotoxigenic staphylococci have grown and produced toxins. Within 1–6 h after ingestion of staphylococcal enterotoxin (SEs)-contaminated foods, victims experience nausea, abdominal cramps, vom-

iting, and diarrhea (Archer & Young, 1988; Garthright, Archer, & Kvenberg, 1988). Although EO water has been proved to be effective against *Staphylococcus aureus*, trace amounts of enterotoxin produced by the bacteria may remain active after disinfection. Suzuki, Itakura, Watanabe, and Ohta (2002a) reported that exposure of 70 ng, or 2.6 pmol, of staphylococcal enterotoxin A (SEA) in 25 μL of phosphate buffer saline (PBS) to a 10-fold volume of EO water, or 64.6×10^3-fold molar excess of HOCl in EO water, caused a loss of immuno-reactivity between SEA and a specific anti-SEA antibody. Native PAGE indicated that EO water caused fragmentation of SEA, and amino acid analysis indicated a loss in amino acid content, in particular Met, Tyr, Ile, Asn, and Asp. EO water denatures SEA through an oxidative reaction caused by OH radicals and reactive chlorine. Thus, EO water might be useful as a preventive measure against food-borne disease caused by SEA.

Suzuki et al. (2002b) also reported that EO water could sterilize *Aspergillus parasiticus* and eliminate the mutagenicity of aflatoxin AFB_1 by the OH radical originating from HOCl. Exposing *A. parasiticus* at an initial density of 10^3 spores in 10 μL to a 50-fold volume (500 μL) of EO water containing 390 μmol HOCl for 15 min at room temperature resulted in a complete inhibition of fungal growth. Three nanomoles of AFB_1 showed a high mutagenicity for both *Salmonella* Typhimurium TA98 and TA100 strains, but this mutagenicity was reduced markedly after exposure to 20-fold molar amount of HOCl in the EO water in both TA98 and TA100. However, foods contain compounds such as proteins, lipids, vitamins, minerals, color, etc., and concerning food soundness, it may not necessarily be appropriate to apply EO water to wash food materials.

8. EO water used as a disinfectant in the food industry

8.1. Use of EO water for food processing equipment

EO water has been used as a disinfectant for food processing equipment (Table 2). Venkitanarayanan et al. (1999a) reported EO water could be used as an effective method for eliminating food-borne pathogens on cutting boards. EO water (pH of 2.53, ORP of 1178 mV and chlorine of 53 mg/L) could also reduce *Enterobacter aerogenes* and *S. aureus* on glass, stainless, steel, glazed ceramic tile, unglazed ceramic tile and vitreous china surfaces. Immersion of these surfaces in EO water for 5 min with agitation (50 rpm) reduced populations of *E. aerogenes* and *S. aureus* on the tested surfaces to <1 CFU/cm^2 (Park et al., 2002b). *Listeria monocytogenes* is a food-borne pathogen that can lead to potentially life-threatening listeriosis in high-risk populations. Listeriosis outbreaks have been associated with processed foods and the formation of *L. monocytogenes* biofilms in the processing environment is an important source for secondary contamination (Carpentier & Chassaing, 2004).

Frank and Koffi (1990) and Lee and Frank (1991) earlier reported that *L. monocytogenes* biofilms are resistant to chlorine, acid anionic and quaternary ammonium sanitizers, so that inadequate cleaning and sanitation of food processing surfaces may lead to spread of the pathogen throughout the entire processing plant. Kim et al. (2001) investigated the resistance of *L. monocytogenes* biofilms on stainless steel surfaces to EO water (pH of 2.60, ORP of 1160 mV and chlorine of 56 mg/L) and found that a 300-s treatment on a stainless steel surface, could reduce the *L. monocytogenes* from 1.9×10^{10} CFU/82.5 cm^2 to below detection levels (5 CFU/coupon). However, it took 300 s of exposure to 200 mg/L chlorine solution to achieve the same result. Ayebah et al. (2005) recently inactivated *L. monocytogenes* biofilms on stainless steel surfaces with a combination of ER and EO water. They found that ER water alone did not significantly reduce the *L. monocytogenes* biofilms. Treatment with EO water for only 30–120 s reduced the viable bacteria populations in biofilms by 4.3–5.2 log CFU per coupon (2 by 5 cm), whereas the combined treatment of ER water followed by EO water could produce an additional reduction by 0.3–1.2 log CFU per coupon.

Stainless steel has been the most commonly used material for food contact surfaces in the food industry. Ayebah and Hung (2005) reported that EO water (pH of 2.42, ORP of 1077 mV and free chlorine of 50 mg/L) and modified EO water (pH of 6.12, ORP of 774 mV and free chlorine of 50 mg/L) did not have any adverse effect on stainless steel for a period of 8 days.

The effect of EO water in reducing bacteria in the pipelines of the milking system has been investigated (Walker et al., 2005a, 2005b). A 10 min wash with 60 °C ER water followed by a 10 min wash with 60 °C EO water successfully removed all detectable bacteria from the non-porous milk contact surfaces and ATP residue tests were negative. These results indicated that EO water has the potential to be used as a cleaning and sanitizing agent for cleaning in place (CIP) cleaning of on-farm milking systems.

8.2. Use of EO water for vegetables

Electrolyzed water has been used to inactivate pathogens on fresh produce (Table 3). Izumi (1999) has demonstrated that EO water is usable for cleaning fresh-cut carrots, bell peppers, spinach, Japanese radish and potatoes. The pre-cut produces, treated with EO water (pH 6.8, 20 mg/L free chlorine) by dipping, rinsing or dipping/blowing, showed a bacterial reduction by 0.6–2.6 logs CFU/g. The EO water containing 50 mg/L chlorine had a stronger bactericidal effect than that containing 15 or 30 mg/L chlorine. The treatment did not cause discoloration of fresh-cut produces. Rinsing EO water (50 mg/L) treated fresh-cut produces with fresh water did not increase the bacterial reduction due to the additive effects of the sequential treatment. Koseki et al. (2004b) reported that cucumbers washed with

Table 2
Inactivation of food-borne pathogens on food processing materials by electrolyzed oxidizing water

Processing materials	Immersion condition	Indicator	Effectiveness	EO water property				Ref.
				pH	ORP (mV)	Free chlorine (mg/L)	Temperature (°C)	
Kitchen cutting board	10 min	*Escherichia coli* O157:H7	++	2.50	1163	87	23	Venkitanarayanan et al. (1999a)
Kitchen cutting board	20 min	*Escherichia coli* O157:H7	++	2.56	1165	80	23	Venkitanarayanan et al. (1999a)
Kitchen cutting board	10 min	*Escherichia coli* O157:H7	+++	2.58	1161	87	35	Venkitanarayanan et al. (1999a)
Kitchen cutting board	20 min	*Escherichia coli* O157:H7	+++	2.56	1162	90	35	Venkitanarayanan et al. (1999a)
Kitchen cutting board	5 min	*Escherichia coli* O157:H7	++	2.46	1154	87	45	Venkitanarayanan et al. (1999a)
Kitchen cutting board	10 min	*Escherichia coli* O157:H7	+++	2.51	1157	93	45	Venkitanarayanan et al. (1999a)
Kitchen cutting board	5 min	*Escherichia coli* O157:H7	+++	2.29	1147	45	55	Venkitanarayanan et al. (1999a)
Kitchen cutting board	20 min	*Listeria monocytogenes*	+++	2.50	1156	72	23	Venkitanarayanan et al. (1999a)
Kitchen cutting board	10 min	*Listeria monocytogenes*	++	2.38	1156	66	35	Venkitanarayanan et al. (1999a)
Kitchen cutting board	10 min	*Listeria monocytogenes*	++	2.33	1150	52	45	Venkitanarayanan et al. (1999a)
Glass	5 min	*Enterobacter aerogenes*	++	2.53	1178	53	23	Park et al. (2002a)
Glass	5 min and 50 rpm	*Enterobacter aerogenes*	+++	2.53	1178	53	23	Park et al. (2002a)
Stainless steel	5 min	*Enterobacter aerogenes*	++	2.53	1178	53	23	Park et al. (2002a)
Stainless steel	5 min and 50 rpm	*Enterobacter aerogenes*	+++	2.53	1178	53	23	Park et al. (2002a)
Glazed ceramic tile	5 min	*Enterobacter aerogenes*	++	2.53	1178	53	23	Park et al. (2002a)
Glazed ceramic tile	5 min and 50 rpm	*Enterobacter aerogenes*	+++	2.53	1178	53	23	Park et al. (2002a)
Unglazed ceramic tile	5 min	*Enterobacter aerogenes*	++	2.53	1178	53	23	Park et al. (2002a)
Unglazed ceramic tile	5 min and 50 rpm	*Enterobacter aerogenes*	+++	2.53	1178	53	23	Park et al. (2002a)
Vitreous china	5 min	*Enterobacter aerogenes*	++	2.53	1178	53	23	Park et al. (2002a)
Vitreous china	5 min and 50 rpm	*Enterobacter aerogenes*	+++	2.53	1178	53	23	Park et al. (2002a)
Glass	5 min	*Staphylococcus aureus*	+	2.53	1178	53	23	Park et al. (2002a)
Glass	5 min and 50 rpm	*Staphylococcus aureus*	+++	2.53	1178	53	23	Park et al. (2002a)
Stainless steel	5 min	*Staphylococcus aureus*	+	2.53	1178	53	23	Park et al. (2002a)
Stainless steel	5 min and 50 rpm	*Staphylococcus aureus*	+++	2.53	1178	53	23	Park et al. (2002a)
Glazed ceramic tile	5 min	*Staphylococcus aureus*	+	2.53	1178	53	23	Park et al. (2002a)
Glazed ceramic tile	5 min and 50 rpm	*Staphylococcus aureus*	+++	2.53	1178	53	23	Park et al. (2002a)
Unglazed ceramic tile	5 min	*Staphylococcus aureus*	+	2.53	1178	53	23	Park et al. (2002a)
Unglazed ceramic tile	5 min and 50 rpm	*Staphylococcus aureus*	+++	2.53	1178	53	23	Park et al. (2002a)
Vitreous china	5 min	*Staphylococcus aureus*	+	2.53	1178	53	23	Park et al. (2002a)
Vitreous china	5 min and 50 rpm	*Staphylococcus aureus*	+++	2.53	1178	53	23	Park et al. (2002a)
Stainless steel	0.5 min	*Listeria monocytogenes* biofilms	++	2.40	1163	47	23	Ayebah et al. (2005)
Stainless steel	1 min	*Listeria monocytogenes* biofilms	++	2.40	1163	47	23	Ayebah et al. (2005)
Stainless steel	2 min	*Listeria monocytogenes* biofilms	++	2.40	1163	47	23	Ayebah et al. (2005)
Stainless steel	0.5 min	*Listeria monocytogenes* biofilms	++	2.38	1169	84	23	Ayebah et al. (2005)
Stainless steel	1 min	*Listeria monocytogenes* biofilms	++	2.38	1169	84	23	Ayebah et al. (2005)
Stainless steel	2 min	*Listeria monocytogenes* biofilms	++	2.38	1169	84	23	Ayebah et al. (2005)
Stainless steel	1 min	*Listeria monocytogenes* biofilms	+++	2.6	1160	56	23	Kim et al. (2001)
Stainless steel	5 min	*Listeria monocytogenes* biofilms	+++	2.6	1160	56	23	Kim et al. (2001)

+++, bacterial reduction being more than 6 log CFU/ per unit; ++, bacterial reduction being between 2 and 6 log CFU/ per unit; +, bacterial reduction being less than 2 log CFU/ per unit.

Table 3
Inactivation of food-borne pathogens on vegetables by electrolyzed oxidizing water

Vegetables	Immersion condition	Indicator	Effectiveness	EO water property				Ref.
				pH	ORP (mV)	Free chlorine (mg/L)	Temperature (°C)	
Carrot	EO 4 min	Aerobic bacteria counts	++	6.8	–	20	23	Izumi (1999)
Spinach	EO 4 min	Aerobic bacteria counts	+++	6.8	–	20	23	Izumi (1999)
Bell pepper	EO 4 min	Aerobic bacteria counts	+	6.8	–	20	23	Izumi (1999)
Japanese radish	EO 4 min	Aerobic bacteria counts	+	6.8	–	20	23	Izumi (1999)
Potato	EO 4 min	Aerobic bacteria counts	+	6.8	–	20	23	Izumi (1999)
Cucumber	EO 5 min	Aerobic bacteria counts	++	2.6	1130	32	23	Koseki et al. (2004b)
Cucumber	EO 5 min	Coliform bacteria	+++	2.6	1130	32	23	Koseki et al. (2004b)
Cucumber	EO 5 min	Fungi	+++	2.6	1130	32	23	Koseki et al. (2004b)
Cucumber	EO 5 min+23 °C ER 5 min	Aerobic bacteria counts	+++	2.6	1130	32	23	Koseki et al. (2004b)
Cucumber	EO 5 min+23 °C ER 5 min	Coliform bacteria	+++	2.6	1130	32	23	Koseki et al. (2004b)
Cucumber	EO 5 min+23 °C ER 5 min	Fungi	+++	2.6	1130	32	23	Koseki et al. (2004b)
Lettuce	EO 10 min	Aerobic bacteria counts	+++	2.6	1140	30	23	Koseki et al. (2001)
Lettuce	EO 1 min+23 °C ER 1 min	Aerobic bacteria counts	+++	2.6	1140	30	23	Koseki et al. (2001)
Lettuce	EO 5 min	*Escherichia coli* O157:H7	++	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	EO 5 min+ EO 5 min	*Escherichia coli* O157:H7	++	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	20 °C ER 5 min+ EO 5 min	*Escherichia coli* O157:H7	++	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	EO 5 min	*Salmonella* sp.	++	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	EO 5 min+ EO 5 min	*Salmonella* sp.	++	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	20 °C ER 5 min+ EO 5 min	*Salmonella* sp.	++	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	EO 1 min	*Escherichia coli* O157:H7	+	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	EO 5 min	*Escherichia coli* O157:H7	++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	1 min EO	*Escherichia coli* O157:H7	+	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	5 min EO	*Escherichia coli* O157:H7	++	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	1 min EO	*Escherichia coli* O157:H7	+++	2.6	–	40	50	Koseki et al. (2004c)
Lettuce	5 min EO	*Escherichia coli* O157:H7	++++	2.6	–	40	50	Koseki et al. (2004c)
Lettuce	1 min EO	*Salmonella* sp.	+	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	5 min EO	*Salmonella* sp.	++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	1 min EO	*Salmonella* sp.	+	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	5 min EO	*Salmonella* sp.	++	2.6	–	40	20	Koseki et al. (2004c)
Lettuce	1 min EO	*Salmonella* sp.	+++	2.6	–	40	50	Koseki et al. (2004c)
Lettuce	5 min EO	*Salmonella* sp.	+++	2.6	–	40	50	Koseki et al. (2004c)
Lettuce	20 °C ER 1 min +1 or 5 min EO	*Escherichia coli* O157:H7	++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	20 °C ER 5 min +1 or 5 min EO	*Escherichia coli* O157:H7	++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	50 °C ER 1 min +1 or 5 min EO	*Escherichia coli* O157:H7	+++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	50 °C ER 5 min +1 or 5 min EO	*Escherichia coli* O157:H7	+++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	20 °C ER 1 min +1 or 5 min EO	*Salmonella* sp.	++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	20 °C ER 5 min +1 or 5 min EO	*Salmonella* sp.	++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	50 °C ER 1 min +1 or 5 min EO	*Salmonella* sp.	+++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	50 °C ER 5 min +1 or 5 min EO	*Salmonella* sp.	+++	2.6	–	40	4	Koseki et al. (2004c)
Lettuce	1 min EO	*Escherichia coli* O157:H7	++++	2.5	1030	45	22	Park et al. (2001)
Lettuce	3 min EO	*Escherichia coli* O157:H7	++++	2.5	1030	45	22	Park et al. (2001)
Lettuce	1 min EO	*Listeria monocytogenes*	+++	2.5	1030	45	22	Park et al. (2001)
Lettuce	3 min EO	*Listeria monocytogenes*	++++	2.5	1030	45	22	Park et al. (2001)
Alfalfa seeds	3 hr	*Salmonella* sp.	++	2.4	1081	84	23	Kim et al. (2003)

(*continued on next page*)

Table 3 (*continued*)

Vegetables	Immersion condition	Indicator	Effectiveness	EO water property				Ref.
				pH	ORP (mV)	Free chlorine (mg/L)	Temperature (°C)	
Alfalfa seeds	3 hr	Non-*Salmonella* microflora	++	2.4	1081	84	23	Kim et al. (2003)
Alfalfa sprouts	10 min EO & sonication	*Salmonella* sp.	++	2.4	1081	84	23	Kim et al. (2003)
Alfalfa sprouts	10 min EO & sonication	Non-*Salmonella* microflora	+	2.4	1081	84	23	Kim et al. (2003)
Alfalfa sprouts	10 min EO & seed coat removal	*Salmonella* sp.	++	2.4	1081	84	23	Kim et al. (2003)
Alfalfa sprouts	10 min EO & seed coat removal	Non-*Salmonella* microflora	++	2.4	1081	84	23	Kim et al. (2003)
Alfalfa sprouts	10 min EO & sonication & seed coat removal	*Salmonella* sp.	+++	2.4	1081	84	23	Kim et al. (2003)
Alfalfa sprouts	10 min EO & sonication & seed coat removal	Non-*Salmonella* microflora	++	2.4	1081	84	23	Kim et al. (2003)
Alfalfa seeds	15 min EO	*Salmonella* sp.	++	2.5	1079	70	23	Stan and Daeschel (2003)
Alfalfa seeds	60 min EO	*Salmonella* sp.	++	2.6	1076	66.8	23	Stan and Daeschel (2003)

++++, bacterial reduction being more than 4 log CFU/ per unit; +++, bacterial reduction being between 2 and 4 CFU/ per unit; ++, bacterial reduction being between 1 and 2 CFU/ per unit; +, bacterial reduction being less than 1 log CFU/ per unit. –, not measured.

