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Advanced research on the health benefit of reduced water☆

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In Japan, research on functional water, especially on reduced water, is developing at a rapid pace. Reduced water such as electrochemically reduced water and natural reduced water can scavenge reactive oxygen species in cultured cells. Reduced waters are expected to have preventive and positive effects on oxidative stress-related diseases such as diabetes, cancer, arteriosclerosis, neurodegenerative diseases, and side effects of hemodialysis. It has been suggested that the active agents in reduced water are hydrogen (atoms and molecules), mineral nanoparticles, and mineral nanoparticle hydrides.

Introduction: electrochemically reduced water is beneficial for health

In the field of food science and technology, water is an important ingredient influencing taste, rheology and preservation of foods. Research on functional foods is currently popular; however, it is not yet well known that drinking water also has physiological functions, and that there are some health-beneficial effects of water (Shirahata, 2002, 2004). In the past decade, the decrease in the quality of tap water because of pollution of the global environment over time has become a major social problem. Air pollution affects water in soils, rivers, and farm products by acid rain. Chemicals in polluted water are considered to generate oxidative stress in the placenta of pregnant women, and this can cause various types of diseases in newborns (Obolenskaya *et al*., 2010).

The human body is approximately 60–80% water. The function of water in the body is mainly classified as follows. (1) The water molecule itself: flowing water affects cellular function and both development and functions of organs (Hirokawa, Tanaka, Okada, & Takeda, 2006; Hove *et al*., 2003), and hydration and Brownian movement of water are fundamentally important for protein function (Iwaki, Iwane, Shimokawa, Cooke, & Yanagida, 2009); (2) atoms and molecules derived from water molecules, such as protons (H^+), hydrogen atoms (active hydrogen [H]), hydrogen anions (H^-), hydrogen molecules (H_2), oxygen molecules (O_2), and reactive oxygen species (ROS); and (3) molecules dissolved in water, such as mineral ions, mineral nanoparticles, organic and inorganic compounds, and gases.

Functional water is activated water exhibiting specific functions. There are many activation methods such as electrolysis, treatment with a magnetic field, light irradiation, ultrasonication, bubbling with gases, strong water flow and collision, and treatment with some types of minerals or rocks. Functional water is defined by The Functional Water Association of Japan as water in which both treatment and function have been scientifically demonstrated or reproducible useful functions have been demonstrated among artificially treated waters. Among functional waters, electrolyzed water has been mostly investigated. Electrochemically reduced water (ERW) is produced near a cathode and electrochemically oxidized water (EOW) is produced near an anode. Potable ERW is a health-beneficial water as discussed here. EOW is also termed electrolyzed acidic water and is functional water exhibiting a sterilizing action, mainly due to hypochlorous acid, chlorine gas, and ozone (Bari, Sabina, Isobe, Uemura, & Isshiki, 2003) (Fig. 1A).

Potable ERW (pH 8–10) is popular as a health-beneficial water in Japan. ERW is also termed alkaline electrolyzed water, alkali-ionic water, alkaline cathodic water, and alkaline ionized water, based on its physicochemical and physiological aspects. ERW exhibits an alkaline pH, is hydrogen molecule-rich, and has a negative oxidation–reduction potential (ORP) and reactive oxygen species (ROS)-scavenging activity (Shirahata *et al*., 2007). Studies on the functions of ERW were initiated in Japan in 1931, and its application to agriculture was first

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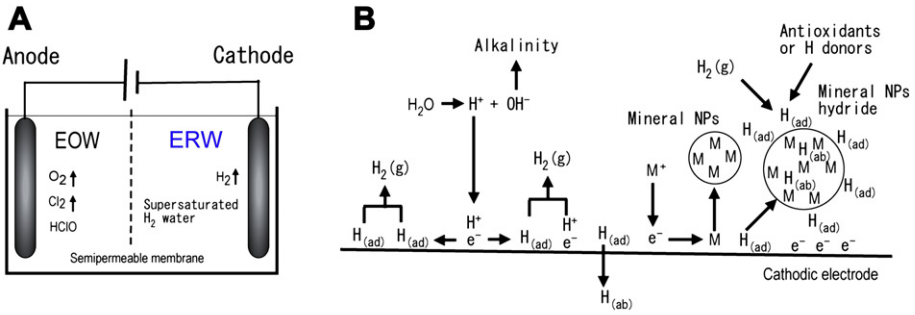

C

	Control >10000	ERW	ERW >10000	ERW <10000
[Pt] (ppb)	N.D.	0.19	12.04	2.16

N.D., not detectable

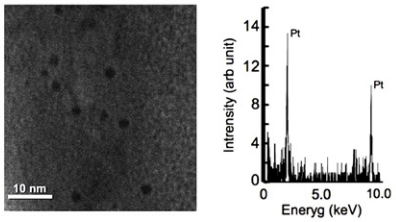

Fig. 1. Electrochemical preparation of reduced water, mineral nanoparticles and mineral hydrides. A. Principle of preparation of electrolyzed water. Electrochemically reduced water nearby a cathode is hydrogen molecule-rich water. Electrolyzed oxidized water nearby an anode contains oxygen gas, chloride gas, and hypochlorous acid if the original water contains Cl^- ions. B. Chemical reactions on the surface of a platinum electrode. Proton dissociated from water is reduced to adsorbed H atoms (H_{ad}) on the surface of the platinum plate. H_{ad} is changed to H_2. H_{ad} is absorbed into Pt metal to produce absorbed H (H_{ab}). Mineral ions in the original water are reduced to metal atoms and then self-organized to mineral nanoparticles. Mineral nanoparticles protected by organic protectors are stable and dispersed in water for a long time. Mineral nanoparticles adsorb or absorb H atoms in the presence of H_2- or H-donors such as organic antioxidants. C. ERW contains Pt nanoparticles. ERW was prepared from 2 mM NaOH solution by electrolysis at a direct current of 100 V for 1 h, as reported by Ye *et al.* (2008). The ERW was ultra-filtrated with a cut size of 10,000. The Pt content of fractions of more than 10,000 Da and less than 10,000 Da was determined with ICP-MS. The control is 2 mM NaOH solution before electrolysis. D. The left panel shows a photograph (×5000) of the fraction of ERW (>10,000 Da) determined by a transmission electron microscope. The right panel shows energy depressive X-ray spectrometer analysis of the left panel, indicating that the nanoparticles are composed of Pt.