ER water (pH of 11.3, ORP of −870 mV) for 5 min and then soaked in EO water (pH of 2.6, ORP of 1130 mV and free chlorine of 30 mg/L) for 5 min showed a reduction in aerobic mesophiles. This treatment had at least 2 log CFU per cucumber greater reduction than that only soaked in EO water (30 mg/L free chlorine), ozonated water (5 mg/L ozone) or sodium hypochlorite solution (NaOCl, 150 mg/L free chlorine) for 10 min. In studies on sequential wash treatment, Koseki et al. (2001) also found that a 2-log CFU/g reduction in aerobic bacteria counts for both the lettuce treated with ER water for 1 min followed by the treatment with EO water for 1 min and the lettuce treated with acidic EO water alone for 10 min; however, repeated EO water treatment did not show a significant increase of bacterial reduction. Koseki et al. (2004c) used mildly heated (50 °C) ER water to treat lettuce for 5 min, and then used chilled (4 °C) EO water to treat for 1 or 5 min. They found the treatment could reduce both *E. coli* O157:H7 and *Salmonella* at a level of 3–4 log CFU/g. Wang, Feng, and Luo (2004) washed fresh-cut cilantro with ozonated water for 5 min followed with a EO water (pH of 2.45, ORP of 1130 mV and free chlorine of 16.8 mg/L) for 5 min and found that the sequential wash is effective in reducing initial microbial count and slowing microbial growth during storage.

Lettuce with smooth surfaces have been used for the investigation of the effectiveness of EO water on bacterial reduction. Park et al. (2001) observed that shaking lettuce with EO water (45 mg/L free chlorine) at 100 rpm for 3 min significantly decreased mean populations of *E. coli* O157:H7 and *L. monocytogenes* by 2.41 and 2.65 log CFU per lettuce leaf, respectively, when compared with sterile H_2O treatment. The result was in agreement with that of Izumi (1999) who pointed out that EO water (50 mg/L of free chlorine) treatment of shredded lettuce did not significantly affect the quality characteristics such as color and general appearance. Yang et al. (2003) suggested that fresh-cut lettuce dipped in EO water (pH 7) containing 300 mg/L of free chlorine for 5 min could not only keep the best visual quality but also achieve a 2-log CFU/g reduction for *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes*.

Koseki and Itoh (2001) suggested that the best temperature for distribution of fresh-cut vegetables with reduced microbial population is 1 °C. Ice is an inexpensive material for preserving fresh produces and fish. Koseki, Fujiwara, and Itoh (2002) treated lettuce with frozen EO water (pH of 2.5, ORP of 1148 mV and free chlorine of 20.5 mg/L) and stored in a styrene-foam container for 24 h. The results indicated that a 1.5-log CFU/g aerobic bacteria counts reduction on lettuce was due to an increased chlorine gas concentration from frozen EO water. In order to check the effectiveness of Cl_2 concentration and volume or weight ratio of vegetables to frozen EO water, Koseki, Isobe, and Itoh (2004a) prepared a EO-ice by freezing EO water at −40 °C. The EO water with 20, 50, 100 and 200 mg/L of free chlorine could generate ice with 30, 70, 150 and

240 mg/L of Cl_2, respectively. EO-ice generating 70–240 mg/L Cl_2 significantly reduced *L. monocytogenes* by 1.5 log CFU/g during 24 h storage. EO-ice generating 70–150 mg/L of Cl_2 reduced *E. coli* O157:H7 cell counts by 2.0 log CFU/g. Although higher concentration with 240 mg/L of Cl_2 showed a significantly higher reduction of *E. coli* O157:H7 by 2.5 log CFU/g, accompanied by physiological disorder resembling leaf burn. The weight ratio of EO-ice to lettuce was >10. Chlorine at a level below 150 mg/L did not affect the surface color of the lettuce.

Sprouts have been associated with a number of foodborne illnesses in recent years. *E. coli* O157:H7, *Salmonella* spp. and *B. cereus* have been responsible for several sprout-associated outbreaks worldwide (Taormina, Beuchat, & Slusker, 1999). Sprouts are produced under warm and humid condition, pathogens can grow rapidly during seed germination increasing the likelihood of infections. Beuchat, Ward, and Pettigrew (2001) reported populations of *Salmonella* exceeding 10^6 CFU/g could occur on alfalfa sprouts produced from contaminated seeds. Although the use of 20,000 mg/L $Ca(OCl)_2$ for treatment of seeds intended for sprout production has been recommended (NACMCF, 1999), the use of high concentrations of $Ca(OCl)_2$ both generated worker safety concerns and significantly reduced seed germination rates (70% versus 90–96%) (Kim et al., 2003). Studies have demonstrated that 64.5 mg/L free chlorine in EO water treatment reduced *E. coli* O157:H7 population on alfalfa sprouts (initial population was about 6 log CFU/g) by 1.05 log CFU/g (91.1%) for 2 min treatment, while the reduction was by 2.72 log CFU/g (99.8%) for 64 min treatment. EO water treatment did not cause any visible damage to the sprouts (Sharma & Demirci, 2003). Kim et al. (2003) reported that treatment of seeds with 20,000 mg/L $Ca(OCl)_2$ reduced the population of *Salmonella* and non-*salmonella* to undetectable levels on culture media, but an amount >6 log CFU/g of *Salmonell*a was still recovered from sprouts generated from these seeds. However, the combination of EO water (84 mg/L free chlorine) and sonication treatment had a better reduction on *Salmonella* and non-*salmonella* populations than that by using EO water alone. Removal of seed coats by sonication might have detached cells that were attached or entrapped in sprouts, thus making the pathogen more susceptible to the EO water. The combined treatment achieved 2.3 and 1.5 log CFU/g greater reductions than EO water alone in populations of *Salmonella* and non-*salmonella* microflora, respectively (Kim et al., 2003).

8.3. Use of EO water for fruits

Postharvest decay of fruits causes economic loss to the fruit industry. In studies on surface sterilization of fruits, Al-Haq et al. (2001) found that EO water could prevent peach from decay and it could be used as an important alternative to liquid sterilants. Al-Haq et al. (2002) later found that EO water immediately reacted with *Botryosphaeria berengeriana* that presented on the first few layers of the pear surface and could not control growth of bacteria that entered into the fruit deeper than 2 mm. No chlorine-induced phytotoxicity on the treated fruit was observed. Both EO water containing 200 and 444 mg/L free chlorine significantly reduce the populations of *E. coli* O157:H7, *S. enteritidis* and *L. monocytogenes* on the surfaces of tomatoes without affecting sensory quality (Bari et al., 2003; Deza et al., 2003).

Patulin is a mycotoxin mainly found in apples and their products that are contaminated with the common storage-rot fungus *Penicillium expansum* (Brian, Elson, & Lowe, 1956; Harwig, Chen, Kennedy, & Scott, 1973). The uses of 100% and 50% EO water containing 60 mg/L free chlorine could decrease *P. expansum* viable spore populations by greater than 4 and 2 log units of aqueous suspension and wounded apples (Okull & Laborde, 2004). EO water did not control brown rot in wound-inoculated fruits, but reduced disease incidence. In contrast to the present results for smooth fruits, on treatment of the surface of the strawberry with 30 mg/L free chlorine EO water and 150 mg/L NaOCl, aerobic mesophiles were reduced by less than 1 log CFU per strawberry after washing in ER water (pH of 11.3, ORP of −870 mV) for 5 min and then with EO water (pH of 2.6, ORP of 1130 mV and free chlorine of 30 mg/L) for 5 min, EO water (30 mg/L free chlorine), ozonated water (5 mg/L ozone) and sodium hypochlorite solution (NaOCl, 150 mg/L free chlorine) for 10 min, respectively. These results can be attributed to the surface structure of the strawberry fruit. There are many achenes (seeds) that render its surface structure uneven and complex (Koseki et al., 2004b). These studies showed that the efficacy of EO water as a sanitizing agent was dependent on the surface structure of fruit treated.

8.4. Use of EO water for poultry and meat

Egg shell can serve as a vehicle for transmission of human pathogens. Due to the fecal matter in the nesting place, the wash water during manipulation, or during packaging process, the shell may become contaminated with *E. coli* O157:H7, *Salmonella* sp., *L. monocytogenes* and *Yersinia enterocolitica* (Gabriela, Maria, Lidia, & Ana, 2000; Moore & Madden, 1993; Schoeni & Doyle, 1994). Elimination of pathogens in hatchery facilities has been usually done by applying of formaldehyde and glutaraldehyde gas or fogging hydrogen peroxide. However, these disinfectants may pose high risk for human and chick health. Russell (2003) found that EO water (pH of 2.1, ORP of 1150 mV and free chlorine of 8 mg/L) with an electrostatic spraying system could completely eliminate *S.* Typhimurium, *S. aureus* and *L. monocytogenes* on egg shells.

Efficacy of EO water in reducing pathogens on poultry has been investigated in recent years (Table 4). Park et al. (2002a) reported that for chicken wings (50 ± 5 g) inoculated with *Campylobacter jejuni*, soaking in EO water (pH of 2.57, ORP of 1082 mV and free chlorine of

Table 4
Inactivation of food-borne pathogens on poultry and meat by electrolyzed oxidizing water

Materials	Immersion condition	Indicator	Effectiveness	EO water property				Ref.
				pH	ORP (mV)	Free chlorine (mg/L)	Temperature (°C)	
Chicken wing	10 min EO	*Campylobacter jejuni*	+++	2.5	1082	51.6	23	Park et al. (2002a)
Chicken wing	30 min EO	*Campylobacter jejuni*	+++	2.5	1082	51.6	23	Park et al. (2002a)
Chicken wing	10 min EO	*Campylobacter jejuni*	+++	2.6	1092	53.3	4	Park et al. (2002a)
Chicken wing	30 min EO	*Campylobacter jejuni*	+++	2.6	1092	53.3	4	Park et al. (2002a)
Broiler carcasses	45 min EO	Aerobic bacteria counts	++	2.6	1150	50	4	Fabrizio et al. (2002)
Broiler carcasses	45 min EO	*Salmonella* Typhimurium	+	2.6	1150	50	4	Fabrizio et al. (2002)
Broiler carcasses	45 min EO	*Escherichia coli*	++	2.6	1150	50	4	Fabrizio et al. (2002)
Broiler carcasses	45 min EO	Coliform bacteria	++	2.6	1150	50	4	Fabrizio et al. (2002)
Chicken carcasses	40 min EO	*Campylobacter jejuni*	+++	2.8	1165	39.5	23	Kim et al. (2005)
Pork belly	15 s spray with EO	Aerobic bacteria counts	++	2.6	1150	50	23	Fabrizio and Cutter (2004)
Pork belly	15 s spray with EO	*Escherichia coli*	++	2.6	1150	50	23	Fabrizio and Cutter (2004)
Pork belly	15 s spray with EO	Coliform bacteria	++	2.6	1150	50	23	Fabrizio and Cutter (2004)
Pork belly	15 s spray with EO	*Salmonella* Typhimurium	++	2.6	1150	50	23	Fabrizio and Cutter (2004)
Pork belly	15 s spray with EO	*Listeria monocytogenes*	++	2.6	1150	50	23	Fabrizio and Cutter (2004)
Pork belly	15 s spray with EO	*Campylobacter coli*	++	2.6	1150	50	23	Fabrizio and Cutter (2004)
Cattle hide	10 s spray with ER & 10 s spray with EO	Aerobic bacteria counts	+++	2.4	–	70	60	Bosilevac et al. (2005)
Cattle hide	10 s spray with ER & 10 s spray with EO	*Enterobacteriaceae* counts	++++	2.4	–	70	60	Bosilevac et al. (2005)
Frankfurter	15 min EO	*Listeria monocytogenes*	++	2.3	1150	45	25	Fabrizio and Cutter (2005)
Frankfurter	15 min spray with EO	Aerobic bacteria counts	+	2.3	1150	45	25	Fabrizio and Cutter (2005)
Frankfurter	15 min spray with EO	*Listeria monocytogenes*	+	2.3	1150	45	25	Fabrizio and Cutter (2005)
Ham	15 min spray with EO	*Listeria monocytogenes*	+	2.3	1150	45	25	Fabrizio and Cutter (2005)

++++, bacterial reduction being more than 4 log CFU/ per unit; +++, bacterial reduction being between 2 and 4 CFU/ per unit; ++, bacterial reduction being between 1 and 2 CFU/ per unit; +, bacterial reduction being less than 1 log CFU/ per unit. –, not measured.

Table 5
Inactivation of food-borne pathogens on seafood fields by electrolyzed oxidizing water

Materials	Immersion condition	Indicator	Effectiveness	EO water property				Ref.
				pH	ORP (mV)	Free chlorine (mg/L)	Temperature (°C)	
Salmon fillet	64 min EO	*Escherichia coli*	+	2.6	1150	90	22	Ozer and Demirci (2006)
Salmon fillet	64 min EO	*Escherichia coli*	++	2.6	1150	90	35	Ozer and Demirci (2006)
Salmon fillet	64 min EO	*Listeria monocytogenes*	+	2.6	1150	90	22	Ozer and Demirci (2006)
Salmon fillet	64 min EO	*Listeria monocytogenes*	++	2.6	1150	90	35	Ozer and Demirci (2006)
Tilapia	1 min EO	*Escherichia coli*	+	2.4	1159	120	23	Huang et al. (2006a)
Tilapia	5 min EO	*Escherichia coli*	++	2.4	1159	120	23	Huang et al. (2006a)
Tilapia	10 min EO	*Escherichia coli*	++	2.4	1159	120	23	Huang et al. (2006a)
Tilapia	1 min EO	*Vibrio parahaemolyticus*	++	2.4	1159	120	23	Huang et al. (2006a)
Tilapia	5 min EO	*Vibrio parahaemolyticus*	++	2.4	1159	120	23	Huang et al. (2006a)
Tilapia	10 min EO	*Vibrio parahaemolyticus*	++	2.4	1159	120	23	Huang et al. (2006a)
Dirty fish retailer in fish market	1 min EO	Aerobic bacteria counts	++++	2.2	1145	200	23	Huang et al. (2006a)
Dirty fish retailer in fish market	1 min EO	Aerobic bacteria counts	++	2.5	1120	100	23	Huang et al. (2006a)
Dirty fish retailer in fish market	5 min EO	Aerobic bacteria counts	+++	2.5	1120	100	23	Huang et al. (2006a)
Dirty fish retailer in fish market	10 min EO	Aerobic bacteria counts	+++	2.5	1120	100	23	Huang et al. (2006a)
Dirty fish retailer in fish market	1 min EO	Aerobic bacteria counts	++	2.7	1090	50	23	Huang et al. (2006a)
Dirty fish retailer in fish market	5 min EO	Aerobic bacteria counts	++	2.7	1090	50	23	Huang et al. (2006a)
Dirty fish retailer in fish market	10 min EO	Aerobic bacteria counts	++	2.7	1090	50	23	Huang et al. (2006a)
Tuna fillet	5 min EO (150 rpm)	Aerobic bacteria counts	++	2.5	1105	50	23	Huang et al. (2006b)
Tuna fillet	5 min EO (150 rpm) & CO	Aerobic bacteria counts	+	2.5	1105	50	23	Huang et al. (2006b)
Tuna fillet	5 min EO (150 rpm)	Aerobic bacteria counts	++	2.2	1135	100	23	Huang et al. (2006b)
Tuna fillet	5 min EO (150 rpm) & CO	Aerobic bacteria counts	++	2.2	1135	100	23	Huang et al. (2006b)
Stainless steel containing seafood residue	5 min EO	*Listeria monocytogenes*	+++	2.5	1150	50	23	Liu et al. (2006b)
Ceramic title containing seafood residue	5 min EO	*Listeria monocytogenes*	+++	2.5	1150	50	23	Liu et al. (2006b)
Floor tile containing seafood residue	5 min EO	*Listeria monocytogenes*	++	2.5	1150	50	23	Liu et al. (2006b)

(*continued on next page*)

Table 5 (*continued*)

Materials	Immersion condition	Indicator	Effectiveness	EO water property				Ref.
				pH	ORP (mV)	Free chlorine (mg/L)	Temperature (°C)	
Natural rubber latex glove containing seafood residue	5 min EO	*Listeria monocytogenes*	+++	2.6	1125	40	23	Liu and Su (2006b)
Natural latex glove containing seafood residue	5 min EO	*Listeria monocytogenes*	++	2.6	1125	40	23	Liu and Su (2006b)
Nitrile containing seafood residue	5 min EO	*Listeria monocytogenes*	++	2.6	1125	40	23	Liu and Su (2006b)
Latex (disposable) containing seafood residue	5 min EO	*Listeria monocytogenes*	+++	2.6	1125	40	23	Liu and Su (2006b)
Nitrile (disposable) containing seafood residue	5 min EO	*Listeria monocytogenes*	+++	2.6	1125	40	23	Liu and Su (2006b)
Above 5 clean food processing gloves	5 min EO	*Listeria monocytogenes*	++++	2.6	1125	40	23	Liu and Su (2006b)

++++, bacterial reduction being more than 4 log CFU/ per unit; +++, bacterial reduction being between 2 and 4 CFU/ per unit; ++, bacterial reduction being between 1 and 2 CFU/ per unit; +, bacterial reduction being less than 1 log CFU/ per unit.

50 mg/L) with 100 rpm agitation for 30 min has achieved reduction by 3 log CFU/g. Since pathogens were attached to a water-skin interfaces and further entrapped in folds, crevices and follicles, no viable cell of *C. jejuni* was recovered in EO water after treatment. Kim, Hung, and Russell (2005) recommended to spray-wash chicken with ER water before defeathering and evisceration to reduce the potential cross-contamination. However, combining immersion with spray-washing did not significantly improve the bactericidal effect of EO water as compared to the immersion-only treatment. Fabrizio et al. (2002) reported that spray-washing with EO water, ozone, 2% acetic acid (AA) or 10% trisodium phosphate (TSP) did not show any significant microbicidal effectiveness. However, spray-washing with ER water followed by immersion in EO water had a better effectiveness than spraying with AA and TSP followed by immersion in chlorine solution at the end of a 7-day refrigerated storage.

Fabrizio and Cutter (2004) had recently examined the spray-washing with EO water for 15 s to disinfect pork bellies inoculated with feces containing *L. monocytogenes*, *S.* Typhimurium and *Campylobacter coli*. This study demonstrated that a 15-s spraying with EO water (pH of 2.4, ORP of 1160 mV and free chlorine of 50 mg/L) had the ability to reduce the populations of *L. monocytogenes*, *S.* Typhimurium and *C. coli* (1.23, 1.67 and 1.81, respectively) on the pork surfaces and inferred that longer contact times might strengthen the disinfection effectiveness. For sterilizing hides of cattle before slaughtering, Bosilevac, Shackelford, Brichta, and Koohmaraie (2005) reported that sequentially applied ER water and EO water containing 70 mg/L free chlorine at 60 °C for a 10-s spraying could reduce aerobic bacteria counts by 3.5 log CFU/100 cm^2 and reduced *Enterobacteriaceae* counts by 4.3 log CFU/100 cm^2. Recently, Fabrizio and Cutter (2005) dipped or sprayed frankfurters and ham inoculated with *L. monocytogenes* with EO water (pH of 2.3, ORP of 1150 mV and free chlorine of 45 mg/L) and/or ER water for 30 min. No significant difference ($p < 0.05$) between treatments on Hunter $L*$, $a*$, $b*$ values for frankfurters and ham at the end of 7 days storage at 4 °C was found. The results indicated that EO water has no detrimental "bleaching" effects on the surface of tested read-to-eat meats.

8.5. Use of EO water for seafood

Using EO water for inactivating bacteria in raw seafood have been reported (Table 5). Ozer and Demirci (2006) found that treating raw salmon with EO water (pH of 2.6, ORP of 1150 mV and free chlorine of 90 mg/L) at 35 °C for 64 min resulted in a 1.07 log CFU/g (91.1%) and 1.12 log CFU/g (92.3%) reduction in *E. coli* O157:H7 and *L. monocytogenes*, respectively. Recently, Liu and Su (2006) stated that gloves used in handling food for protection of the worker and seller could become a carrier of pathogens through the contact of raw materials or contaminated surfaces. However, applications of EO water follow-

ing a thorough cleaning greatly reduced *L. monocytogenes* population on gloves and seafood processing plants. Soaking inoculated gloves in EO water (pH of 2.6, ORP of 1125 mV and free chlorine of 40 mg/L) at room temperature for 5 min completely eliminated *L. monocytogenes* on gloves (>4.46 log CFU/cm^2) (Liu & Su, 2006). The treatment by immersion in EO water containing 50 mg/L chlorine for 5 min significantly reduced *L. monocytogenes* on tested surfaces (3.73 log/25 cm^2 on stainless steel sheet, 4.24 log/25 cm^2 on ceramic tile and 1.52 log/25 cm^2 on floor tile) (Liu, Duan, & Su, 2006). Huang et al. (2006a) also reported that EO water was a very effective sanitizer used for cleaning fish contacting surfaces in traditional grocery stores and fish markets, so that secondary bacterial contamination could be prevented. EO water was especially effective in reducing the population of *E. coli* and *V. parahaemolyticus* contamination on tilapia.

In order to prolong the shelf life of yellow-fin tuna (*Thunnus albacares*) during refrigerated and frozen storage, combination of EO water and CO gas were applied. Huang, Shiau, Hung, and Hwang (2006b) reported that tuna treated with a combination of EO water containing 100 mg/L chlorine and CO gas could immediately result in the lowest APC. EO water containing 50 mg/L or 100 mg/L chlorine combined with CO gas treatment in tuna fish steak would be an effective method for enhancing the hygienic quality and freshness for tuna meat and extending refrigerated storage time. The efficiency of EO water on the growth and toxicity of the dinoflagellates *Alexandrium minutum*, *Alexandrium catenella* and *Gymnodinium catenatum* has been studied in our laboratory. It was found that EO water very effectively killed toxic dinoflagillates and destroyed toxicity.

9. Conclusions

Since EO water is considered to be a solution containing HOCl, the application of EO water can be fitted into the regulations for hypochlorous (HOCl). In 2002, Japan had officially approved EO water as a food additive (Yoshida, Achiwa, & Katayose, 2004). Electrolyzed water generator has also been approved for applications in the food industry by the US Environmental Protection Agency (EPA) (Park et al., 2002b).

Although EO water has advantages as a disinfectant for use in many food products, relevant topics in EO water deserve future research. These may include the methods for expanding the usages of EO water in food processing plant and the application in HACCP and SSOP systems. Since bactericidal effects of the EO water may be reduced in the presence of organic matter due to the formation of monochloramines, techniques to avoid these matters need to be researched. Furthermore, the sensory characteristics of food processed may be affected by degradation of contaminants in the food during the application of EO water need to be further studied.

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Clinical Studies and Research on Electrolyzed-Reduced Water

Fluid replacement promotes optimal physical performance:

Adequate fluid replacement helps maintain hydration and, promotes the health, safety, and optimal physical performance of individuals participating in regular physical activity.

Med Sci Sports Exercise
1996 Jan;28(1):i-vii.
American College of Sports Medicine position stand. Exercise and fluid replacement.

Convertino VA, Armstrong LE, Coyle EF, Mack GW, Sawka MN, Senay LC Jr, Sherman WM.

It is the position of the American College of Sports Medicine that adequate fluid replacement helps maintain hydration and, therefore, promotes the health, safety, and optimal physical performance of individuals participating in regular physical activity. This position statement is based on a comprehensive review and interpretation of scientific literature concerning the influence of fluid replacement on exercise performance and the risk of thermal injury associated with dehydration and hyperthermia.

Based on available evidence, the American College of Sports Medicine makes the following general recommendations on the amount and composition of fluid that should be ingested in preparation for, during, and after exercise or athletic competition: 1) It is recommended that individuals consume a nutritionally balanced diet and drink adequate fluids during the 24-hr period before an event, especially during the period that includes the meal prior to exercise, to promote proper hydration before exercise or competition.

2) It is recommended that individuals drink about 500 ml (about 17 ounces) of fluid about 2 h before exercise to promote adequate hydration and allow time for excretion of excess ingested water.

3) During exercise, athletes should start drinking early and at regular intervals in an attempt to consume fluids at a rate sufficient to replace all the water lost through sweating (i.e., body weight loss), or consume the maximal amount that can be tolerated.

4) It is recommended that ingested fluids be cooler than ambient temperature [between 15 degrees and 22 degrees C (59 degrees and 72 degrees F])] and flavored to enhance palatability and promote fluid replacement. Fluids should be readily available and served in containers that allow adequate volumes to be ingested with ease and with minimal interruption of exercise.

5) Addition of proper amounts of carbohydrates and/or electrolytes to a fluid replacement solution is recommended for exercise events of duration greater than 1 h since it does not significantly impair water delivery to the body and may enhance performance. During exercise lasting less than 1 h, there is little evidence of physiological or physical performance differences between consuming a carbohydrate-electrolyte drink and plain water.

6) During intense exercise lasting longer than 1 h, it is recommended that carbohydrates be ingested at a rate of 30-60 g.h(-1) to maintain oxidation of carbohydrates and delay fatigue. This rate of carbohydrate intake can be achieved without compromising fluid delivery by drinking 600-1200 ml.h(-1) of solutions containing 4%-8% carbohydrates (g.100 ml(-1)). The carbohydrates can be sugars (glucose or sucrose) or starch (e.g., maltodextrin).

7) Inclusion of sodium (0.5-0.7 g.l(-1) of water) in the rehydration solution ingested during exercise lasting longer than 1 h is recommended since it may be advantageous in enhancing palatability, promoting fluid retention, and possibly preventing hyponatremia in certain individuals who drink excessive quantities of fluid. There is little

physiological basis for the presence of sodium in n oral rehydration solution for enhancing intestinal water absorption as long as sodium is sufficiently available from the previous meal.

Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage.

Biochem Biophys Res Commun.
1997 May 8
234(1):269-74.

Shirahata S, Kabayama S, Nakano M, Miura T, Kusumoto K, Gotoh M, Hayashi H, Otsubo K, Morisawa S, Katakura Y.

Institute of Cellular Regulation Technology, Graduate School of Genetic Resources Technology, Kyushu University, Fukuoka, Japan. sirahata@grt.kyushu-u.ac.jp

Active oxygen species or free radicals are considered to cause extensive oxidative damage to biological macromolecules, which brings about a variety of diseases as well as aging. The ideal scavenger for active oxygen should be 'active hydrogen'. 'Active hydrogen' can be produced in reduced water near the cathode during electrolysis of water.

Reduced water exhibits high pH, low dissolved oxygen (DO), extremely high dissolved molecular hydrogen (DH), and extremely negative redox potential (RP) values. Strongly electrolyzed-reduced water, as well as ascorbic acid, (+)-catechin and tannic acid, completely scavenged O.-2 produced by the hypoxanthine-xanthine oxidase (HX-XOD) system in sodium phosphate buffer (pH 7.0). The superoxide dismutase (SOD)-like activity of reduced water is stable at 4 degrees C for over a month and was not lost even after neutralization, repeated freezing and melting, deflation with sonication, vigorous mixing, boiling, repeated filtration, or closed autoclaving, but was lost by opened autoclaving or by closed autoclaving in the presence of tungsten trioxide which efficiently adsorbs active atomic hydrogen.

Water bubbled with hydrogen gas exhibited low DO, extremely high DH and extremely low RP values, as does reduced water, but it has no SOD-like activity. These results suggest that the SOD-like activity of reduced water is not due to the dissolved molecular hydrogen but due to the dissolved atomic hydrogen (active hydrogen).

Although SOD accumulated H2O2 when added to the HX-XOD system, reduced water decreased the amount of H2O2 produced by XOD. Reduced water, as well as catalase and ascorbic acid, could directly scavenge H2O2. Reduced water suppresses single-strand breakage of DNA b active oxygen species produced by the Cu(II)-catalyzed oxidation of ascorbic acid in a dose-dependent manner, suggesting that reduced water can scavenge not only O2.- and H2O2, but also 1O2 and .OH.

PMID: 9169001 [PubMed - indexed for MEDLINE]

The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis.

Biophys Chem. 2004
Jan 1;107(1):71-82.

Hanaoka K, Sun D, Lawrence R, Kamitani Y, Fernandes G.
Bio-REDOX Laboratory Inc. 1187-4, Oaza-Ueda, Ueda-shi, Nagano-ken 386-0001, Japan. hanak@rapid.ocn.ne.jp

We reported that reduced water produced by electrolysis enhanced the antioxidant effects of proton donors such as ascorbic acid (AsA) in a previous paper.

We also demonstrated that reduced water produced by electrolysis of 2 mM NaCl solutions did not show antioxidant effects by itself. We reasoned that the enhancement of antioxidant effects may be due to the increase of the ionic product of water as solvent. The ionic product of water (pKw) was estimated by measurements of pH and by a neutralization titration method. As an indicator of oxidative damage, Reactive Oxygen Species- (ROS) mediated DNA strand breaks were measured by the conversion of supercoiled phiX-174 RF I double-strand DNA to open and linear forms. Reduced water had a tendency to suppress single-strand breakage of DNA induced by reactive oxygen species produced by H2O2/Cu (II) and HQ/Cu (II) systems. The enhancement of superoxide anion radical dismutation activity can be explained by changes in the ionic product of water in the reduced water.

PMID: 14871602 [PubMed - in process]

Oxygen Radical Absorbance Capacity--High-ORAC Foods May Slow Aging

Agricultural Research Service, USDA, February 8, 1999
Foods that score high in an antioxidant analysis called ORAC may protect cells and their components from oxidative damage, according to studies of animals and human blood at the Agricultural Research Services Human Nutrition Research Center on Aging at Tufts in Boston. ARS is the chief scientific agency of the U.S. Department of Agriculture.
ORAC, short for oxygen radical absorbance capacity, is a test tube analysis that measures the total antioxidant power of foods and other chemical substances. Early findings suggest that eating plenty of high-ORAC fruits and vegetables, such as spinach and blueberries, may help slow the processes associated with aging in both body and brain. If these findings are borne out in further research, young and middle-aged people may be able to reduce risk of diseases of aging (including senility) simply by adding high-ORAC foods to their diets, said ARS Administrator Floyd P. Horn.
In the studies,eating plenty of high-ORAC foods:

- Raised the antioxidant power of human blood 10 to 25%
- Prevented some loss of long-term memory and learning ability in middle-aged rats
- Maintained the ability of brain cells in middle-aged rats to respond to a chemical stimulus-a function that normally decreases with age.
- Protected rats' tiny blood vessels (capillaries) against oxygen damage.

Nutritionist Ronald L. Prior contends, "If we can show some relationship between ORAC intake and health outcome in people, I think we may reach a point where the ORAC value will become a new standard for good antioxidant protection." (See the table at the bottom for ORAC values of fruits and vegetables.)
The thesis that oxidative damage culminates in many of the maladies of aging is well accepted in the health community. The evidence has spurred skyrocketing sales of antioxidant vitamins. But several large trials have had mixed results. It may be that combinations of nutrients found in foods have greater protective effects than each nutrient taken alone, said Guohua (Howard) Cao, a physician and chemist who developed the ORAC assay.
He and Prior have seen the ORAC value of human blood rise in two studies. In the first, eight women gave blood after separately ingesting spinach, strawberries, and red wine (all high-ORAC foods) or taking 1,250 milligrams of vitamin C. A large serving of fresh spinach produced the biggest rise in the women's blood antioxidant scores (up to 25 percent) followed by vitamin C, strawberries, and lastly, red wine. In the second study, men and women had a 13- to 15-percent increase in the antioxidant power of their blood after doubling their daily fruit and vegetable intake compared to what they consumed before the study. Just doubling intake, without regard to ORAC scores of the fruits and vegetables, more than doubled the number of ORAC units the volunteers consumed, Prior reported.
Early evidence for the protecting power of these diets comes from rat studies by Prior, Cao, and colleagues. Rats fed daily doses of blueberry extract for six weeks before being subjected to two days of pure oxygen apparently suffered

much less damage to the capillaries in and around their lungs, Prior said. The fluid that normally accumulates in the pleural cavity surrounding the lungs was much lower compared to the group that didnt get blueberry extract.
Neuroscientist James Joseph and psychologist Barbara Shukitt-Hale at the center tested middle-aged rats that had eaten diets fortified with spinach, strawberry extract, or vitamin E for nine months.
A daily dose of spinach extract prevented some loss of long-term memory and learning ability normally experienced by the 15-month-old rats, said Shukitt-Hale. Spinach was also the most potent in protecting different types of nerve cells in two separate parts of the brain against the effects of aging. These cells were significantly more responsive when the animals ate diets fortified with high-ORAC foods, especially spinach, compared to unfortified diets, Joseph said. The spinach group scored twice as responsive as the control animals. Why spinach is more effective than strawberries (which score higher in the ORAC assay) is still a mystery. The researchers conjecture that it may be due to specific compounds or a specific combination of them in the greens.
Top-Scoring Fruits and Vegetables ORAC units per 100 grams (about 3.5 ounces) Prunes 5770 Raisins 2830 Blueberries 2400 Blackberries 2036 Kale 1770 Strawberries 1540 Spinach 1260 Raspberries 1220 Brussels Sprouts 980 Plums 949 Alfalfa Sprouts 930 Broccoli flowers 890 Beets 840 Red Grapes 785 Oranges 750 Red Bell Peppers 710 Cherries 670 Kiwi Fruit 602 Pink Grapefruit 483 Onion 450 Corn 400 Eggplant 390

Use of Ionized water in hypochlorhydria or achlorhydria

Prof. Kuninaka Hironage, Head of Kuninaka Hospital
"Too many fats in the diets, which lead to the deposition of cholesterol on the blood vessels, which in turn constrict the blood flow, cause most illnesses such as high blood pressure. In accordance with the theory of Professor Gato of Kyushu University on Vitamin K (because vitamin K enables the blood calcium to increase), or the consumption of more antioxidant water, the effectiveness of the increase in the calcium in high blood pressure is most significant. The consumption of alkaline antioxidant water for a period of 2 to 3 months, I have observed the blood pressure slowly drop, due to the water's solvent ability, which dissolves the cholesterol in the blood vessels."

Use of Ionized water for gynecological conditions

Prof. Watanabe Ifao, Watanabe Hospital
"Ionized alklaine antioxidant water improves body constituents and ensures effective healing to many illnesses. The uses of antioxidant water in gynecological patients have proved to be very effective. The main reason for its effectiveness is that this water can neutralize toxins.
When given antioxidant water to pre-eclamptic toxemia cases, the results are most significant. During my long years of servicing the pre-eclamptic toxemia cases, I found that the women with pre-eclamptic toxemia who consumed antioxidant water tend to deliver healthier babies with stronger muscles. A survey report carried out on babies in this group showed intelligence above average."