attempted in 1954. In 1960, it was applied to medical care as a health-beneficial water, and in 1966, the Ministry of Health, Labour and Welfare of Japan admitted that ERW was effective for chronic diarrhea, indigestion, abnormal gastrointestinal fermentation, antacid, and hyperacidity, and it authorized an ERW-producing device for home-use (see the homepage of the Association of Alkaline Ionized Water Apparatus: http://www.3aaa.gr.jp/english/alkali/kl.html). In 1994, to mainly promote electrolyzed water use in society, the Functional Water Foundation was established with the support of the Ministry of Health, Labour and Welfare of Japan. Hayakawa (1999) reported that rats administrated alkali-ionic water for 8 weeks exhibited a significantly lower amount of total short chain fatty acids in the appendix than that in control rats; however, alkali-ionic water did not affect the flora of intestinal bacteria. Rats administrated ERW of pH 10 had a more negative ORP in the intestine than that in control rats. A double-blind placebo-controlled study on the effects of alkali-ionized water was performed using subjects who had abdominal symptoms such as pyrosis, dysphoria, abdominal distension, chronic diarrhea, and constipation from January 1996 to January 1999. The placebo control water was purified water obtained from tap water using an activated charcoal filter, which was then electrolyzed to obtain alkali-ionized water. The number of patients was 84 in the alkali-ionized water group and 79 in the purified water group. The patients drank at least 0.5 L of alkali-ionized water of pH 9.5 or purified water per day for 2 weeks. The results showed that alkali-ionized water significantly improved the abdominal complaints. In particular, chronic diarrhea patients who drank alkali-ionized water showed a significantly higher improvement efficacy of 94.1% compared with those who drank purified water (64.7%) (Tashiro, Kitahora, Fujiyama, & Banba, 2000). When the Drugs, Cosmetics and Medical Instruments Act of Japan was revised in 2005, a device for preparation of ERW was re-authorized based on considerable scientific evidence as a home managed medical device. The purpose of use was recognized to generate potable alkaline electrolyzed water for the improvement of gastrointestinal symptoms. The Japanese Society for Functional Water was established in 2001, and active studies on various functional waters including ERW have been performed to date.

Mechanism of action of reduced water containing hydrogen and mineral nanoparticles as a newly recognized ROS scavenger

Clinical data suggested that ERW improved oxygen stress-related diseases (Hayashi & Kawamura, 2002). The authors reported that ERW scavenged ROS and inhibited ROS-induced DNA damage *in vitro* (Shirahata *et al.*,

1997). Electrolysis of water produces a strong reducing circumstance in the vicinity of a cathode, because most voltage is applied in the very narrow water layer nearby the cathode, forming very high electric field. Platinum-coated titanium electrodes are often used for electrolysis of water in the commercial ERW-producing apparatus. On the cathodic platinum plate, hydrogen atoms (active hydrogen) and hydrogen molecules are generated. Mineral nanoparticles and mineral nanoparticle hydrides are also formed as shown in Fig. 1B. Actually we found that ERW prepared from NaOH solution contained a small amount of Pt nanoparticles (Fig. 2C and D). Synthesized Pt nanoparticles scavenged $O_2^{\cdot -}$, $^{\cdot}OH$, and H_2O_2 (Hamasaki *et al.*, 2008; Kajita *et al.*, 2007) (Fig. 2). Synthesized Pt nanoparticles also activated hydrogen molecules to hydrogen atoms by their catalysis action. Natural reduced waters (NRWs) such as Hita Tenryosui water in Japan and Nordenau water in Germany also exhibited ROS-scavenging activities (Li *et al.*, 2002). We propose an active hydrogen mineral nanoparticles hypothesis of reduced water to explain the mechanism of action of both ERW and NRW (Fig. 3). See supplementary information for detailed discussion.

Anti-diabetic effect of reduced water

According to a national health and nutrition survey in 2007, 22.1 million people, amounting for one sixth of the entire population, are patients with diabetes or people with suspected diabetes in Japan. Diabetes mellitus is mainly classified into two types: type 1 insulin dependent diabetes mellitus and type 2 non-insulin dependent diabetes mellitus. Type 1 diabetes mellitus is caused by insulin deficiency due to the oxidative damage of pancreatic β cells attacked by immune cells. Type 2 diabetes mellitus is also strongly associated with the oxidative damage of myotube and adipocyte cells due to stress, hyperphagia, and lack of exercise. ERW, Hita Tenryosui water and Nordenau water have been shown to scavenge intracellular ROS in a hamster pancreatic β cell line HIT-T15 cells, and remarkably accelerate the secretion of insulin. The oxidative damage induced by alloxan, a type 1 diabetes inducer, is suppressed by ERW and NRW in cells and in alloxan-induced type 1 diabetes model mice (Li *et al.*, 2002, 2005, 2010, 2011).

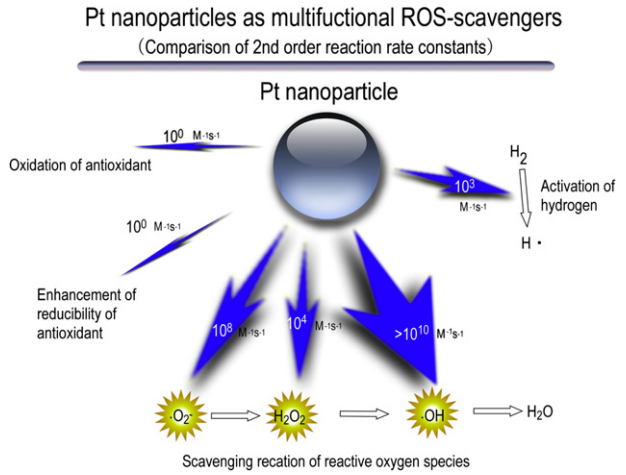

Fig. 2. Multi-functional ROS-scavenging activity of Pt nanoparticles. The 2nd order reaction rates of synthesized Pt nanoparticles of 2–3 nm were determined as reported by Hamasaki *et al.* (2008). Pt nanoparticles exhibit superoxide anion radical-scavenging activity as well as SOD enzyme activity. They also exhibit catalase-like activity. The hydroxyl radical-scavenging activity of Pt nanoparticles is as strong as that of ascorbic acid, one of the strongest scavengers. Pt nanoparticles activate hydrogen molecules to active hydrogen and stimulate the reducibility of antioxidants. The autoxidation activity of Pt nanoparticles on antioxidants is weak. The numbers show *Ks* values of second reaction rate constants.