CLINICAL Improvements Obtained From The Intake Of Reduced Water

Extracts from " Presentation At The Eight Annual International Symposium On man And His Environment in Health And Disease" on February 24th 1990, at The Grand Kempinski Hotel, Dalls, Texas, USA by Dr. H. Hayashi, M.D. and Dr. M Kawamura, M.D., on : -

(THE CONCEPT OF PREHEPATIC MEDICINES)

Since the introduction of alkaline ionic water in our clinic in 1985, we have had the following interesting clinical experiences in the use of this type of water. By the use of alkaline ionic water for drinking and the preparation of meals for our in-patients, we have noticed :-
Declines in blood sugar levels in diabetic patients.
Improvements in peripheral circulation in diabetic gangrene.
Declines in uric acid levels in patients with gout.
Improvements in liver function exams in hepatic disorders.
Improvements in gastroduodenal ulcers and prevention of their recurrences.
Improvements in hypertension and hypotension.
Improvements in allergic disorders such as asthma, urticaria, rhinites and atopic dermatitis.
Improvements in persistent diarrhoea which occurred after gastrectomy.
Quicker improvements in post operative bower paralysis.
Improvements in serum bilirubin levels in new born babies.
Being confirming clinical improvements, we have always observed changes of stools of the patients, with the colour of their feces changing from black-brown colour to a brighter yellow-brown one, and the odour of their feces becoming almost negligible.
The number of patients complaining of constipation also decreased markedly. The change of stool findings strongly suggests that alkaline ionic water intake can decrease the production of putrefied or pathogenic metabolites.
Devices to produce reduced water were introduced into our clinic in May 1985. Based on the clinical experiences obtained in the past 15 years, it can be said that introduction of electrolyzed-reduced water for drinking and cooking purpose for in-patients should be the very prerequisite in our daily medical practices. Any dietary recipe cannot be a scientific one if property of water is not taken by the patients is not taken into consideration.
The Ministry of Health and Welfare in Japan announced in 1965 that the intake of reduced water is effective for restoration of intestinal flora metabolism.

Toxin Neutralization

Prof. Kuwata Keijiroo, Doctor of Medicine
"In my opinion, the wonder of antioxidant water is the ability to neutralize toxins; but it is not a medicine. The difference is that medicine can only apply to individual cases, whereas the antioxidant water can be consumed generally and its neutralizing power is something which is very much unexpected. Now, in brief, let me introduce to you a heart disease case and how it was cured.
The patient was a 35 years old male suffering from vascular heart disease. For 5 years, his sickness deteriorated. He was in the Setagays Government Hospital for treatment.

During those 5 years, he had been in and out of the hospital 5 to 6 times. He had undergone high tech examinations such as angiogram by injecting VINYL via the vein into the heart. He consulted and sought treatment from many good doctors where later he underwent a major surgical operation. Upon his discharge from the hospital, he quit his job to convalesce. However, each time when his illness relapsed, the attack seemed to be even more severe.
Last year, in August, his relatives were in despair and expected he would not live much longer. It so happened at that time that the victim's relative came across antioxidant water processor. His illness responded well and he is now on the road to recovery."

(In the United States, cardiovascular diseases account for more than one-half of the approximate 2 million deaths occurring each year.... It is estimated that optimal conditioning of drinking water could reduce this cardiovascular disease mortality rate by as much as 15 percent in the United States) From: Report of the Safe Drinking Water Committee of the National Academy of Sciences, 1977

Eczema

Prof. Tamura Tatsuji, Keifuku Rehabilitation Center
"Eczema is used to describe several varieties of skin conditions, which have a number of common features. The exact causes of eczema are not fully understood. I many cases, eczema can be attributed to external irritants. Let me

introduce a patient who recovered from skin disease after consuming the antioxidant water. This patient suffered 10 years of eczema and could not be cured effectively even under specialist treatment. This patient, who is 70 years of age, is the president of a vehicle parts company. After the war, his lower limbs suffered acute eczema, which later became chronic. He was repeatedly treated in a specialist skin hospital.

The left limb responded well to treatment, but not so on the right limb. He suffered severe itchiness, which, when scratched led to bleeding. During the last 10 years, he was seen and treated by many doctors. When I first examined him, his lower limb around the joints was covered with vesicles. Weeping occurred owing to serum exuding from the vesicles.

I advised him to try consuming antioxidant water. He bought a unit and consumed the antioxidant water religiously and used the acidic water to bathe the affected areas. After 2 weeks of treatment the vesicles dried up. The eczema was completely cleared without any relapse after 1½ month."

Allergies

Prof. Kuninaka Hironaga, Head of Kuninaka Hospital
"Mr. Yamada, the head of Police Research Institute, suffered from severe allergy. He was treated repeatedly by skin specialist, but with no success. Then he started consuming antioxidant water. The allergy responded very well and was soon completely cured. No relapse had occurred, although he had taken all kinds of food. He was most grateful and excited about this treatment.

As for myself, I had also suffered severe allergy. Ever since I began to consume antioxidant water, the allergy has recovered. Since then, I started a research on the effectiveness of antioxidant water.

I discovered that most allergies are due to acidification of body condition and is also related to consuming too much meat and sugar. In every allergy case, the patient's antioxidant minerals are excessively low which in turn lower the body resistance significantly. The body becomes overly sensitive and develops allergy easily. To stabilize the sensitivity, calcium solution in injected into the vein. Therefore, it is clear that the antioxidant water has ionic calcium, which can help alleviate allergy.

The ionic calcium not only enhances the heart, urination, and neutralization of toxins but controls acidity. It also enhances the digestive system and liver function. This will promote natural healing power and hence increase its resistance to allergy. In some special cases of illness, which do not respond to drugs, it is found, it is found to respond well to antioxidant water."

Digestive Problems

Prof. Kogure Keizou, Kogure Clinic of Juntendo Hospital

"The stomach is readily upset both by diseases affecting the stomach and by other general illnesses. In addition, any nervous tension or anxiety frequently causes gastric upset.

The important role of antioxidant water in our stomach is to neutralize the secretion and strengthen its functions. Usually, after consuming the antioxidant water for 1 to 3 minutes, the gastric juice increase to 1½ times. For those suffering from achlorhydria (low in gastric juice) the presence of antioxidant water will stimulate the stomach cells to secrete more gastric juice. This in turn enhances digestion and absorption of minerals. However, those with hyperchlorhydria (high in gastric juice), the antioxidant water neutralizes the excessive gastric juice. Hence, it does not create any adverse reaction. According to the medical lecturer from Maeba University, the pH of the gastric secretion will still remain normal when antioxidant water is consumed. This proves the ability of the antioxidant water to neutralize as well as to stimulate the secretion."

Effects of Alkaline Ionized Water on Spontaneously diabetic GK-rats fed Sucrose

Diabetes
TWO ABSTRACTS and ONE REPORT ON DIABETES / ALKALINE WATER RESEARCH

Jin Man Kim Division of Life Science, R&D center, Sunkyong Industries, Kazuhito Yokoyama Department of Public Health, Faculty of Medicine, The University of Tokyo

This study was carried out to evaluate the effects of alkaline ionized water (AIW) on spontaneously diabetic GK-rats fed sucrose for aggravation of diabetes mellitus.

One half of the 32 GK rats was given AIW and the other was given tap water (TW). These two groups were further divided into two subgroups by fed with or without 30% sucrose solution (8 in each group). In blood glucose level, sucrose fed TW group was significantly higher than the other groups. Sucrose fed both AIW and TW groups were significantly increased in body weight as compared to TW group. In serum malondialdehyde (MDA), a marker of lipid peroxide, sucrose fed TW group was significantly higher than AIW and TW groups.

It is suggested that AIW (Alkaline Ionized Water) supplementation may inhibit the increase of blood glucose and lipid peroxide levels in diabetes mellitus.

Protective mechanism of reduced water against alloxan-induced pancreatic β-cell damage: Scavenging effect against reactive oxygen species

Cytotechnology 40: 139–149, 2002. Netherlands.139

Yuping Li1, Tomohiro Nishimura1, Kiichiro Teruya1, et al , Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, Fukuoka, Japan; 2 Nihon Trim Co. Ltd., 1-8-34 Oyodonaka, Kita-ku, Osaka, Japan: 3 Hita TenryosuiCo. Ltd., 647 Nakanoshima, Hita, Oita, Japan; 4 Center for Holistic Medicine and Naturopathy, Schmallenberg-Nordenau, Germany Author for correspondence; E-mail: sirahata@grt.kyushu-u.ac.jp

Abstract

Reactive oxygen species (ROS) cause irreversible damage to biological macromolecules, resulting in many diseases.Reduced water (RW) such as hydrogen-rich electrolyzed reduced water and natural reduced waters like Hita Tenryosui water in Japan and Nordenau water in Germany that are known to improve various diseases, could protect a hamster pancreatic β cell line, HIT-T15 from alloxan-induced cell damage. Alloxan, a diabetogenic compound, is used to induce type 1 diabetes mellitus in animals. Its diabetogenic effect is exerted via the production of ROS. Alloxan-treated HIT-T15 cells exhibited lowered viability, increased intracellular ROS levels, elevated cytosolic free Ca2+ concentration, DNA fragmentation, decreased intracellular ATP levels and lowering of glucose-stimulated release of insulin. RWcompletely prevented the generation of alloxan-inducedROS, increase of cytosolic Ca2+ concentration, decrease of intracellular ATP level, and lowering of glucose-stimulated insulin release, and strongly blocked DNA fragmentation, partially suppressing the lowering of viability of alloxan-treated cells. Intracellular ATP levels and glucose-stimulated insulin secretion were increased by RW to 2–3.5 times and 2–4 times, respectively, suggesting that RW enhances the glucose-sensitivity and glucose response of β-cells. The protective activity of RWwas stable at 4 ◦C for over a month, but was lost by autoclaving. These results suggest that RW protects pancreatic β-cells from alloxan-induced cell damage by preventing alloxan-derived ROS generation. RW may be useful in preventing alloxan-induced type 1-diabetes mellitus.

Diabetes

Prof. Kuwata Keijiroo, Doctor of Medicine

"When I was serving in the Fire Insurance Association, I used to examine many diabetic patients. Besides treating them with drugs, I provided them with antioxidant water. After drinking antioxidant water for one month, 15 diabetic patients were selected and sent to Tokyo University for further test and observations.
Initially, the more serious patients were a bit apprehensive about the treatment. When the antioxidant water was consumed for some time, the sugar in the blood and urine ranged from a ratio of 300 mg/l to 2 mg / dc. There was a time where the patient had undergone 5 to 6 blood tests a day and detected to be within normal range. Results also

showed that even 1 ½ hour after meals, the blood sugar and urine ratio was 100 mg/dc: 0 mg/dc . The sugar in the urine has completely disappeared."

NOTE: More Americans than ever before are suffering from diabetes, with the number of new cases averaging almost 800,000 each year. The disease has steadily increased in the United States since 1980, and in 1998, 16 million Americans were diagnosed with diabetes (10.3 million diagnosed; 5.4 million undiagnosed). Diabetes is the seventh leading cause of death in the United States, and more than 193,000 died from the disease and its related complications in 1996. From: U. S. Department of Health and Human Services, October 13, 2000 Fact Sheet.

Use of Ionized water in treating Acidosis

Prof. Hatori Tasutaroo, Head of Akajiuiji Blood Centre, Yokohama Hospital, Faitama District
"Due to a higher standard of living, our eating habits have changed. We consume too much proteins, fats and sugar. The excess fats and carbohydrates are in the body as fats. In the present lifestyles, Americans are more extravagant on food compared to the Japanese. Due to this excessive intake obesity is a significant problem. Normally, one out of five males and one out of four females is obese.

The degree of "burn-out" in food intake largely depends on the amount on intake of vitamins and minerals. When excessive intake of proteins, carbohydrates and fats occurs, the requirement for vitamins and minerals increases. However, there is not much research carried out pertaining to the importance of vitamins and minerals.

Nowadays, many people suffer from acidification that leads to diabetes, heart diseases, cancer, live and kidney diseases. If our food intake can be completely burned off, then there is no deposition of fats. Obviously, there will be no acidification problem and hence there should not be any sign of obesity.

The antioxidant water contains an abundance of ionic calcium. This ionic calcium helps in the "burn-off" process. By drinking antioxidant water, it provides sufficient minerals for our body. As a result, we do not need to watch our diet to stay slim.

Hence, antioxidant water is a savior for those suffering from obesity and many adult diseases, providing good assistance in enhancing good health."

REDUCED WATER FOR PREVENTION OF DISEASES

Dr.Sanetaka Shirahata
Graduate school of Genetic Resources Technology, Kyushu University,
6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan.
It has long been established that reactive oxygen species (ROS) cause many types
of damage to biomolecules and cellular structures, that, in turn result in the development of a variety of pathologic states such as diabetes, cancer and aging. Reduced water is defined as anti-oxidative water produced by reduction of water. Electrolyzed reduced water (ERW) has been demonstrated to be hydrogen-rich water and can scavenge ROS in vitro (Shirahata et al., 1997). The reduction of proton in water to active hydrogen (atomic hydrogen, hydrogen radical) that can scavenge ROS is very easily caused by a weak current, compared to oxidation of hydroxyl ion to oxygen molecule. Activation of water by magnetic field, collision, minerals etc. will also produce reduced water containing active hydrogen and/or hydrogen molecule. Several natural waters such as Hita Tenryosui water drawn from deep underground in Hita city in Japan, Nordenau water in Germany and Tlacote water in Mexico are known to alleviate various diseases. We have developed a sensitive method by which we can detect active hydrogen existing in reduced water, and have demonstrated that not only ERW but also natural reduced waters described above contain active hydrogen and scavenge ROS in cultured cells. ROS is known to cause reduction of glucose uptake by

inhibiting the insulin-signaling pathway in cultured cells. Reduced water scavenged intracellular ROS and stimulated glucose uptake in the presence or absence of insulin in both rat L6 skeletal muscle cells and mouse 3T3/L1 adipocytes. This insulin-like activity of reduced water was inhibited by wortmannin that is specific inhibitor of PI-3 kinase, a key molecule in insulin signaling pathways. Reduced water protected insulin-responsive cells from sugar toxicity and improved the damaged sugar tolerance of type 2 diabetes model mice, suggesting that reduced water may improve insulin-independent diabetes mellitus. Cancer cells are generally exposed to high oxidative stress. Reduced water cause impaired tumor phenotypes of human cancer cells, such as reduced growth rate, morphological changes, reduced colony formation ability in soft agar, passage number-dependent telomere shortening, reduced binding abilities of telomere binding proteins and suppressed metastasis. Reduced water suppressed the growth of cancer cells transplanted into mice, demonstrating their anti-cancer effects in vivo. Reduced water will be applicable to not only medicine but also food industries, agriculture, and manufacturing industries.

Shirahata, S. et al.: Electrolyzed reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochem. Biophys. Res. Commun., 234, 269174, 1997.

Clinical evaluation of alkaline ionized water for abdominal complaints: Placebo controlled double blind tests

by Hirokazu Tashiro, Tetsuji Hokudo, Hiromi Ono, Yoshihide Fujiyama, Tadao Baba (National Ohkura Hospital, Dept. of Gastroenterology; Institute of Clinical Research, Shiga University of Medical Science, Second Dept. of Internal Medicine)

Effect of alkaline ionized water on abdominal complaints was evaluated by placebo controlled double blind tests. Overall scores of improvement using alkaline ionized water marked higher than those of placebo controlled group, and its effect proved to be significantly higher especially in slight symptoms of chronic diarrhoea and abdominal complaints in cases of general malaise. Alkaline ionized water group did not get interrupted in the course of the test, nor did it show serious side effects nor abnormal test data. It was confirmed that alkaline ionized water is safer and more effective than placebos.

Summary
Effect of alkaline ionized water on abdominal complaints was clinically examined by double blind tests using clean water as placebo. Overall improvement rate was higher for alkaline ionized water group than placebo group and the former proved to be significantly more effective than the other especially in cases of slight symptoms. Examining improvement rate for each case of chronic diarrhoea, constipation and abdominal complaints, alkaline ionized water group turned out to be more effective than placebo group for chronic diarrhoea, and abdominal complaints. The test was stopped in one case of chronic diarrhoea, among placebo group due to exacerbation, whereas alkaline ionized water group did not stop testing without serious side effects or abnormal test data in all cases. It was confirmed that alkaline ionized water is more effective than clean water against chronic diarrhoea, abdominal complaints and overall improvement rate (relief of abdominal complaints) and safer than clean water.

Introduction
Since the approval of alkaline ionized water electrolyzers by Pharmaceutical Affairs Law in 1966 for its antacid effect and efficacy against gastrointestinal disorders including hyperchylia, indigestion, abnormal gastrointestinal fermentation and chronic diarrhoea, they have been extensively used among patients. However, medical and scientific evaluation of their validity is not established. In our study, we examined clinical effect of alkaline ionized water on gastrointestinal disorders across many symptoms in various facilities. Particularly, we studied safety and usefulness of alkaline ionized water by doubleblind tests using clean water as a control group.

Test subjects and methods

163 patients (34 men, 129 women, age 21 to 72, average 38.6 years old) of indigestion, abnormal gastrointestinal fermentation (with abnormal gas emission and rugitus) and abdominal complaints caused by irregular dejection (chronic diarrhoea, or constipation) were tested as subjects with good informed consent. Placebo controlled double blind tests were conducted using alkaline ionized water and clean water at multiple facilities. An alkaline ionized water electrolyzer sold commercially was installed with a pump driven calcium dispenser in each of the subject

homes. Tested alkaline ionized water had pH at 9.5 and calcium concentration at 30ppm. Each subject in placebo group used a water purifier that has the same appearance as the electrolyzer and produces clean water.

The tested equipment was randomly assigned by a controller who scaled off the key code which was stored safely until the tests were completed and the seal was opened again.