ERW, Hita Tenryosui water and Nordenau water scavenge ROS in rat L6 myotube cells and enhance sugar uptake (Oda *et al.*, 1999). Nordenau water and Hita Tenryosui water promote the phosphorylation of the insulin receptor *via* suppression of the activity of tyrosine protein phosphatase, which is a redox-sensitive protein, and activate PI3 kinase and Akt, as well as promote the translocation of the sugar transport carrier GLUT4 to the cell membrane to promote sugar uptake (Shirahata *et al.*, 2001, 2007). These waters also alleviate sugar tolerance damage in type 2 diabetes model mice (Gadek & Shirahata, 2002; Osada *et al.*, 2010). ERW derived from tap water improves the symptoms of diabetes model mice (Jin *et al.*, 2006; Kim, Jung, Uhm, Leem, & Kim, 2007; Kim & Kim, 2006).

It has been reported that in 45% of 411 type 2 diabetes patients (mean age, 71.5 years) who drank 2 L of Nordenau water per day, blood glucose and HbA1c levels were significantly decreased after drinking this water for 6 days. These levels were further decreased after long term drinking. Additionally, blood cholesterol, low density lipoprotein (LDL), and creatinine levels were significantly decreased and high density lipoprotein levels were significantly increased. Drinking this water for a longer period resulted in an increase in the percentage of patients who improved (Gadek, Hamasaki, & Shirahata, 2009; Gadek, Li, & Shirahata, 2006). In an open clinical test performed at the First Central Hospital in Jilin Cangchun City in China, 65 patients with diabetes and 50 patients with hyperlipidemia drank 2 L of Hita Tenryosui water per day for 2 months. This resulted in a significant decrease in blood sugar levels in 89% of patients with diabetes. Additionally, blood triglyceride and total cholesterol levels in 92% of patients with hyperlipidemia were significantly decreased (Osada *et al.*, 2010). Furthermore, a double-blind random clinical trial for 29 patients with type 2 diabetes was performed at the Fukuoka Tokusyukai Hospital, Fukuoka city, Japan. Urinary 8-OH dG (an internal oxidation marker) levels of patients who drank 1 L of Hita Tenryosui water per day for 6 months were significantly decreased (Matsubayashi, Hisamoto, Murao, & Hara, 2008). In addition, in a double-blind clinical trial with 100 subjects performed at Hiroshima University from November 2008 to September

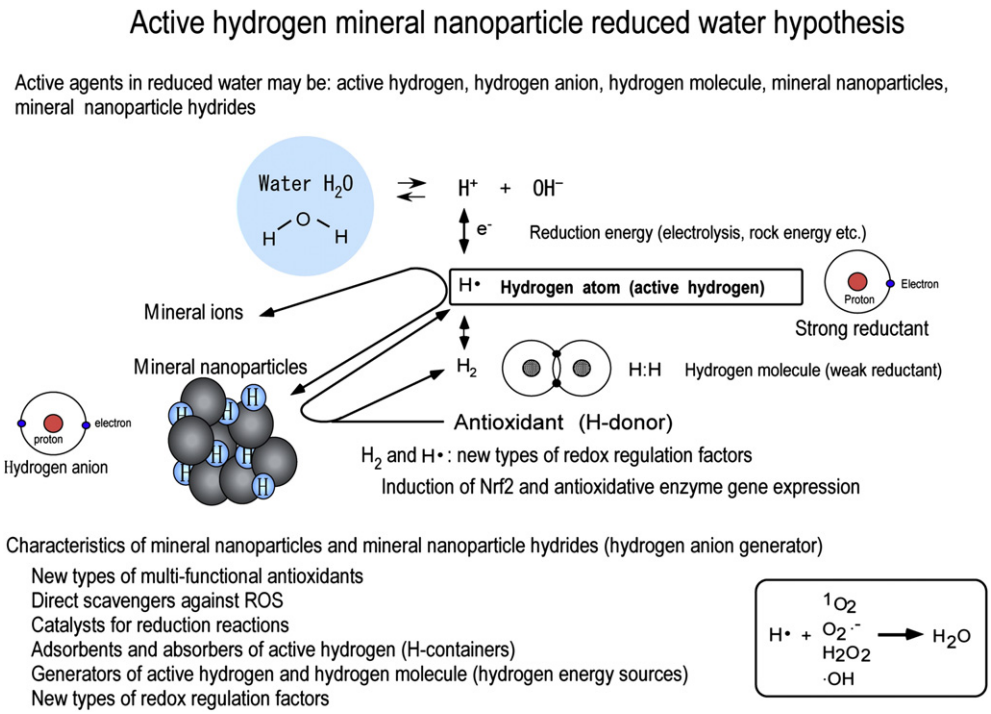

Fig. 3. Active hydrogen mineral nanoparticle reduced water hypothesis. As a common mechanism in both ERW and NRW, water is reduced by electric energy, rock energy and other energy to produce active hydrogen (H atom) and mineral nanoparticles. H atoms produce hydrogen molecules, which are weak reductants, but can function as H-donors. Mineral nanoparticles sustain reduction energy, because they gradually dissociate to mineral ions, releasing electrons. Mineral nanoparticles directly scavenge $O_2^{\cdot-}$, $^{\cdot}OH$ and H_2O_2 by catalysis mechanisms. Mineral nanoparticles stimulate H atom release from many organic substances such as antioxidants and methanol to enhance reducibility. Mineral nanoparticle hydrides can release hydrogen anions, which can function as a reductant.