Water samples were given to each patient in the amount of 200ml in the morning with the total of 50OmI or more per day for a month. Before and after the tests, blood, urine and stool were tested and a log was kept on the subjective symptoms, bowel movements and accessory symptoms. After the tests, the results were analyzed based on the log and the test data.

Test Results

1. Symptom
Among 163 tested subjects, alkaline ionized water group included 84 and placebo group 79. Background factors such as gender, age and basal disorders did not contribute to significant difference in the results.

2. Overall improvement rate

As to overall improvement rate of abdominal complaints, alkaline ionized water group had 2 cases of outstanding improvement (2.5%), 26 cases of fair improvement (32.1%), 36 cases of slight improvement (44.4%), 13 cases of no change (16%) and 4 cases of exacerbation (4.9%), whereas placebo group exhibited 4 (5.2%), 19 (24.7%), 27 (35.1%), 25 (32.5%) and 2 cases (2.6%) for the same category. Comparison between alkaline ionized water and placebo groups did not reveal any significant difference at the level of 5% significance according to the Wilcoxon test, although alkaline ionized water group turned out to be significantly more effective than placebo group at the level of p value of 0.22.

Examining overall improvement rates by a 7, 2 test (with no adjustment for continuity) between the effective and noneffective groups, alkaline ionized water group had 64 (79%) of effective cases and 17 cases (21%) of non effective cases, whereas placebo group had 50 (64.9%) and 27 (35.1%) cases respectively. The result indicated that alkaline ionized water group was significantly more effective than placebo group at the level of p value of 0.0.48.

Looking only at 83 slight cases of abdominal complaints, overall improvement rate for alkaline ionized water group

(45 cases) was composed of 11 cases (242%) of fair improvement, 22 cases (48.9%) of slight improvement, 17 cases (44.7%) of no change and 3 cases (6.7%) of exacerbation, whereas placebo group (38 cases) had 3 (7.8%), 17 (44.7%), 17 (44.7%) and 1 (2.6%) cases for the same category. Alkaline ionized water group was significantly more effective than placebo group according to the comparison between the groups (p value = 0.033).

3. Improvement rate by basal symptom

Basal symptoms were divided into chronic diarrhea, constipation and abdominal complaints (dyspepsia) and overall improvement rate was evaluated for each of them to study effect of alkaline ionized water. In case of chronic diarrhoea, alkaline ionized water group resulted in 94.1% of effective cases and 5.9% of non effective cases. Placebo group came up with 64,7% effective and 35.3% non effective. These results indicate alkaline ionized water group proved to be significantly more effective than placebo group. In case of slighter chronic diarrhoea, comparison between groups revealed that alkaline ionized water group is significantly more effective than placebo group (p=0.015). In case of constipation, alkaline ionized water group consisted of 80.5% of effective and 19.5% of non effective cases, whereas placebo group resulted in 73.3% effective and 26.3 non effective. As to abdominal complaints (dyspepsia), alkaline ionized water group had 85.7% of effective and 14.3% non effective cases while placebo group showed 47.1% and 62.9% respectively. Alkaline ionized water group proved to be significantly more effective than placebo group (p=0.025).

4. Safety

Since one case of chronic diarrhoea, in placebo group saw exacerbation, the test was stopped. There was no such cases in alkaline ionized water group. Fourteen cases of accessory symptoms, 8 in alkaline ionized water group and 6 in placebo group, were observed, none of which were serious. 31 out of 163 cases (16 in alkaline ionized water group, 15 in placebo group) exhibited fluctuation in test data, although alkaline ionized water group did not have any problematic fluctuations compared to placebo group. Two cases in placebo group and one case in alkaline ionized water group have seen K value of serum climb up and resume to normal value after re testing which indicates the value changes were temporary.

Conclusion

As a result of double blind clinical tests of alkaline ionized water and clean water, alkaline ionized water was proved to be more effective than clean water against chronic diarrhea, abdominal complaints (dyspepsia) and overall improvement rate (relief from abdominal complaints). Also, safety of alkaline ionized water was confirmed which clinically verifies its usefulness.

Selective stimulation of the growth of anaerobic microflora in the human intestinal tract by electrolyzed reducing water

Vorobjeva NV, Med Hypotheses. 2005;64(3):543-6.

96-99% of the "friendly" or residential microflora of intestinal tract of humans consists of strict anaerobes and only 1-4% of aerobes. Many diseases of the intestine are due to a disturbance in the balance of the microorganisms inhabiting the gut. The treatment of such diseases involves the restoration of the quantity and/or balance of residential microflora in the intestinal tract. It is known that aerobes and anaerobes grow at different oxidation-reduction potentials (ORP). The former require positive E(h) values up to +400 mV. Anaerobes do not grow unless the E(h) value is negative between -300 and -400 mV. In this work, it is suggested that prerequisite for the recovery and maintenance of obligatory anaerobic microflora in the intestinal tract is a negative ORP value of the intestinal milieu. Electrolyzed reducing water with E(h) values between 0 and -300 mV produced in electrolysis devices possesses this property. Drinking such water favours the growth of residential microflora in the gut. A sufficient array of data confirms this idea. However, most researchers explain the mechanism of its action by an antioxidant properties destined to detox the oxidants in the gut and other host tissues. Evidence is presented in favour of the hypothesis that the primary target for electrolyzed reducing water is the residential microflora in the gut.

Physiological effects of alkaline ionized water: Effects on metabolites produced by intestinal fermentation

by Takashi Hayakawa, Chicko Tushiya, Hisanori Onoda, Hisayo Ohkouchi, Harul-~to Tsuge (Gifu University, Faculty of Engineering, Dept. of Food Science)

We have found that long-term ingestion of alkaline ionized water (AIW) reduces cecal fermentation in rats that were given highly fermentable commercial diet (MF: Oriental Yeast Co., Ltd.). In this experiment, rats were fed MF and test water (tap water, AIW with pH at 9 and 10) for about 3 months. Feces were collected on the 57th day, and the rats were dissected on the 88th day. The amount of ammonium in fresh feces and cecal contents as well as fecal free-glucose tended to drop down for the AIW group. In most cases, the amount of free-amino acids in cecal contents did not differ sign- icantly except for cysteine (decreased in AIW with pH at 10) and isoleucine (increased in AIW with pH at 10).

Purpose of tests
Alkaline ionized water electrolyzers have been approved for manufacturing in 1965 by the Ministry of Health and Welfare as medical equipment to produce medical substances. Alkaline ionized water (AIW) produced by this equipment is known to be effective against gastrointestinal fermentation, chronic diarrhea, indigestion and hyperchylia as well as for controlling gastric acid.*1 This is mainly based on efficacy of the official calcium hydroxide. *2 By giving AIW to rats for a comparatively long time under the condition of extremely high level of

intestinal fermentation, we have demonstrated that AIW intake is effective for inhibition of intestinal fermentation when its level is high based on some test results where AIW worked against cecal hypertrophy and for reduction in the amount of short-chain fatty acid that is the main product of fermentation.*3 We have reported that this is caused by the synergy between calcium level generally contained in AIW (about 50ppm) and the value of pH, and that frequency of detecting some anaerobic bacteria tends to be higher in alkaline ionized water groups than the other, although the bacteria count in the intestine does not have significant difference. Based on these results, we made a judgment that effect of taking AIW supports part of inhibition mechanism against abnormal intestinal fermentation, which is one of the claims of efficacy that have been attributed to alkaline ionized water electrolyzers. *4 On the other hand, under the dietary condition of low intestinal fermentation, AIW uptake does not seem to inhibit fermentation that leads us to believe that effect of AIW uptake is characteristic of hyper-fermentation state. Metabolites produced by intestinal fermentation include indole and skatole in addition to organic acids such as short-chain fatty acid and lactic acid as well as toxic metabolites such as ammonium, phenol and pcresol. We do not know how AIW uptake would affect the production of these materials. In this experiment, we have tested on ammonium production as explained in the following sections.

Testing methods

Four-week-old male Wistar/ST Clean rats were purchased from Japan SLC Co., Ltd. and were divided into 3 groups of 8 each after preliminary breeding. AIW of pH 9 and 10 was produced by an electrolyzer Mineone ROYAL NDX3 1 OH by Omco Co., Ltd. This model produces AIW by electrolyzing water with calcium lactate added. On the last day of testing, the rats were dissected under Nembutal anesthesia to take blood from the heart by a heparin-treated syringe. As to their organs, the small intestines, cecum and colon plus rectum were taken out from each of them. The cecurn was weighed and cleaned with physiological saline after its contents were removed, and the tissue weight was measured after wiping out moisture. Part of cecal contents was measured its pH, and the rest was used to assay ammonium concentration. The amount of ammonium contained in fresh feces and cecal contents was measured by the Nessler method after collecting it in the extracted samples using Conway's micro-diffusion container. Fecal free-glucose was assayed by the oxygen method after extraction by hot water. Analysis of free amino acids contained in cecal contents was conducted by the Waters PicoTag amino acid analysis system.

Test results and analyses

No difference was found in the rats' weight gain, water and feed intake and feeding efficiency, nor was any particular distinction in appearance identified. The length of the small intestines and colon plus rectum tended to decline in AIW groups. PH value of cecal contents was higher and the amount of fecal free-glucose tended to be lower in AIW groups than the control group. Since there was no difference in fecal discharge itself, the amount of free-glucose discharged per day was at a low level. The amount of discharged free-glucose in feces is greater when intestinal fermentation is more intensive, which indicates that intestinal fermentation is more inhibited in AIW groups than the control group. Ammonium concentration in cecal contents tends to drop down in AIW groups (Fig. 1). This trend was most distinctive in case of fresh feces of one of AIW groups with pH 10 (Fig.2) AIW uptake was found to be inhibitory against ammonium production. In order to study dynamics of amino acids in large intestines, we examined free amino acids in the cecal contents to find out that cysteine level is low in AIW groups whereas isoleucine level is high in one of AIW groups with pH 10, although no significant difference was identified for other amino acids.

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Effects of alkaline ionized water on formation

& maintenance of osseous tissues

by Rei Takahashi Zhenhua Zhang Yoshinori Itokawa
(Kyoto University Graduate School of Medicine, Dept. of Pathology and Tumor Biology, Fukui Prefectural University)

Effects of calcium alkaline ionized water on formation and maintenance of osseous tissues in rats were examined. In the absence of calcium in the diet, no apparent calcification was observed with only osteoid formation being prominent. Striking differences were found among groups that were given diets with 30% and 60% calcium. Rats raised by calcium ionized water showed the least osteogenetic disturbance. Tibiae and humeri are more susceptible to calcium deficiency than femora. Theses results may indicate that calcium in drinking water effectively supplements osteogenesis in case of dietary calcium deficiency. The mechanism involved in osteoid formation such as absorption rate of calcium from the intestine and effects of calcium alkaline ionized drinking water on maintaining bone structure in the process of aging or under the condition of calcium deficiency is investigated.

Osteoporosis that has lately drawn public attention is defined as "conditions of bone brittleness caused by reduction in the amount of bone frames and deterioration of osseous microstructure." Abnormal calcium metabolism has been considered to be one of the factors to contribute to this problem, which in turn is caused by insufficient calcium take in, reduction in enteral absorption rate of calcium and increase in the amount of calcium in urinal discharge. Under normal conditions, bones absorb old bones by regular metabolism through osteoid formation to maintain their strength and function as supporting structure. It is getting clear that remodeling of bones at the tissue level goes through the process of activation, resorption, reversal, matrix synthesis and mineralization. Another important function of bones is storing minerals especially by coordinating with intestines and kidneys to control calcium concentration in the blood. When something happens to this osteo metabolism, it results in abnormal morphological changes. Our analyses have been focusing mostly on the changes in the amount of bones to examine effects of calcium alkaline ionized water on the reaction system of osteo metabolism and its efficiency. Ibis time, however, we studied it further from the standpoint of histology. In other words, we conducted comparative studies on morphological and kinetic changes of osteogenesis by testing alkaline ionized water, tap water and solution of lactate on rats.
Three week old male Wistar rats were divided into 12 groups by conditions of feed and drinking water. Feeds were prepared with 0%, 30%, 60% and 100% of normal amount of calcium and were given freely. Three types of drinking water, tap water (city water, about 6ppm of Ca), calcium lactate solution (Ca=40ppm) and alkaline ionized water (Ca =40ppm, pH=9, produced by an electrolyzer NDX 4 LMC by Omco OMC Co., Ltd.) were also given keely. Rats' weight, amount of drinking water and feed as well as the content of Ca in drinking water were assayed every day. On the 19th and 25th days of testing, tetracycline hydrochloride was added to the feed for 48 hours so as to bring its concentration to 30mg/kg. On the 30th day, blood samples were taken under Nembutal anesthesia, and tibiae, humeri and femora were taken out to make non decalcified samples. Their conditions of osteoid formation and rotation were observed using Villanueva bone stain and Villanueva goldner stain.

Three groups that were given different types of drinking water and the same amount of Ca in the feed were compared to find out no significant difference in the rate of weight gain and intakes of feed and drinking water. Alkaline ionized water group had significantly greater amount of tibiae and humeri with higher concentration of calcium in the bones.

The group of 0% calcium in the feed saw drastic increase in the amount of osteoid. There was not much difference by types of drinking water. Almost no tetracycline was taken into tibiae and humeri, although a small amount was identified in ferora. As a result, osteogenesis went as far as osteoid formation, but it was likely that decalcification has not happened yet, or most of newly formed bones were absorbed.

As to the groups of 30% and 60% calcium in the feed, increase in the area of tetracycline take in was more identifiable with higher clarity in descending order of alkaline ionized water, calcium lactate solution and tap water groups. Especially in case of tap water group, irregularity among the areas of tetracycline take in was distinctive. The group of 100% calcium in the feed saw some improvements in osteogenesis in descending order of alkaline ionized water, calcium lactate solution and tap water. In any case, bone formation seemed to be in good condition at near normal level.

Alkaline ionized water was regarded to be effective for improvements of osteogenesis under the conditions of insufficient calcium in the feed. Also, the extent. of dysosteogenesis differed by the region. That is, tibiae and humeri tend to have more significant dysosteogenesis than femora.

In addition, there is a possibility that osteo metabolism varies depending on enteral absorption rate of calcium, adjustment of discharge from kidneys and functional adjustment of accessory thyroid in the presence of alkaline ionized water. We are now studying its impact on calcium concentration in the blood. We are also examining whether it is possible to deter bone deterioration by testing on fast aging mouse models.

Magnesium and calcium in drinking water and cardiovascular mortality

Excerpt from:
Scand J Work Environ Health 1991;17:91-4

Ragnar Rylander, MD, Håkan Bonevik, MD, Eva Rubenowitz, MD
Department of Environmental Hygiene, University of Göteborg, Göteborg, Sweden.

Data on the hardness of drinking water were collected from 27 municipalities in Sweden where the drinking water quality had remained unchanged for more than 20 years. Analyses were made of the levels of lead, cadmium, calcium, and magnesium. These water-quality data were compared with the age-adjusted mortality rate from ischemic heart and cerebrovascular disease for the period 1969-1978. Lead and cadmium were not present in detectable amounts except in one water sample. A statistically significant inverse relationship was present between hardness and mortality from cardiovascular disease for both sexes. Mortality caused by ischemic heart disease was inversely related to the magnesium content, particularly for the men ($P<0.01$). The rather small set of data supports results from previous studies suggesting that a high magnesium level in drinking water reduces the risk for death from ischemic heart disease, especially among men, although the possible importance of confounding factors needs further evaluation.
Key terms: cerebrovascular disease, ischemic heart disease, magnesium, water hardness.
Several epidemiologic investigations performed during recent decades have demonstrated an inverse relationship between water hardness and death from cardiovascular disease. The first observation was made in 1957 (1) and was subsequently elaborated upon in investigations in many other countries (2-4). A particularly relevant study was reported by Crawford et al (5), who followed the mortality rate in 11 English cities where the water hardness had changed between 1950 and 1960. Hardness had increased in five cities and decreased in six. Mortality from cardiovascular disease increased about 10% in the general population during the period of study. In the cities where hardness had decreased, mortality had increased by 20%....

Evaluation of ionized calcium as a nutrient

Chen H, Kimura M, Zhu Z, Itokawa Y, The 11th symposium on Trace Nutrients Research, Japan Trace Nutrients Research Society, p131-138, 1994.

Summary: To clarify effect of ionized calcium water for drinking water in rats, 36 Male Wister rats weighing about 50g were randomly divided into 6 groups, and given following diet and drinking water : (1) Ca-sufficient diet, tap-water; (2) Ca-sufficient diet, tap-water;(3) Ca-sufficient diet, calcium lactate added-ionized calcium-water : (4) Ca-deficient diet, calcium lactate added-water ; (5) Ca deficient diet, calcium lactate added-water :(6) Ca-deficient diet, calcium lactate added ionized calcium-water. The diets were given by paired-feeding method 4 weeks and drinking water was ad libitum. The significant change of calcium concentration in the rats were was follows; Ca concentration of plasma, spleen, of plasma, spleen, kidney, testis and tibia in Ca deficient groups (4), (5), (6) were significantly low compared with these in Ca sufficient groups (1),(2),(3) Ca concentration in brain of groups (4),(5),(6) was low compared to these in groups (2), Ca concentration in heart and muscle of group (4) was low compared to Ca deficient groups (1),(2),(3), but these in group (5) drank Ca added-water was recovered and these in group (6) drank ionized-Ca-water was higher than these in any other groups. Ca concentration of liver in groups (4) were significantly lower than that in group (1),(3) and Ca concentration of liver in Ca deficient rats (groups (5),(6)) drank Ca-added-water were high compared to these in group (4). In 24 hours urine discharge of group (2) was high compared with groups (4), (5), (6). These results suggest that ionized Ca in drinking water may be active for intestinal absorption.

Calcium and magnesium in drinking water
and risk of death from cerebrovascular disease.

MEDLINE ABSTRACT
Author: Yang CY
Author Affiliation: School of Public Health, Kaohsiung Medical College, Taiwan, Republic of China.
chunyuh*cc.kmc.edu.tw
Source: Stroke 1998 Feb; 29(2):411-4

BACKGROUND AND PURPOSE: Many studies have demonstrated a negative association between mortality from cardiovascular or cerebrovascular diseases and water hardness. This report examines whether calcium and magnesium in drinking water are protective against cerebrovascular disease.

METHODS: All eligible cerebrovascular deaths (17133 cases) of Taiwan residents from 1989 through 1993 were compared with deaths from other causes (17133 controls), and the levels of calcium and magnesium in drinking water of these residents were determined. Data on calcium and magnesium levels in drinking water throughout Taiwan were obtained from the Taiwan Water Supply Corporation. The control group consisted of people who died from other causes, and the controls were pair matched to the cases by sex, year of birth, and year of death.
RESULTS: The adjusted odds ratios (95% confidence interval) were 0.75 (0.65 to 0.85) for the group with water magnesium levels between 7.4 and 13.4 mg/L and 0.60 (0.52 to 0.70) for the group with magnesium levels of 13.5 mg/L or more. After adjustment for magnesium levels in drinking water, there was no difference between the groups with different levels of calcium.
CONCLUSIONS: The results of the present study show that there is a significant protective effect of magnesium intake from drinking water on the risk of cerebrovascular disease. This is an important finding for the Taiwan water industry and human health.

Reduced hemodialysis-induced oxidative stress in end-stage renal disease patients by electrolyzed reduced water

Huang KC, Yang CC, Lee KT, Chien CT
Department of Family Medicine, National Taiwan University College of Medicine and National Taiwan University Hospital, Taipei, Taiwan.
KIDNEY INTERNATIONAL.
2003 Aug; 64(2):704-14.
BACKGROUND: Increased oxidative stress in end-stage renal disease (ESRD) patients may oxidize macromolecules and consequently lead to cardiovascular events during chronic hemodialysis. Electrolyzed reduced water (ERW) with reactive oxygen species (ROS) scavenging ability may have a potential effect on reduction of hemodialysis-induced oxidative stress in ESRD patients. METHODS: We developed a chemiluminescence emission spectrum and high-performance liquid chromatography analysis to assess the effect of ERW replacement on plasma ROS (H_2O_2 and HOCl) scavenging activity and oxidized lipid or protein production in ESRD patients undergoing hemodialysis. Oxidized markers, dityrosine, methylguanidine, and phosphatidylcholine hydroperoxide, and inflammatory markers, interleukin 6 (IL-6), and C-reactive protein (CRP) were determined. RESULTS: Although hemodialysis efficiently removes dityrosine and creatinine, hemodialysis increased oxidative stress, including phosphatidylcholine hydroperoxide, and methylguanidine. Hemodialysis reduced the plasma ROS scavenging activity, as shown by the augmented reference H_2O_2 and HOCl counts (Rh202 and Rhocl, respectively) and decreased antioxidative activity (expressed as total antioxidant status in this study). ERW administration diminished hemodialysis-enhanced Rh202 and Rhocl, minimized oxidized and inflammatory markers (CRP and IL-6), and partly restored total antioxidant status during 1-month treatment. CONCLUSION: This study demonstrates that hemodialysis with ERW administration may efficiently increase the H_2O_2- and HOCl-dependent antioxidant defense and reduce H_2O_2- and HOCl-induced oxidative stress.

CLINICAL STUDIES OF ALKALINE WATER

Ionized water is known by various names:

Reduced water
Electrolyzed water
Alkaline /Acid water
Microwater

There have been many studies by Doctors in Japanese Hospitals on the Benefits of using Alkaline Water. Below are a few now translated into English and available to the public.

- Fluid replacement promotes optimal physical performance.
- Electrolyzed-reduced water scavenges active oxygen & protects DNA from oxidative damage.
- The mechanism of the enhanced antioxidant effects of reduced water produced by electrolysis.
- Antimicrobial interventions to reduce Salmonella species on poultry
- Treatment of Escherichia coli inoculated alfalfa sprouts with electrolyzed oxidizing water
- Inactivation of E. coli & Listeria on plastic kitchen cutting boards by electrolyzed oxidizing water.
- The bactericidal effects of electrolyzed oxidizing water on bacterial strains in hospital infections
- Effect of electrolyzed water on wound healing.
- Effect of electrolyzed oxidizing water on excised burn-wounds in rats
- Decomposition of ethylene, a flower-senescence hormone, with electrolyzed anode water.
- Use of Ionized water in hypochlorhydria, achlorhydria, reduction of high blood pressure
- Use of Ionized water for gynecological conditions
- Clinical Improvements obtained from the uptake of Ionized Water
- Alkaline ionized water for abdominal complaints: Placebo controlled double blind tests
- Physiological effects of alkaline ionized water: intestinal fermentation
- Effects of calcium alkaline ionized water on formation and maintenance of osseous tissues
- Reduced Water for Prevention of Disease
- Use of Ionized water in heart disease and toxins.
- Use of Ionized water in skin disease.
- Use of Ionized water in allergies.
- Use of Ionized water in diebetes treatment

- Use of Ionized water in treating Acidosis
- Environmental electroshemistry of water

The following information is sourced from various peer reviewed literature as well as various Internet sites. This information is for educational purposes only and is not meant to cure or treat any disease or illness. Consult your doctor for specialised medical advice.

Adequate fluid replacement helps maintain hydration and, promotes the health, safety, and optimal physical performance of individuals participating in regular physical activity. American College of Sports Medicine position stand.

Article on need for adequate water when exercising.

Med Sci Sports Exercise
1996 Jan;28(1):i-vii.

Convertino VA, Armstrong LE, Coyle EF, Mack GW, Sawka MN, Senay LC Jr, Sherman WM.

It is the position of the American College of Sports Medicine that adequate fluid replacement helps maintain hydration and, therefore, promotes the health, safety, and optimal physical performance of individuals participating in regular physical activity. This position statement is based on a comprehensive review and interpretation of scientific literature concerning the influence of fluid replacement on exercise performance and the risk of thermal injury associated with dehydration and hyperthermia.
Based on available evidence, the American College of Sports Medicine makes the following general recommendations on the amount and composition of fluid that should be ingested in preparation for, during, and after exercise or athletic competition:
1) It is recommended that individuals consume a nutritionally balanced diet and drink adequate fluids during the 24-hr period before an event, especially during the period that includes the meal prior to exercise, to promote proper hydration before exercise or competition.
2) It is recommended that individuals drink about 500 ml (about 17 ounces) of fluid about 2 hours before exercise to promote adequate hydration and allow time for excretion of excess ingested water.
3) During exercise, athletes should start drinking early and at regular intervals in an attempt to consume fluids at a rate sufficient to replace all the water lost through sweating (i.e., body weight loss), or consume the maximal amount that can be tolerated.
4) It is recommended that ingested fluids be cooler than ambient temperature (between 15 degrees and 22 degrees C or 59 degrees and 72 degrees F) and flavored to enhance palatability and promote fluid replacement. Fluids should be readily available and served in containers that allow adequate volumes to be ingested with ease and with minimal interruption of exercise.
5) Addition of proper amounts of carbohydrates and/or electrolytes to

a fluid replacement solution is recommended for exercise events of duration greater than 1 hour since it does not significantly impair water delivery to the body and may enhance performance. During exercise lasting less than 1 hour, there is little evidence of physiological or physical performance differences between consuming a carbohydrate-electrolyte drink and plain water.
6) During intense exercise lasting longer than 1 hr, it is recommended that carbohydrates be ingested at a rate of 30-60 g.h(-1) to maintain oxidation of carbohydrates and delay fatigue. This rate of carbohydrate intake can be achieved without compromising fluid delivery by drinking 600-1200 ml.hr(-1) of solutions containing 4%-8% carbohydrates (g.100 ml(-1)). The carbohydrates can be sugars (glucose or sucrose) or starch (e.g., maltodextrin).
7) Inclusion of sodium (0.5-0.7 g.1(-1) of water) in the rehydration solution ingested during exercise lasting longer than 1 hr is recommended since it may be advantageous in enhancing palatability, promoting fluid retention, and possibly preventing hyponatremia in certain individuals who drink excessive quantities of fluid. There is little physiological basis for the presence of sodium in an oral rehydration solution for enhancing intestinal water absorption as long as sodium is sufficiently available from the previous meal.

Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage.

Use of Alkaline water with low ORP to reduce Radical Damage

Biochem Biophys Res Commun.
1997 May 8;234(1):269-74.

Shirahata S, Kabayama S, Nakano M, Miura T, Kusumoto K, Gotoh M, **Hayashi H**, Otsubo K, Morisawa S, Katakura Y.

Institute of Cellular Regulation Technology, Graduate School of Genetic Resources Technology, Kyushu University, Fukuoka, Japan. sirahata@grt.kyushu-u.ac.jp

Active oxygen species or free radicals are considered to cause extensive oxidative damage to biological macromolecules, which brings about a variety of diseases as well as aging. The ideal scavenger for active oxygen should be 'active hydrogen'. 'Active hydrogen' can be produced in reduced water near the cathode during electrolysis of water. Reduced water exhibits high pH, low dissolved oxygen (DO), extremely high dissolved molecular hydrogen (DH), and extremely negative redox potential (RP) values. Strongly electrolyzed-reduced water, as well as ascorbic acid, (+)-catechin and tannic acid, completely scavenged O.-2 produced by the hypoxanthine-xanthine oxidase (HX-XOD) system in sodium phosphate buffer (pH 7.0). The superoxide dismutase (SOD)-like activity of reduced water is

stable at 4 degrees C for over a month and was not lost even after neutralization, repeated freezing and melting, deflation with sonication, vigorous mixing, boiling, repeated filtration, or closed autoclaving, but was lost by opened autoclaving or by closed autoclaving in the presence of tungsten trioxide which efficiently adsorbs active atomic hydrogen. Water bubbled with hydrogen gas exhibited low DO, extremely high DH and extremely low RP values, as does reduced water, but it has no SOD-like activity. These results suggest that the SOD-like activity of reduced water is not due to the dissolved molecular hydrogen but due to the dissolved atomic hydrogen (active hydrogen). Although SOD accumulated H2O2 when added to the HX-XOD system, reduced water decreased the amount of H2O2 produced by XOD. Reduced water, as well as catalase and ascorbic acid, could directly scavenge H2O2.
Reduced water suppresses single-strand breakage of DNA b active oxygen species produced by the Cu(II)-catalyzed oxidation of ascorbic acid in a dose-dependent manner, suggesting that reduced water can scavenge not only O2.- and H2O2, but also 1O2 and .OH.

PMID: 9169001 [PubMed - indexed for MEDLINE]

The mechanism of the enhanced antioxidant effects against superoxide anion radicals of reduced water produced by electrolysis.

Effect of Alkaline Water on Free Radicals

Biophys Chem. 2004
Jan 1;107(1):71-82.

Hanaoka K, Sun D, Lawrence R, Kamitani Y, Fernandes G.

Bio-REDOX Laboratory Inc. 1187-4, Oaza-Ueda, Ueda-shi, Nagano-ken 386-0001, Japan.
hanak@rapid.ocn.ne.jp

We reported that reduced water produced by electrolysis enhanced the antioxidant effects of proton donors such as ascorbic acid (AsA) in a previous paper.
We also demonstrated that reduced water produced by electrolysis of 2 mM NaCl solutions did not show antioxidant effects by itself. We reasoned that the enhancement of antioxidant effects may be due to the increase of the ionic product of water as solvent. The ionic product of water (pKw) was estimated by measurements of pH and by a neutralization titration method. As an indicator of oxidative damage, Reactive Oxygen Species- (ROS) mediated DNA strand breaks were measured by the conversion of supercoiled phiX-174 RF I double-strand DNA to open and linear forms. Reduced water had a tendency

to suppress single-strand breakage of DNA induced by reactive oxygen species produced by H2O2/Cu (II) and HQ/Cu (II) systems. The enhancement of superoxide anion radical dismutation activity can be explained by changes in the ionic product of water in the reduced water.

PMID: 14871602 [PubMed - in process]

Comparison of electrolyzed oxidizing water with various antimicrobial interventions to reduce Salmonella species on poultry.

Use of Acid Water to reduce Foodborne Pathogens

Poult Sci.
2002 Oct;81(10):1598-605.

Fabrizio KA, Sharma RR, Demirci A, Cutter CN.

Department of Food Science, The Pennsylvania State University, University Park 16802, USA.

Foodborne pathogens in cell suspensions or attached to surfaces can be reduced by electrolyzed oxidizing (EO) water; however, the use of EO water against pathogens associated with poultry has not been explored.
In this study, acidic EO water [EO-A; pH 2.6, chlorine (CL) 20 to 50 ppm, and oxidation-reduction potential (ORP) of 1,150 mV], basic EO water (EO-B; pH 11.6, ORP of -795 mV), CL, ozonated water (OZ), acetic acid (AA), or trisodium phosphate (TSP) was applied to broiler carcasses inoculated with Salmonella Typhimurium (ST) and submerged (4 C, 45 min), spray-washed (85 psi, 25 C, 15 s), or subjected to multiple interventions (EO-B spray, immersed in EO-A; AA or TSP spray, immersed in CL). Remaining bacterial populations were determined and compared at Day 0 and 7 of aerobic, refrigerated storage. At Day 0, submersion in TSP and AA reduced ST 1.41 log10, whereas EO-A water reduced ST approximately 0.86 log10. After 7 d of storage, EO-A water, OZ, TSP, and AA reduced ST, with detection only after selective enrichment. Spray-washing treatments with any of the compounds did not reduce ST at Day 0. After 7 d of storage, TSP, AA, and EO-A water reduced ST 2.17, 2.31, and 1.06 log10, respectively. ST was reduced 2.11 log10 immediately following the multiple interventions, 3.81 log10 after 7 d of storage. Although effective against ST, TSP and AA are costly and adversely affect the environment.
This study demonstrates that EO water can reduce ST on poultry surfaces following extended refrigerated storage.

PMID: 12412930 [PubMed - indexed for MEDLINE]

Treatment of Escherichia coli (O157:H7) inoculated alfalfa seeds and sprouts with electrolyzed oxidizing water.

Acid Water and Food Sanitation

Int J Food Microbiol.
2003 Sep 15;86(3):231-7.

Department of Agricultural and Biological Engineering, Pennsylvania State University, University Park, PA 16802, USA.

Electrolyzed oxidizing water is a relatively new concept that has been utilized in agriculture, livestock management, medical sterilization, and food sanitation.
Electrolyzed oxidizing (EO) water generated by passing sodium chloride solution through an EO water generator was used to treat alfalfa seeds and sprouts inoculated with a five-strain cocktail of nalidixic acid resistant Escherichia coli O157:H7. EO water had a pH of 2.6, an oxidation-reduction potential of 1150 mV and about 50 ppm free chlorine. The percentage reduction in bacterial load was determined for reaction times of 2, 4, 8, 16, 32, and 64 min. Mechanical agitation was done while treating the seeds at different time intervals to increase the effectiveness of the treatment. Since E. coli O157:H7 was released due to soaking during treatment, the initial counts on seeds and sprouts were determined by soaking the contaminated seeds/sprouts in 0.1% peptone water for a period equivalent to treatment time. The samples were then pummeled in 0.1% peptone water and spread plated on tryptic soy agar with 5 microg/ml of nalidixic acid (TSAN). Results showed that there were reductions between 38.2% and 97.1% (0.22-1.56 log(10) CFU/g) in the bacterial load of treated seeds. The reductions for sprouts were between 91.1% and 99.8% (1.05-2.72 log(10) CFU/g).
An increase in treatment time increased the percentage reduction of E. coli O157:H7. However, germination of the treated seeds reduced from 92% to 49% as amperage to make EO water and soaking time increased. EO water did not cause any visible damage to the sprouts.

PMID: 12915034 [PubMed - indexed for MEDLINE]

The following information is sourced from various peer reviewed literature as well as various Internet sites. This information is for educational purposes only and is not meant to cure or treat any disease or illness. Consult your doctor for specialised medical advice.

Inactivation of Escherichia coli (O157:H7) and Listeria monocytogenes on plastic kitchen cutting boards by electrolyzed oxidizing water.

Use of Acid Water to clean Plastic Cutting Boards

Venkitanarayanan KS, Ezeike GO, Hung YC, Doyle MP.

Department of Animal Science, University of Connecticut, Storrs 06269, USA.

One milliliter of culture containing a five-strain mixture of Escherichia coli O157:H7 (approximately 10(10) CFU) was inoculated on a 100-cm2 area marked on unscarred cutting boards.
Following inoculation, the boards were air-dried under a laminar flow hood for 1 h, immersed in 2 liters of electrolyzed oxidizing water or sterile deionized water at 23 degrees C or 35 degrees C for 10 or 20 min; 45 degrees C for 5 or 10 min; or 55 degrees C for 5 min. After each temperature-time combination, the surviving population of the pathogen on cutting boards and in soaking water was determined. Soaking of inoculated cutting boards in electrolyzed oxidizing water reduced E. coli O157:H7 populations by > or = 5.0 log CFU/100 cm2 on cutting boards. However, immersion of cutting boards in deionized water decreased the pathogen count only by 1.0 to 1.5 log CFU/100 cm2. Treatment of cutting boards inoculated with Listeria monocytogenes in electrolyzed oxidizing water at selected temperature-time combinations (23 degrees C for 20 min, 35 degrees C for 10 min, and 45 degrees C for 10 min) substantially reduced the populations of L. monocytogenes in comparison to the counts recovered from the boards immersed in deionized water. E. coli O157:H7 and L. monocytogenes were not detected in electrolyzed oxidizing water after soaking treatment, whereas the pathogens survived in the deionized water used for soaking the cutting boards. This study revealed that **immersion of kitchen cutting boards in electrolyzed oxidizing water could be used as an effective method for inactivating foodborne pathogens on smooth, plastic cutting boards.**

PMID: 10456736 [PubMed - indexed for MEDLINE]

The bactericidal effects of electrolyzed oxidizing water on bacterial strains involved in hospital infections.