2009, when 2 L of Hita Tenryosui water was drunk a day, it was found to have anti-metabolic syndrome effects such as a significant decrease in starved blood sugar levels, blood pressure, total cholesterol, LDL cholesterol, GOT, γ-GTP, and triglyceride levels, arteriosclerosis index, and uric acid levels, and a significant increase in leptin levels, as well as improvement of constipation (Higashikawa, Kuriya, Noda, & Sugiyama, 2009). In the clinical trial, it was suggested that drinking 2 L of natural mineral water per day itself was beneficial for health.

Other physiological functions of reduced water

Elongation effect on the lifespan of nematodes

A recent theory on aging suggests that there are adequate ROS levels in living organisms to elongate lifespan, and both insufficient and excess ROS levels shorten the lifespan (Brewer, 2009). We have reported that ERW significantly extends the lifespan of nematodes (*Caenorhabditis elegans*) by scavenging ROS in nematodes (Yan *et al.*, 2010). The active agent responsible for the lifespan extension in ERW is suggested to be Pt nanoparticles of ppb levels, but not hydrogen molecules (Yan *et al.*, 2011). It has also been reported that Pt nanoparticles of an optimum concentration extend the lifespan of nematodes by scavenging ROS (Kim *et al.*, 2008; Kim, Shirasawa, & Miyamoto, 2010).

Anti-cancer effects

ERW causes telomere shortening in cancer cells (Shirahata *et al.*, 1999). It suppresses tumor angiogenesis by scavenging intracellular ROS and suppressing the gene expression and secretion of vascular endothelial growth factor (Ye *et al.*, 2008). ERW suppresses the growth of cancer cells and microorganisms (Hamasaki *et al.*, 2005; Komatsu *et al.*, 2001) and induces apoptosis together with glutathione in human leukemia HL60 cells (Tsai, Hsu, Chen, Ho, & Lu, 2009). ERW induces differentiation of K562 cells to megakaryocytes (Komatsu *et al.*, 2003), and when supplemented with Pt nanoparticles, it strongly suppresses the two step transformation of NIH3T3 cells by a carcinogen (Nishikawa *et al.*, 2005).

Anti-arteriosclerosis effects

ERW suppresses the Cu^{2+}-catalyzed oxidation of human LDL and suppresses triglyceride levels in mice fed high fat foods (Abe *et al.*, 2010). Hydrogen-supplemented water also suppresses arteriosclerosis (see the supplemental information).

Anti-neurodegenerative effects

ERW suppresses neural cell death by oxidative stress (Kashiwagi *et al.*, 2005). Hydrogen-supplemented water also exhibits various anti-neurodegenerative disease effects (see the supplemental information).

Application of ERW to electrolyzed water hemodialysis

Recently, the application of ERW to hemodialysis has been intensively investigated to establish a new dialysis method using ERW (Huang, Yang, Lee, & Chien, 2003; Huang *et al*., 2006, 2010; Nakayama *et al*., 2007, 2009, 2010; Zhu *et al*., 2011).

Suppressive effect of the side effects of anti-cancer drugs

Hydrogen-supplemented water suppresses the side effects of anti-cancer drugs (see the supplemental information).

Aquaporin penetration of water

Kitagawa, Liu, and Ding (2011) recently reported that extractable organic solvents and freeze-labile special components in Hita Tenryosui water promote aquaporin activity in penetration of water into cells. Such components moving via aquaporin are suggested to activate cellular immune responses, which allow prevention and/or treatment of some chronic diseases.

Other effects

Naito *et al*. (2002) reported that chronic administration with electrolyzed alkaline water inhibits aspirin-induced gastric mucosal injury in rats. Hydrogen-enriched electrolyzed water has been demonstrated to be safe in mutagenicity, genotoxicity and subchronic oral toxicity (Saitoh, Harata, Mizuhashi, Nakajima, & Miwa, 2010). ERW exhibits hepatoprotective effects against CCl_4-induced liver damage in mice (Tsai *et al*., 2009). Ionized alkaline water improves the symptoms of metabolic acidosis in experimental animals (Abo-Enein, Gheith, Barakat, Nour, & Sharaf, 2009). ERW also exhibits an anti-hangover effect (Park *et al*., 2009). Silica hydride found in Funza water in Pakistan suppressed carbon tetrachloride-induced hepatotoxicity in mice (Tsu *et al*., 2010).

Recently, many papers have been published on the suppressive effects of hydrogen molecules contained in ERW on oxidative stress-related diseases (see the supplementary information). The major functions of reduced water are summarized in Fig. 4.

Conclusions and perspective

Accumulating evidence has shown that reduced waters are health beneficial and they suppress oxidative stress-related diseases such as diabetes, cancer, arteriosclerosis, neurodegenerative diseases, and the side effects of hemodialysis. The mechanisms of action of reduced water for scavenging ROS are considered to be complicated. ERW contains hydrogen molecules and mineral nanoparticles. Hydrogen molecules and active hydrogen may be new redox regulation factors that can induce the gene expression of antioxidative enzymes. Hydrogen molecules may be converted to active hydrogen by catalyst action of metal nanoparticles to exhibit more potent reducibility. Mineral nanoparticles themselves are new types of multifunctional antioxidants. Mineral hydride nanoparticles, which are H-donors as well as organic antioxidants like ascorbic acid, are also candidates of active agents in reduced waters. NRW may have one or some of the active agents described above. Further investigation on activation methods of water by electricity, magnetic fields or light are likely to contribute to the development of energy-rich waters, which will be beneficial for human health. Reduced water may suppress harmful effects of environmental pollution on the embryo in pregnant women by purifying amniotic fluid and blood. Reduced water might also contribute to the food industry by improving the taste, texture and preservation of foods. In industries, the usage of ERW is