Acid Water and Hospital Infections

Vorobjeva NV, Vorobjeva LI, Khodjaev EY.

Artif Organs.
2004 Jun;28(6):590-2.

Department of Physiology of Microorganisms, Biology Faculty, Moscow State University, Lenin Hills 1/12, Moscow 119992, Russia. nvvorobjeva@mail.ru

The study is designed to investigate bactericidal actions of electrolyzed oxidizing water on hospital infections.
Ten of the most common opportunistic pathogens are used for this study. Cultures are inoculated in 4.5 mL of electrolyzed oxidizing (EO) water or 4.5 mL of sterile deionized water (control), and incubated for 0, 0.5, and 5 min at room temperature. At the exposure time of 30 s the EO water completely inactivates all of the bacterial strains, with the exception of vegetative cells and spores of bacilli which need 5 min to be killed. The results indicate that **electrolyzed oxidizing water may be a useful disinfectant for hospital infections**, but its clinical application has still to be evaluated.
PMID: 15153153 [PubMed - in process]

Effect of electrolyzed oxidizing water and hydrocolloid occlusive dressings on excised burn-wounds in rats.

Use of Acid Water on Burns

Chin J Traumatol
2003 Aug 1;6(4):234-7.

Xin H, Zheng YJ, Hajime N, Han ZG.

Department of Thoracic Surgery, China-Japan Union Hospital, Jilin University, Jilin 130031, China. xinhua7254@yahoo.com.cn

OBJECTIVE: To study the efficacy of electrolyzed oxidizing water (EOW) and hydrocolloid occlusive dressings in the acceleration of epithelialization in excised burn-wounds in rats.
METHODS: Each of the anesthetized Sprague-Dawley rats (n=28) was subjected to a third-degree burn that covered approximately 10% of the total body surface area. Rats were assigned into four groups: Group I (no irrigation), Group II (irrigation with physiologic saline), Group III (irrigation with EOW) and Group IV (hydrocolloid occlusive dressing after EOW irrigation). Wounds were observed macroscopically until complete epithelialization was present, then the epithelialized wounds were examined microscopically. RESULTS: Healing of the burn wounds was the fastest in Group IV treated with hydrocolloid occlusive dressing together with EOW. Although extensive regenerative epidermis was seen in each Group, the proliferations of lymphocytes and macrophages associated with dense collagen deposition were more extensive in Group II, III and IV than in Group I. These findings were particularly evident in Group III and IV.
CONCLUSIONS: **Wound Healing may be accelerated by applying a hydrocolloid occlusive dressing on burn surfaces after they are cleaned with electrolyzed oxidating water.**

PMID: 12857518 [PubMed - indexed for MEDLINE]

Effect of electrolyzed water on wound healing.

Acid Water for Burns

Artif Organs.
2000 Dec;24(12):984-7.

Yahagi N, Kono M, Kitahara M, Ohmura A, Sumita O, Hashimoto T, Hori K, Ning-Juan C, Woodson P, Kubota S, Murakami A, Takamoto S.

Department of Anesthesiology, Teikyo University Mizonokuchi Hospital, Tokyo, Japan. naokiyah@aol.com

Electrolyzed water accelerated the healing of full-thickness cutaneous wounds in rats, but only anode chamber water (acid pH or neutralized) was effective. Hypochlorous acid (HOCl), also produced by electrolysis, was ineffective, suggesting that these types of electrolyzed water enhance wound healing by a mechanism unrelated to the well-known antibacterial action of HOCl. One possibility is that reactive oxygen species, shown to be electron spin resonance spectra present in anode chamber water, might trigger early wound healing through fibroblast migration and proliferation.

PMID: 11121980 [PubMed - indexed for MEDLINE]

Decomposition of ethylene, a flower-senescence hormone, with electrolyzed anode water.

Acid Water used to extend Flower Life

Biosci Biotechnol Biochem.
2003 Apr;67(4):790-6.

Harada K, Yasui K.

Department of Research and Development, Hokkaido Electric Power Co., Inc., 2-1 Tsuishikari, Ebetsu, Hokkaido 067-0033, Japan. kharada@h1.hotcn.ne.jp

Electrolyzed anode water (EAW) markedly extended the vase life of cut carnation flowers.
Therefore, a flower-senescence hormone involving ethylene decomposition by EAW with potassium chloride as an electrolyte was investigated. Ethylene was added externally to EAW, and the reaction between ethylen and the available chlorine in EAW was examined.

EAW had a low pH value (2.5), a high concentration of dissolved oxygen, and extremely high redox potential (19.2 mg/l and 1323 mV, respectively) when available chlorine was at a concentration of about 620 microns. The addition of ethylene to EAW led to ethylene decomposition, and an equimolar amount of ethylene chlorohydrine with available chlorine was produced. The ethylene chlorohydrine production was greatly affected by the pH value (pH 2.5, 5.0 and 10.0 were tested), and was faster in an acidic solution. Ethylene chlorohydrine was not produced after ethylene had been added to EAW at pH 2.6 when available chlorine was absent, but was produced after potassium hypochlorite had been added to such EAW. The effect of the pH value of EAW on the vase life of cut carnations was compatible with the decomposition rate of ethylene in EAW of the same pH value. These results suggest that the effect of Electrolyzed Anode Water on the vase life of cut carnations was due to the decomposition of ethylene to ethylene chlorohydrine by chlorine from chlorine compounds.

PMID: 12784619 [PubMed - indexed for MEDLINE]

Use of Ionized water in hypochlorhydria or achlorhydria

Alkaline Water and Reducing Cholestrol

Prof. Kuninaka Hironage, Head of Kuninaka Hospital

"Too many fats in the diets, which lead to the deposition of cholesterol on the blood vessels, which in turn constrict the blood flow, cause most illnesses such as high blood pressure.
In accordance with the theory of Professor Gato of Kyushu University on Vitamin K *(because vitamin K enables the blood calcium to increase)*, or the consumption of more antioxidant water, the effectiveness of the increase in the calcium in high blood pressure is most significant.
With the consumption of alkaline antioxidant water for a period of 2 to 3 months, I have observed the blood pressure slowly drop, due to the water's solvent ability, which dissolves the cholesterol in the blood vessels.

Use of Ionized water for gynecological conditions

Alkaline Water Use in Illness Recovery

Prof. Watanabe Ifao, Watanabe Hospital

"**Ionized alkaline antioxidant water improves body constituents and ensures effective healing to many illnesses**. The uses of antioxidant water in gynecological patients have proved to be very effective. The main reason for its effectiveness is that this water can neutralize toxins.

When given antioxidant water to pre-eclamptic toxemia cases, the results are most significant. During my long years of servicing the pre-eclamptic toxemia cases, I found that the women with pre-eclamptic toxemia who consumed antioxidant water tend to deliver healthier babies with stronger muscles. A survey report carried out on babies in this group showed intelligence above average."

Toxin Neutralisation

Alkaline Water used to Neutralize Toxins

Prof. Kuwata Keijiroo, Doctor of Medicine

In my opinion, the wonder of antioxidant water is the ability neutralizes toxins, but it is not a medicine. The difference is that the **medicine can only apply to each and individual case, whereas the antioxidant water can be consumed generally** and its neutralizing power is something which is very much unexpected. Now, in brief, let me introduce to you a heart disease case and how it was cured.

The patient was a 35 years old male suffering from vascular heart disease. For 5 years, his sickness deteriorated. He was in the Setagays Government Hospital for treatment.

During those 5 years, he had been in and out of the hospital 5 to 6 times. He had undergone high tech examinations such as angiogram by injecting VINYL via the vein into the heart. He consulted and sought treatment from many good doctors where later he underwent a major surgical operation. Upon his discharge from the hospital, he quit his job to convalesce. However, each time when his illness relapsed, the attack seemed to be even more severe.

Last year, in August, his relatives were in despair and expected he would not live much longer. It so happened at that time that the victim's relative came across an antioxidant alkaline water processor... His illness responded well and he is now on the road to recovery."
In the United States, cardiovascular diseases account for more than one-half of the approximate 2 million deaths occurring each year. **It is estimated that optimal conditioning of drinking water could reduce this cardiovascular disease mortality rate by as much as**

15 percent.
From: Report of the Safe Drinking Water Committee of the National Academy of Sciences, 1977

Eczema

Alkaline Water and Eczema

Prof. Tamura Tatsuji, Keifuku Rehabilitation Center

"Eczema is used to describe several varieties of skin conditions, which have a number of common features.
The exact cause or causes of eczema are not fully understood. In many cases, eczema can be attributed to external irritants.

Let me introduce a patient who recovered from skin disease after consuming the antioxidant water. This patient suffered 10 years of eczema and could not be cured effectively even under specialist treatment. This patient, who is 70 years of age, is the president of a vehicle spare parts company. After the war, his lower limbs suffered acute eczema, which later became chronic. He was repeatedly treated in a specialist skin hospital.

The left limb responded well to treatment, but not so on the right limb. He suffered severe itchiness, which, when scratched led to bleeding. During the last 10 years, he was seen and treated by many doctors. When I first examined him, his lower limb around the joints was covered with vesicles. Weeping occurred owing to serum exuding from the vesicles.

I advised him to try consuming antioxidant water. He bought a unit and consumed the antioxidant water religiously and used the acidic water to bathe the affected areas. After 2 weeks of treatment the vesicles dried up. The eczema completely cleared without any relapse after 1½ month."

Allergies

Alkaline Water for Allergy Treatment

Prof. Kuninaka Hironaga, Head of Kuninaka Hospital

"Mr. Yamada, the head of the Police Research Institute, suffered from severe allergy. He was treated repeatedly by a skin specialist, but with no success. Then he started consuming antioxidant water. The allergy responded very well and was soon completely cured. No relapse had occurred, although he had taken all kinds of food. He was most grateful and excited about this treatment.

As for myself, I had also suffered severe allergy. From the time I began to consume antioxidant water, the allergy has not returned. Since then, I started research on the effectiveness of antioxidant water.

I discovered that **most allergies are due to acidification of body condition** and is also related to consuming too much meat and sugar. In every allergy case, the patient's antioxidant minerals are excessively low which in turn lower the body resistance significantly. The body becomes overly sensitive and develops allergy easily. To stabilize the sensitivity, calcium solution in injected into the vein. Therefore, it is clear that antioxidant water, with ionic calcium, can help alleviate allergy.

The ionic calcium not only enhances the heart, urination, and neutralization of toxins but controls acidity. It also enhances the digestive system and liver function. This will promote natural healing power and hence increase its resistance to allergy. In some special cases of illness, which do not respond to drugs, they are found to respond well to antioxidant water."

Digestive Problems

Alkaline Water and Stomach Disease

Prof. Kogure Keizou, Kogure Clinic of Juntendo Hospital

"The stomach is readily upset both by diseases affecting the stomach and by other general illnesses. In addition, any nervous tension or anxiety frequently causes gastric upset or vague symptoms.

The important role of antioxidant water in our stomach is to neutralize the secretion and strengthen it's functions. Usually, after consuming the antioxidant water for 1 to 3 minutes, the gastric juice increase to 1½ times. For those suffering from hypochlorhydria or achlorhydria (low in gastric juice) the presence of antioxidant water will stimulate the stomach cells to secrete more gastric juice. This in turn enhances digestion and absorption of minerals.

However, on the other hand, those with hyperchlorhydria (high in gastric juice), the antioxidant water neutralizes the excessive gastric

juice. Hence, it does not create any adverse reaction.

According to the medical lecturer from Maeba University, the pH of the gastric secretion will still remain normal when antioxidant water is consumed. This proves that the ability of the antioxidant water is able to neutralize as well as to stimulate the secretion."

Diabetes

Alkaline Water for Diabetes

Prof. Kuwata Keijiroo, Doctor of Medicine

"When I was serving in the Fire Insurance Association, I used to examine many diabetic patients. Besides treating them with drugs, I provided them with antioxidant water. After drinking antioxidant water for one month, 15 diabetic patients were selected and sent to Tokyo University for further test and observations.

Initially, the more serious patients were a bit apprehensive about the treatment. When the antioxidant water was consumed for some time, the sugar in the blood and urine ranged from a ratio of 300 mg/l to 2 mg / dc. There was a time where the patients had undergone 5 to 6 blood tests a day and detected to be within normal range. Results also showed that even 1 ½ hour after meals, the blood sugar and urine ratio was 100 mg/dc: 0 mg/dc . The sugar in the urine had completely disappeared.

NOTE:
More Americans than ever before are suffering from diabetes, with the number of new cases averaging almost 800,000 each year. The disease has steadily increased in the United States since 1980, and in 1998, 16 million Americans were diagnosed with diabetes (10.3 million diagnosed; 5.4 million undiagnosed). Diabetes is the seventh leading cause of death in the United States, and more than 193,000 died from the disease and its related complication in 1996.

The greatest increase, 76 percent, occurred in people age 30 to 30. From: U. S. Department of Health and Human Services, October 13, 2000 Fact Sheet.

Use of Ionized water in treating Acidosis

Alkaline Water and Obesity

Prof. Hatori Tasutaroo, Head of Akajiuiji Blood Centre, Yokohama Hospital, Faitama District

"Due to a higher standard of living, our eating habits have changed. We consume too much proteins, fats and sugar. The excess fats and carbohydrates are in the body as fats. In the present lifestyles, Americans are more extravagant on food compared to the Japanese. Due to this excessive intake obesity is a significant problem. Normally, one out of five males and one out of four females is obese.

The degree of "burn-out" in food intake largely depends on the amount on intake of vitamins and minerals. When excessive intake of proteins, carbohydrates and fats occurs, the requirement for vitamins and minerals increases. However, there is not much research carried out pertaining to the importance of vitamins and minerals.

Nowadays, many people suffer from acidification that leads to diabetes, heart diseases, cancer, liver and kidney diseases. If our food intake can be completely burned off, then there is no deposition of fats. Obviously, there will be no acidification problem and hence there should not be any sign of obesity.

The antioxidant water contains an abundance of ionic calcium. This ionic calcium (and other alkalizing minerals) help in the "burn-off" process. By drinking antioxidant water, it provides sufficient minerals for our body.

Hence, **antioxidant water is a savior for those suffering from obesity and many adult diseases**, providing assistance in enhancing good health."

REDUCED WATER FOR PREVENTION OF DISEASES

Health Benefits of Alkaline Water

Dr.Sanetaka Shirahata
Graduate school of Genetic Resources Technology, Kyushu University,

6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan.

It has long been established that reactive oxygen species (ROS) cause many types of damage to biomolecules and cellular structures, that, in turn result in the development of a variety of pathologic states such as diabetes, cancer and aging.
Reduced water is defined as anti-oxidative water produced by reduction of water. Electrolyzed reduced water (ERW) has been demonstrated to be hydrogen-rich water and can scavenge ROS in

vitro (Shirahata et al., 1997). The reduction of proton in water to active hydrogen (atomic hydrogen, hydrogen radical) that can scavenge ROS is very easily caused by a weak current, compared to oxidation of hydroxyl ion to oxygen molecule. Activation of water by magnetic field, collision, minerals etc. will also produce reduced water containing active hydrogen and/or hydrogen molecule. Several natural waters such as Hita Tenryosui water drawn from deep underground in Hita city in Japan, Nordenau water in Germany and Tlacote water in Mexico are known to alleviate various diseases. We have developed a sensitive method by which we can detect active hydrogen existing in reduced water, and have demonstrated that not only ERW but also natural reduced waters described above contain active hydrogen and scavenge ROS in cultured cells. ROS is known to cause reduction of glucose uptake by inhibiting the insulin-signaling pathway in cultured cells. Reduced water scavenged intracellular ROS and stimulated glucose uptake in the presence or absence of insulin in both rat L6 skeletal muscle cells and mouse 3T3/L1 adipocytes. This insulin-like activity of reduced water was inhibited by wortmannin that is specific inhibitor of PI-3 kinase, a key molecule in insulin signaling pathways. Reduced water protected insulin-responsive cells from sugar toxicity and improved the damaged sugar tolerance of type 2 diabetes model mice, suggesting that reduced water may improve insulin-independent diabetes mellitus.
Cancer cells are generally exposed to high oxidative stress. Reduced water cause impaired tumor phenotypes of human cancer cells, such as reduced growth rate, morphological changes, reduced colony formation ability in soft agar, passage number-dependent telomere shortening, reduced binding abilities of telomere binding proteins and suppressed metastasis.
Reduced water suppressed the growth of cancer cells transplanted into mice, demonstrating their anti-cancer effects in vivo. Reduced water is applicable to not only medicine but also food industries, agriculture, and manufacturing industries.

Shirahata, S. et al.: Electrolyzed reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochem. Biophys. Res. Commun., 234, 269174, 1997.

CLINICAL Impovements Obtained From The Intake Of Reduced Water

Results from Drinking Alkaline Water

Extracts from " Presentation At The Eight Annual International Symposium On man And His Environment in Health And Disease" on February 24th 1990, at The Grand Kempinski Hotel, Dalls, Texas, USA by Dr. H. Hayashi, M.D. and Dr. M Kawamura, M.D., on : -

(THE CONCEPT OF PREHEPATIC MEDICINES)

Since the introduction of alkaline ionic water in our clinic in 1985, we have had the following interesting clinical experiences in the use of this type of water. By the use of alkaline ionic water for drinking and the preparation of meals for our in-patients, we have noticed :-

Declines in blood sugar levels in diabetic patients.