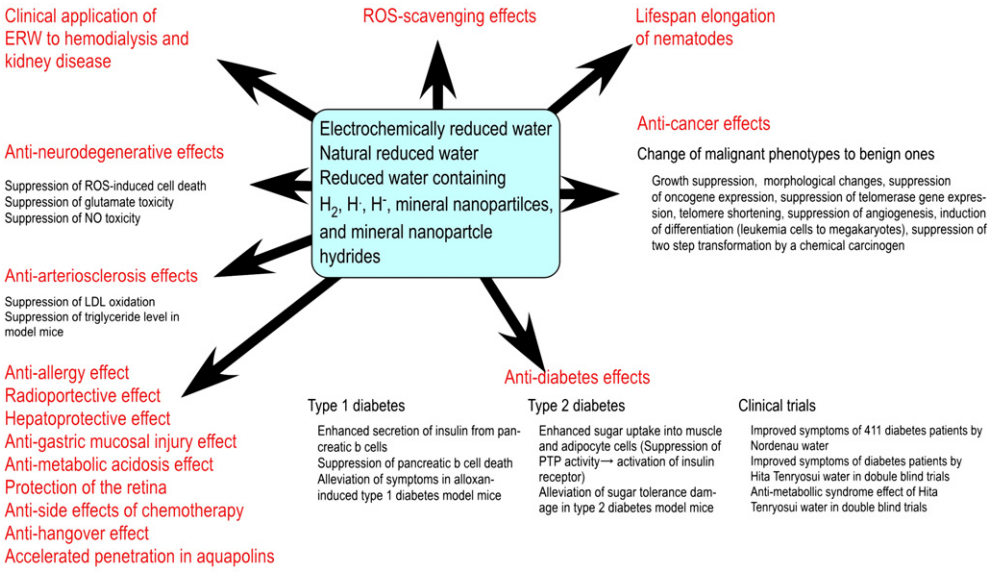

Fig. 4. Variety of functions of reduced water.

expected as washing water to prevent the rust of semiconductors. In the field of environmental remediation, reduced water will prevent the rotting of river and lake water, because the proliferation of bacteria or organisms causing the rotting will be suppressed in a reduced circumstance. Further research on water itself may ultimately reveal the secret of the origin of life.

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Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.tifs.2011.10.009.

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Supplementary information

Advanced research on the health benefit of reduced water

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Mechanism of action of reduced water containing hydrogen and mineral nanoparticles as a newly recognized ROS scavenger

Physiological functions of reduced water as reactive oxygen species scavenger

Reactive oxygen species (ROS) are known to cause various oxidative stress-related diseases and aging (Jomova & Valko 2011). Hayashi (1995) first showed the positive effects of ERW on various diseases and proposed a "Water Regulation Theory" that ERW containing hydrogen molecules improves various diseases by scavenging ROS in the body. Hayashi and Kawamura (2002) reported many positive effects of ERW, based on several thousand clinical observations from 1985 to 2000. The following diseases were improved: blood glucose and glycosylated hemoglobin (HbA1c) levels in diabetes mellitus; the peripheral circulation in diabetic gangrene; uric acid levels in gout; liver function in hepatic disease, cirrhosis of the liver, and hepatitis; gastroduodenal ulcers and prevention of recurrence; cholesterol levels, hypertension, angina, and myocardial infarction; hypersensitive disorders, atopic dermatitis, asthma, and urticaria; autoimmune disorders, rheumatism collagen disease, and systemic lupus erythematosus Behcet's syndrome, Crohn's disease, ulcerative colitis, and Kawasaki's disease; malignant tumors of the liver, hepatoma, and metastatic tumors.

The authors first demonstrated that ERW derived from a NaCl solution as a simple model of potable ERW derived from tap water scavenges ROS and protects DNA from oxidative damage *in vitro* (Shirahata et al., 1997). Thereafter, we preferably used ERW derived from a NaOH solution produced by a batch-type electrolysis device to avoid the contamination of HOCl from an anodic solution. Lee et al. (2006) also reported that ERW derived from a NH_4Cl solution protects DNA, RNA and proteins against oxidative damage. We found that ERW and natural reduced water (NRW) such as Hita Tenryosui water in Japan and Nordenau water in Germany, which are said to have therapeutic effects, can scavenge intracellular ROS in cultured animal cells (Li et al., 2002).

ERW containing hydrogen molecules

During electrolysis of water, hydrogen molecules are produced on the surface of a cathode and oxygen molecules are produced on the surface of an anode (Fig. 1A). Platinum (Pt)-coated titanium electrodes are favorably used for electrolysis of water in many commercial apparatus, because Pt is safe and can efficiently produce hydrogen

molecules. As shown in Fig. 1B, on the surface of the Pt plate of a cathode, a hydronium ion (H_3O^+) reacts with an electron, producing H_2O and an adsorbed H atom ($H_3O^+ + e^- \rightarrow H_2O + H(ad)$, termed the Volmer step). Two H(ad) can diffuse on the surface of the Pt electrode (spillover of H atoms) and produce H_2 gas ($2H(ad) \rightarrow H_2(g)$, termed the Tafel step) or H(ad) directly reacts with a H atom generated in the Volmer step to produce H_2 gas ($H(ad) + H_3O^+ + e^- \rightarrow H_2(g) + H_2O$, termed the Heyrofsky step). Part of H(ad) is absorbed into the Pt plate to produce mineral hydride containing adsorbed H(ab). Because of its small size, a hydrogen atom can be adsorbed to and absorbed in almost all metals. Recently, these electrode dynamic reactions were clearly demonstrated by a computer simulation method (Otani et al., 2008).

When tap water or natural mineral water is electrolyzed with direct current (e.g., 100 V), voltage is mostly applied on the very thin interface layer (Helmholz layer) between bulk of water and the surface of the Pt plate because of low resistance of bulk water, forming a strong reduction atmosphere with a high electric field in the vicinity of the cathode. In the strong electric field, all protons and mineral ions move with increasing speed and exhibit high reactivity (Hamann et al., 2007). Fresh ERW is supersaturated with hydrogen molecules including hydrogen nanobubbles, which will disappear within 3 h (Kikuchi et al., 2007).

Electrochemical preparation of mineral nanoparticles and mineral hydrides

Drinking water contains various mineral ions. Such mineral ions are easily reduced to mineral atoms on the surface of a cathode. The reduced mineral atoms are self-organized to produce mineral nanoparticles or mineral nanoclusters (Fig. 1B). Electrochemical reduction is one of the general methods of producing mineral nanoparticles or metal nanoclusters (Watzky and Finke, 1997; Aiken III and Finke, 1999). Metal ions with low ionic tendency, such as platinum, gold, vanadium, and palladium, easily form stable mineral nanoparticles, which can be stably dispersed in water for a long time. Mineral nanoparticles formed are gradually oxidized to mineral ions, exhibiting weak reducibility by releasing electrons. Therefore, electric reduction energy is sustained in ERW as a form of mineral nanoparticles for a long time. Metal nanoparticles exhibit high catalytic activities because they contain many active surface atoms, which can sense conditions.

A 1 mM NaOH solution was electrolyzed for 2 h at 100 V by using a batch type TI-200S electrolysis device equipped with platinum-coated titanium electrodes as reported previously (Ye et al., 2008). As shown in Fig. 1C, the ERW contained 0.19 ppb of Pt when determined with ICP-MS. ERW of 10 L was concentrated to 1 L by vacuum evaporation. The concentrated ERW was ultra-filtrated with a membrane of 10,000 Da cut size. The unfiltrated fraction (>10,000) contained 12.04 ppb of Pt and the filtrated fraction (<10,000) contained 2.16 ppb of Pt. Transmission electron microscopy revealed that the unfiltrated fraction contained Pt nanoparticles of 1 to 10 nm sizes (Fig. 1D). The Pt nanoparticles appeared to be derived from the Pt-coated titanium electrodes. As shown in Fig. 1E, synthesized Pt nanoparticles exhibited multifunctional antioxidative activity (Hamasaki et al., 2008). The activity of Pt nanoparticles as superoxide radical-scavengers was comparable with that of super oxide dismutase (SOD) (the second order rate constant *ks* for Pt nanoparticles = 5.03×10^7 M^{-1} s^{-1} and *ks* for SOD = $0.7 - 16 \times 10^8$ M^{-1} s^{-1}), suggesting that the scavenging properties of stable Pt nanoparticles are superior to those of conventional antioxidants. Pt nanoparticles also scavenged hydroxyl radicals

as well as glutathione ($ks > 10^{10}$ $M^{-1}s^{-1}$). The *ks* value of the H_2O_2 decomposition reaction catalyzed by Pt nanoparticles was in the order of 10^4 and 3 orders of magnitude lower than that of catalase. Pt nanoparticles activated H_2 molecules to H atoms with a *ks* value of 10^3 orders. Various antioxidants served as H donors and the reductive reaction by antioxidants was accelerated with *Ks* values of 10^0 orders in the presence of Pt nanoparticles. When an electron acceptor is dissolved oxygen, Pt nanoparticles accelerate autooxidation of antioxidants; however, the *Ks* value is 10^0 orders, and therefore, not high. Overall, Pt nanoparticles are considered to be good multi-functional antioxidants. Potable ERW may contain various types of mineral nanoparticles, which function as ROS scavengers, as well as H_2 activators and H-donors.

A lot of interest has been generated in metal nanoparticles being used as hydrogen storage sources. Because of the small size of hydrogen atoms, they can penetrate among metal atoms of almost all metals and be absorbed into metals, forming metal hydrides. Metal nanoparticles can store a lot of hydrogen atoms. Hydrogen atoms in vanadium hydride have been directly observed by electron microscopy (Findilay et al., 2010). The hydrogen solubility of Pd nanoparticles was reported to be 0.5 H per Pd (Yamauchi & Kitagawa, 2005). Pt nanoparticles of 2.0 nm size have been observed to absorb 0.12 H per Pt under a H_2 atmosphere of less than 0.1 kPa (Isobe et al., 2003; Kitagawa & Yamauchi, 2004). Ions with high ionic tendencies such as Ca^{2+} and Mg^{2+} are reduced to mineral hydrides such as CaH2 and MgH2. CaH2 and MgH2 gradually dissolve in water and produce hydrogen molecules (Tessier et al. 2004). A complex mineral hydride can also gradually produce hydrogen molecules in an aqueous solution (Fan et al., 2011). Because of the existence of abundant hydrogen atoms on the surface of cathodes and supersaturated hydrogen molecules in ERW, it is possible that ERW contains nanoparticles of various mineral hydrides (Fig. 1B).

Natural reduced waters containing mineral nanoparticles and mineral hydrides

There are several natural mineral waters, which are believed to be health beneficial. Lourdes water in France, which was discovered in 1985, is reported to have healing power for various diseases (Stephens, 1910). Tracote water in Mexico, discovered in 1991, has been reported to improve symptoms of various diseases, although there are no scientific reports on this water. Nordenau water in Germany, which is spring water from 300 m underground, was found to be health-beneficial water in 1992 (Gadek and Shirahata, 2002). Hita Tenryosui water in Japan, which is drawn from deep underground by a pump, was also found by us to be health-beneficial in 1997. Hita Tenryosui water and Nordenau water have been demonstrated to scavenge intracellular ROS (Li et al., 2002). We have termed those waters that can scavenge intracellular ROS as natural reduced waters (NRWs).

So why can NRWs scavenge intracellular ROS? Recent research has demonstrated that a great number of microorganisms are living underground at a depth of 5000 m. Similar numbers of microorganisms are also living underground at a depth of several m. These microorganisms are considered to use H_2 energy produced by the reduction of ground water by rock energy at high temperatures. H_2 reacts with CO_2 and produces CH_4 and water (carbon dioxide respiration). CH_4 reacts with SO_4 ions to produce H_2S and CO_2 (sulfate respiration). H_2S reacts with NO_3 ions to produce N_2 and SO_4 ions (nitrate respiration). Such microorganisms using chemical respiration are called lithoautotrophic microorganisms (Martin et al., 2008)(See Supplementary Fig. 1). NRW

drawn up deep from underground may contain some minerals sustaining such hydrogen energy. Hiraoka et al. (2004) recognized the antioxidative activity of ERW and Hita Tenryosui water. However, they reported that hydrogen gas and reductive vanadium ions, which are suggested to be the components responsible for the antioxidant activities in vitro, could not enhance the scavenging ability for ROS in vivo after being drunk and absorbed into the human body (Hiraoka et al., 2006).