Improvements in peripheral circulation in diabetic gangrene.

Declines in uric acid levels in patients with gout.

Improvements in liver function exams in hepatic disorders.

Improvements in gastroduodenal ulcer and prevention of their recurrences.

Improvements in hypertension and hypotension.

Improvements in allergic disorders such as asthma, urticaria, rhinites and atopic dermatitis.

Improvements in persistent diarrhoea which occurred after gastrectomy.

Quicker improvements in post operative bower paralysis.

Improvements in serum bilirubin levels in new born babies.

By confirming clinical improvements, we have always observed changes of stools of the patients, with the colour of their feaces changing from black-brown colour to a brigher yellow-brown one, and the odour of their feaces becoming almost negligible.

The number of patients complaining of constipation also decreased markedly. The change of stool findings strongly suggests that alkaline ionic water intake can decrease the production of putrefied or pathogenic metabolites.

Devices to produce reduced water were introduced into our clinic in May 1985. Based on the clinical experiences obtained in the past 15 years, it can be said that introduction of electrolyzed-reduced water for drinking and cooking purpose for in-patients should be the very prerequisite in our daily medical practices. Any dietary recipe cannot be a scientific one if property of water is not taken by the patients is not taken into consideration.

The Ministry of Health and Welfare in Japan announced in 1965 that the intake of reduced water is effective for restoration of intestinal flora metabolism.

Clinical evaluation of alkaline ionized water for abdominal complaints: Placebo controlled double blind tests

Alkaline Water and Stomach Complaints

by Hirokazu Tashiro, Tetsuji Hokudo, Hiromi Ono, Yoshihide Fujiyama, Tadao Baba (National Ohkura Hospital, Dept. of Gastroenterology; Institute of Clinical Research, Shiga University of Medical Science, Second Dept. of Internal Medicine)

Effect of alkaline ionized water on abdominal complaints was evaluated by placebo controlled double blind tests. Overall scores of improvement using alkaline ionized water marked higher than those of placebo controlled group, and its effect proved to be significantly higher especially in slight symptoms of chronic diarrhoea and abdominal complaints in cases of general malaise. Alkaline ionized water group did not get interrupted in the course of the test, nor did it show serious side effects nor abnormal test data. It was confirmed that alkaline ionized water is safer and more effective than placebos.

Summary

Effect of alkaline ionized water on abdominal complaints was clinically examined by double blind tests using clean water as placebo. Overall improvement rate was higher for alkaline ionized water group than placebo group and the former proved to be significantly more effective than the other especially in cases of slight symptoms. Examining improvement rate for each case of chronic diarrhoea, constipation and abdominal complaints, alkaline ionized water group turned out to be more effective than placebo group for chronic diarrhoea, and abdominal complaints. The test was stopped in one case of chronic diarrhoea, among placebo group due to exacerbation, whereas alkaline ionized water group did not stop testing without serious side effects or abnormal test data in all cases.
It was confirmed that alkaline ionized water is more effective than clean water against chronic diarrhoea, abdominal complaints and overall improvement rate (relief of abdominal complaints) and safer than clean water.

Introduction

Since the approval of alkaline ionized water electrolyzers by Pharmaceutical Affairs Law in 1966 for its antacid effect and efficacy against gastrointestinal disorders including hyperchylia, indigestion, abnormal gastrointestinal fermentation and chronic diarrhoea, they have been extensively used among patients. However, medical and scientific evaluation of their validity is not established. In our study, we examined clinical effect of alkaline ionized water on gastrointestinal disorders across many symptoms in various facilities. Particularly, we

studied safety and usefulness of alkaline ionized water by doubleblind tests using clean water as a control group.

Test subjects and methods

163 patients (34 men, 129 women, age 21 to 72, average 38.6 years old) of indigestion, abnormal gastrointestinal fermentation (with abnormal gas emission and rugitus) and abdominal complaints caused by irregular dejection (chronic diarrhoea, or constipation) were tested as subjects with good informed consent. Placebo controlled double blind tests were conducted using alkaline ionized water and clean water at multiple facilities. An alkaline ionized water electrolyzer sold commercially was installed with a pump driven calcium dispenser in each of the subject homes. Tested alkaline ionized water had pH at 9.5 and calcium concentration at 30ppm. Each subject in placebo group used a water purifier that has the same appearance as the electrolyzer and produces clean water.

The tested equipment was randomly assigned by a controller who scaled off the key code which was stored safely until the tests were completed and the seal was opened again.

Water samples were given to each patient in the amount of 200ml in the morning with the total of 50OmI or more per day for a month. Before and after the tests, blood, urine and stool were tested and a log was kept on the subjective symptoms, bowel movements and accessory symptoms. After the tests, the results were analyzed based on the log and the test data.

Test Results

1. Symptom

Among 163 tested subjects, alkaline ionized water group included 84 and placebo group 79. Background factors such as gender, age and basal disorders did not contribute to significant difference in the results.

2. Overall improvement rate

As to overall improvement rate of abdominal complaints, alkaline ionized water group had 2 cases of outstanding improvement (2.5%), 26 cases of fair improvement (32.1%), 36 cases of slight improvement (44.4%), 13 cases of no change (16%) and 4 cases of exacerbation (4.9%), whereas placebo group exhibited 4 (5.2%), 19 (24.7%), 27 (35.1%), 25 (32.5%) and 2 cases (2.6%) for the same category. Comparison between alkaline ionized water and placebo groups did not reveal any significant difference at the level of 5% significance according to the Wilcoxon test, although alkaline ionized water group turned out to be significantly more effective than placebo group at the level of p value of 0.22.

Examining overall improvement rates by a 7, 2 test (with no adjustment for continuity) between the effective and noneffective groups, alkaline ionized water group had 64 (79%) of effective cases and 17 cases (21%) of non effective cases, whereas placebo group had 50 (64.9%) and 27 (35.1%) cases respectively. The result indicated that alkaline ionized water group was significantly more effective than placebo group at the level of p value of 0.0.48.

Looking only at 83 slight cases of abdominal complaints, overall improvement rate for alkaline ionized water group

(45 cases) was composed of 11 cases (242%) of fair improvement, 22 cases (48.9%) of slight improvement, 17 cases (44.7%) of no change and 3 cases (6.7%) of exacerbation, whereas placebo group (38 cases) had 3 (7.8%), 17 (44.7%), 17 (44.7%) and 1 (2.6%) cases for the same category. Alkaline ionized water group was significantly more effective than placebo group according to the comparison between the groups (p value = 0.033).

3. Improvement rate by basal symptom

Basal symptoms were divided into chronic diarrhea, constipation and abdominal complaints (dyspepsia) and overall improvement rate was evaluated for each of them to study effect of alkaline ionized water. In case of chronic diarrhoea, alkaline ionized water group resulted in 94.1% of effective cases and 5.9% of non effective cases. Placebo group came up with 64.7% effective and 35.3% non effective. These results indicate alkaline ionized water group proved to be significantly more effective than placebo group. In case of slighter chronic diarrhoea, comparison between groups revealed that alkaline ionized water group is significantly more effective than placebo group (p=0.015). In case of constipation, alkaline ionized water group consisted of 80.5% of effective and 19.5% of non effective cases, whereas placebo group resulted in 73.3% effective and 26.3 non effective. As to abdominal complaints (dyspepsia), alkaline ionized water group had 85.7% of effective and 14.3% non effective cases while placebo group showed 47.1% and 62.9% respectively. Alkaline ionized water group proved to be significantly more effective than placebo group (p=0.025).

4. Safety

Since one case of chronic diarrhoea, in placebo group saw exacerbation, the test was stopped. There was no such cases in alkaline ionized water group. Fourteen cases of accessory symptoms, 8 in alkaline ionized water group and 6 in placebo group, were observed, none of which were serious. 31 out of 163 cases (16 in alkaline ionized water group, 15 in placebo group) exhibited fluctuation in test data, although alkaline ionized water group did not have any problematic fluctuations compared to placebo group. Two cases in placebo group and one case in alkaline ionized water group have seen K value of

serum climb up and resume to normal value after retesting which indicates the value changes were temporary.

Conclusion

As a result of double blind clinical tests of alkaline ionized water and clean water, alkaline ionized water was proved to be more effective than clean water against chronic diarrhoea, abdominal complaints (dyspepsia) and overall improvement rate (relief from abdominal complaints). Also, the safety of alkaline ionized water was confirmed which clinically verifies its usefulness.

Physiological effects of alkaline ionized water:
Effects on metabolites produced by intestinal fermentation

Alkaline Water and Intestinal Fermentation

by Takashi Hayakawa, Chicko Tushiya, Hisanori Onoda, Hisayo Ohkouchi, Harul-~to Tsuge
(Gifu University, Faculty of Engineering, Dept. of Food Science)

We have found that long-term ingestion of alkaline ionized water (AIW) reduces cecal fermentation in rats that were given highly fermentable commercial diet (MF: Oriental Yeast Co., Ltd.).
In this experiment, rats were fed MF and test water (tap water, AIW with pH at 9 and 10) for about 3 months. Feces were collected on the 57th day, and the rats were dissected on the 88th day. The amount of ammonium in fresh feces and cecal contents as well as fecal free-glucose tended to drop down for the AIW group. In most cases, the amount of free-amino acids in cecal contents did not differ sign-icantly except for cysteine (decreased in AIW with pH at 10) and isoleucine (increased in AIW with pH at 10).

Purpose of tests

Alkaline ionized water electrolyzers were approved for manufacturing in 1965 by the Ministry of Health and Welfare as medical equipment to produce medical substances. Alkaline ionized water (AIW) produced by this equipment is known to be effective against gastrointestinal fermentation, chronic diarrhea, indigestion and hyperchylia as well as for controlling gastric acid.
*1 This is mainly based on efficacy of the official calcium hydroxide.
*2 By giving AIW to rats for a comparatively long time under the condition of extremely high level of intestinal fermentation, we have demonstrated that AIW intake is effective for inhibition of intestinal fermentation when its level is high based on some test results where AIW worked against cecal hypertrophy and for reduction in the amount of short-chain fatty acid that is the main product of fermentation.
*3 We have reported that this is caused by the synergy between

calcium level generally contained in AIW (about 50ppm) and the value of pH, and that frequency of detecting some anaerobic bacteria tends to be higher in alkaline ionized water groups than the other, although the bacteria count in the intestine does not have significant difference. Based on these results, we made a judgment that effect of taking AIW supports part of inhibition mechanism against abnormal intestinal fermentation, which is one of the claims of efficacy that have been attributed to alkaline ionized water electrolyzers.
*4 On the other hand, under the dietary condition of low intestinal fermentation, AIW uptake does not seem to inhibit fermentation that leads us to believe that effect of AIW uptake is characteristic of hyper-fermentation state. Metabolites produced by intestinal fermentation include indole and skatole in addition to organic acids such as short-chain fatty acid and lactic acid as well as toxic metabolites such as ammonium, phenol and pcresol. We do not know how AIW uptake would affect the production of these materials. In this experiment, we have tested on ammonium production as explained in the following sections.

Testing methods

Four-week-old male Wistar/ST Clean rats were purchased from Japan SLC Co., Ltd. and were divided into 3 groups of 8 each after preliminary breeding. AIW of pH 9 and 10 was produced by an electrolyzer Mineone ROYAL NDX3 1 OH by Omco Co., Ltd. This model produces AIW by electrolyzing water with calcium lactate added. On the last day of testing, the rats were dissected under Nembutal anesthesia to take blood from the heart by a heparin-treated syringe. As to their organs, the small intestines, cecum and colon plus rectum were taken out from each of them. The cecurn was weighed and cleaned with physiological saline after its contents were removed, and the tissue weight was measured after wiping out moisture. Part of cecal contents was measured its pH, and the rest was used to assay ammonium concentration. The amount of ammonium contained in fresh feces and cecal contents was measured by the Nessler method after collecting it in the extracted samples using Conway's micro-diffusion container. Fecal free-glucose was assayed by the oxygen method after extraction by hot water. Analysis of free amino acids contained in cecal contents was conducted by the Waters PicoTag amino acid analysis system.

Test results and analyses

No difference was found in the rats' weight gain, water and feed intake and feeding efficiency, nor was any particular distinction in appearance identified. The length of the small intestines and colon plus rectum tended to decline in AIW groups. PH value of cecal contents was higher and the amount of fecal free-glucose tended to be lower in AIW groups than the control group. Since there was no difference in fecal discharge itself, the amount of free-glucose discharged per day was at a low level. The amount of discharged free-glucose in feces is greater when intestinal fermentation is more intensive, which indicates that

intestinal fermentation is more inhibited in AIW groups than the control group. Ammonium concentration in cecal contents tends to drop down in AIW groups (Fig. 1). This trend was most distinctive in case of fresh feces of one of AIW groups with pH 10 (Fig.2) AIW uptake was found to be inhibitory against ammonium production. In order to study dynamics of amino acids in large intestines, we examined free amino acids in the cecal contents to find out that cysteine level is low in AIW groups whereas isoleucine level is high in one of AIW groups with pH 10, although no significant difference was identified for other amino acids.

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Effects of alkaline ionized water on formation & maintenance of osseous tissues

Alkaline Water for Bone Health

by Rei Takahashi Zhenhua Zhang Yoshinori Itokawa
(Kyoto University Graduate School of Medicine, Dept. of Pathology and Tumor Biology, Fukui Prefectural University)

Effects of calcium alkaline ionized water on formation and maintenance of osseous tissues in rats were examined. In the absence of calcium in the diet, no apparent calcification was observed with only osteoid formation being prominent. Striking differences were found among groups that were given diets with 30% and 60% calcium. Rats raised by calcium ionized water showed the least osteogenetic disturbance. Tibiae and humeri are more susceptible to calcium deficiency than femora. Theses results may indicate that calcium in drinking water effectively supplements osteogenesis in case of dietary calcium deficiency. The mechanism involved in osteoid formation such as absorption rate of calcium from the intestine and effects of calcium alkaline ionized drinking water on maintaining bone structure in the process of aging or under the condition of calcium deficiency is investigated.

Osteoporosis that has lately drawn public attention is defined as "conditions of bone brittleness caused by reduction in the amount of bone frames and deterioration of osseous microstructure. " Abnormal calcium metabolism has been considered to be one of the factors to contribute to this problem, which in turn is caused by insufficient calcium take in, reduction in enteral absorption rate of calcium and increase in the amount of calcium in urinal discharge. Under normal conditions, bones absorb old bones by regular metabolism through osteoid formation to maintain their strength and function as supporting structure. It is getting clear that remodeling of bones at the tissue level goes through the process of activation, resorption, reversal, matrix synthesis and mineralization. Another important function of bones is storing minerals especially by coordinating with intestines and kidneys to control calcium concentration in the blood. When something happens to this osteo metabolism, it results in abnormal morphological changes. Our analyses have been focusing mostly on the changes in the amount of bones to examine effects of calcium alkaline ionized water on the reaction system of osteo metabolism and its efficiency. Ibis time, however, we studied it further from the standpoint of histology. In other words, we conducted comparative studies on morphological and kinetic changes of osteogenesis by testing alkaline ionized water, tap water and solution of lactate on rats.

Three week old male Wistar rats were divided into 12 groups by conditions of feed and drinking water. Feeds were prepared with 0%, 30%, 60% and 100% of normal amount of calcium and were given freely. Three types of drinking water, tap water (city water, about 6ppm of Ca), calcium lactate solution (Ca=40ppm) and alkaline ionized water (Ca =40ppm, pH=9, produced by an electrolyzer NDX 4 LMC by Omco OMC Co., Ltd.) were also given keely. Rats' weight, amount of drinking water and feed as well as the content of Ca in drinking water were assayed every day. On the 19th and 25th days of testing, tetracycline hydrochloride was added to the feed for 48 hours so as to bring its concentration to 30mg/kg. On the 30th day, blood samples were taken under Nembutal anesthesia, and tibiae, humeri and femora were taken out to make non decalcified samples. Their conditions of osteoid formation and rotation were observed using Villanueva bone stain and Villanueva goldner stain.

Three groups that were given different types of drinking water and the same amount of Ca in the feed were compared to find out no significant difference in the rate of weight gain and intakes of feed and drinking water. Alkaline ionized water group had significantly greater amount of tibiae and humeri with higher concentration of calcium in the bones.

The group of 0% calcium in the feed saw drastic increase in the amount of osteoid. There was not much difference by types of drinking water. Almost no tetracycline was taken into tibiae and humeri, although a small amount was identified in ferora. As a result, osteogenesis went as far as osteoid formation, but it was likely that

decalcification has not happened yet, or most of newly formed bones were absorbed.

As to the groups of 30% and 60% calcium in the feed, increase in the area of tetracycline take in was more identifiable with higher clarity in descending order of alkaline ionized water, calcium lactate solution and tap water groups. Especially in case of tap water group, irregularity among the areas of tetracycline take in was distinctive. The group of 100% calcium in the feed saw some improvements in osteogenesis in descending order of alkaline ionized water, calcium lactate solution and tap water. In any case, bone formation seemed to be in good condition at near normal level.

Alkaline ionized water was regarded to be effective for improvements of osteogenesis under the conditions of insufficient calcium in the feed. Also, the extent of dysosteogenesis differed by the region. That is, tibiae and humeri tend to have more significant dysosteogenesis than femora.

In addition, there is a possibility that osteo metabolism varies depending on enteral absorption rate of calcium, adjustment of discharge from kidneys and functional adjustment of accessory thyroid in the presence of alkaline ionized water. We are now studying its impact on calcium concentration in the blood. We are also examining whether it is possible to deter bone deterioration by testing on fast aging mouse models.