Langmür (1927) reported that water molecules inhibited the reassociation of hydrogen atoms to hydrogen molecules. He heated hydrogen gas with moisture at a high temperature to produce pure hydrogen atom gas and invented the hydrogen torch. Based on an investigation on silica hydrides in snowmelt water at Hunza in Pakistan, which are believed to be beneficial for human longevity, Stephanson et al. (2002) synthesized a novel silsequioxane bioencapusulated-hydride compound (named silica hydride) by using H atoms generated from H_2 molecules at a high temperature and they showed that silica hydride slowly liberates hydride ions in an aqueous environment for several weeks. They also showed its ROS scavenging activity without cytotoxicity (Stephanson et al., 2003; Stephanson and Flanagan, 2003a, b; 2004a; 2004b). Recently, a protective effect of silica hydride has also been described against a hepatotoxin, carbon tetrachloride (CCl_4), which induces liver damage in mice via elevating antioxidative enzyme levels (Hsu et al., 2010).

Natural waters including ground and surface waters, tap water and different brands of mineral waters contain various nanoparticles (Wagner et al., 2004; Wigginton et al., 2007; Handy et al., 2008). Nanoparticles (fibrous polysaccharide particles and spherical organic matter) in drinking water are visualized using atomic force and transmission electron microscopy. The total particle number is estimated to be 7–10 x 10^8 particles/ml in conventionally treated drinking water (Kaegi et al., 2008). Moreover, natural waters contain amorphous silicate minerals as a form of nanoparticles, which exert antioxidative potential by the adsorbed hydrogen anion (Kimberly et al., 2001). Although further investigations are required, accumulated data suggest that NRW contains mineral nanoparticles and/or mineral hydrides giving rise to anti-oxidative activity.

ORP of reduced water and the human body

Because entropy gradually increases in nature, all living organisms gain enthalpy by using energy. The reduced state in living organisms is energy-rich and well ordered. Living organisms contain a large amount of various antioxidants to sustain the reduced state in their bodies. ORP exhibits an electron donating or accepting ability of the system. Instead of electrons, hydrogen or oxygen atoms can also be used for reduction and oxidation reactions. ORP monitoring is normally accomplished by using platinum measuring electrodes. Chemicals in water produce chemical reactions with the platinum electrode and oxidize or reduce the platinum band. This process generates a millivolt (mV) signal.

Most organic antioxidants such as ascorbic acid release hydrogen atoms and exhibit low ORP values. However, ORP values gradually increase because of autoxidation of antioxidants. Unstable organic antioxidants produce many chemicals, which function as antioxidants or prooxidants, giving very complex reactions with a platinum electrode. The ORP value depends upon the pH value. ORP values of fish, blood plasma, amniotic fluid, saliva, urine, vegetables, fruits, livestock products, fishery products, and various

types of commercial beverages are all in the reduction system region with respect to the equilibrium ORP level (Agustini et al., 2001; Okouchi et al., 2002). Okouchi et al. (2002) proposed the concept of "vital water" which exhibits low ORP values and is analogous to human body fluid. Hydrogen-rich water exhibits extremely low ORP values of -200 to -800 mV, because hydrogen molecules donate electrons to a platinum electrode. There is a linear relationship between dissolved hydrogen concentration and ORP, suggesting that hydrogen molecules are responsible for negative ORP values of ERW (Shirahata et al., 1997). The ORP value of ERW gradually increases, because hydrogen molecules are lost by gasification.

The ORP values of Hita Tenryosui water and Nordenau water are around +200 mV when determined by an ORP meter using platinum electrode (without correction to standard hydrogen electrode) and they do not contain hydrogen molecules. The waters also exhibit detectable radioactivity, denying a hormesis effect of weak radiation on health. We assume that NRW containing a small amount of reductive mineral nanoparticles may not contribute a negative ORP because of the low sensitivity of ORP meters. ORP values are not considered to be a reliable marker of reduced water.

Raman spectrum of ERW

The Raman spectrum reflects hydrogen bonds in water and the structure of water (Walrafen, 1964). The Raman spectra of ERW derived from $NaHSO_4$ solution and its chemical analogues are substantially different from those of water before electrolysis (Pastukhov and Morozov, 2000). It is assumed that the excess OH^- ions in the catholyte can participate in the formation of the more symmetric and weak hydrogen bond $(O\ldots H\ldots O)^-$. This is equivalent to capture of the excess electron by the associate, which possesses a positive electron affinity because of a cooperative effect, and hydration of this excess electron to the $(H_2O)_n^-$ association. The Raman spectrum is likely to be useful for analysis of energy-rich water.

Mechanism of action of hydrogen molecules as newly recognized ROS scavengers

Recently, it has been reported that hydrogen molecule is a newly discovered ROS scavenger that can specifically and directly scavenge hydroxyl radicals and peroxynitrite radicals (Ohsawa et al., 2007) and improved symptoms in various oxidative stress-related disease animal models (ischemic and reperfusion brain injury: Ohsawa et al., 2007: hepatic injury: Fukuda et al., 2007; myocardial ischemia-reperfusion injury: Hayashida et al., 2008; transplantation-induced intestinal graft injury: Buchholz et al., 2008; neonatal hypoxia-ischemia: Cai et al., 2008; brain learning task: Nagata et al., 2009; Parkinson disease: Fu et al., 2009; side effects of anti-cancer drug: Nakashima-Kamimura et al., 2009; hepatitis: Kajiya et al., 2009; polymicrobial sepsis: Xie et al., 2010; hyperglycemia-enhanced hemorrhagic transformation: Chen et al., 2010a; carbon monoxide poisoning: Shen et al., 2010; traumatic brain injury: Ji et al., 2010; neuroprotection: Domoki et al., 2010; radioprotection: Qian et al., 2010a; lung transplant-induced ischemia/reperfusion injury: Kawamura et al., 2010; cardic ischemia/reperfusion injury: Nakao et al., 2010a; radiation-induced oxidative stress: Schoenfeld et al., 2011; oxidative stress-induced angiogenesis: Kubota et al., 2011; lung injury involving NF-κB: Huang et al., 2011a; spinal cord ischemia-reperfusion injury: Huang et al., 2011b; lung allograft transplantation: Kawamura et al., 2011), cultured cells (Fc epsilon RI-mediated signal

transduction in mast cells: Itoh et al., 2009; bone marrow multipotential stromal cells: Kawasaki et al., 2010; nitric oxide production in macrophanges: Itoh et al., 2011). However, Matchett e al. (2009) reported that hydrogen gas was ineffective on moderate and severe neonatal hypoxia-ischemia rat models. Hydrogen molecules-containing water also improves the symptoms of various oxidative stress-related disease in model animals (atherosclerosis in apoE knockout mice: Ohsawa et al., 2008; neuroprotection in Parkinson disease: Fujita et al., 2009; Cai et al., 2009; lung protection against oxygen toxicity: Zheng et al., 2009; lung injury: Mao et al., 2009; cardiovascular events: Suzuki et al., 2009; myocardium injury: Sun et al., 2009; suppression of growth of cancer cells by Pt nanocolloide-supplemented hydrogen water: Saitoh et al., 2009; neuroprotection: Cai et al., 2009; protection of retina: Oharazawa et al., 2010; chronic allograft nephropathy: Cardinal et al., 2010; Chen et al., 2010; memory function in brain: Li et al., 2010; spinal cord injury: Chen et al., 2010b; pancreatis: Chen et al., 2010c; liver injury: Liu et al., 2010; zymosan-induced generalized inflammation: Xie et al., 2010; lung protection: Zheng et al., 2010; radioprotection: Qian et al., 2010a; 2010b; inflammation: Zhang et al., 2010; senescence-accelerated mice; Gu et al., 2010; renal injury: Shingu et al., 2010; side effects of anti-cancer drug: Kitamura et al., 2010; lung injury: Sun et al., 2011a; carbon monoxide toxicity: Sun et al., 2011b; inhibition of JNK and NF-kappa B activation: Wang et al., 2011; noise-induced hearing loss: Lin et al., 2011; lung injury: Fang et al., 2011) and in human (type 2 diabetes: Kajiyama et al., 2008; Stimulative effect of turmeric on hydrogen production by intestinal bacteria: Shimouchi et al., 2009; metabolic syndrome: Nakao et al., 2010b;).

Taken together, it is obvious that hydrogen molecules attenuate the oxidative stress in animal models, however, only a few papers have been published on the effect of hydrogen molecules in cultured cells. Further detailed investigation on the mechanisms of action of hydrogen molecules is required to reveal whether hydrogen molecules directly or indirectly scavenge ROS in cells and animals, because the reaction rate constant of hydrogen molecules with hydroxyl radicals is very small, as pointed out by Wood & Gradwin (2007). Sato et al. (2008) reported that hydrogen-rich pure water prevents superoxide formation in brain slices. The results are inconsistent with the argument by Ohsawa et al. (2007) that hydrogen molecules directly and specifically scavenge hydroxyl radical and peroxynitrite and do not scavenge superoxide and hydrogen peroxide. A question has been raised that the concentration of hydrogen molecules in blood and tissues may be too low to scavenge ROS directly and specifically.

Hydrogen molecules exist a lot next to oxygen and carbon dioxide in blood except for nitrogen. Rat blood contains several ppb of hydrogen molecules (Ohsawa et al., 2007). The saturated concentration of hydrogen gas (molecule) in water is 1.6 ppm (800 μM) at room temperature. Potable hydrogen-rich waters like ERW produced by commercial devices usually contain hydrogen molecules from 0.08 ppm to 1.6 ppm. When hydrogen-saturated water was placed into stomach of a rat, 10 ppb (5 μM) hydrogen molecules were detected in blood of the heart after 3 min (160 fold decrease) (Nagata et al., 2009). Fujita et al. (2009) reported that 0.08 ppm (40 μM) of hydrogen molecules-containing water could improve the symptoms of Parkinson model mice, although they could not detect hydrogen molecules in the striatum of the brain. The results suggest that hydrogen molecules are effective in a very low concentration of less than 0.5 ppb (0.25 μM) in vivo.

There is a possibility that hydrogen signal transduction pathway exist in cells. It is plausible that living organisms use hydrogen molecules during evolution as a signal substance because hydrogen bacteria having hydrogenase, which catalyze the conversion reaction between hydrogen atoms and molecules, produce a lot of hydrogen in the intestine and oral cavity in the human body (Neale, 1988; Urita et al., 2008; Urita et al., 2009). Are there any cascades of signal pathways in cells to convey the stimulation by hydrogen in vivo? So far, no receptor for oxygen molecules has been found. A variety of human disorders, such as ischemic heart disease, stroke, kidney disease, eventually share the deleterious consequences of a common, hypoxic and oxidative stress. The hypoxia-inducible factor (HIF) is a key player as it activates a broad range of genes protecting cells against hypoxia. HIF regulates about 2% of total genes in human alloy epithelial cells, including erythropoietin, vascular endothelial growth factor, adrenomedullin, matrix metalloproteases, endotheline and nitrogen oxide synthetases (Manalo et al., 2005). Its level is determined by its degradation rate by intracellular oxygen sensors prolyl hydroxylases (PHDs)(Miyata et al., 2011) . PHDs are redox-sensitive proteins, of which the ratio of disulfide bonds is changed depending upon cellular redox status.

The nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) regulates the basal and inducible expression of numerous antioxidant stress genes. Nrf2 regulates many antioxidant genes: GSH biosynthesis, glutathione peroxidases, thioredoxin reductase, thioredoxin, peroxiredoxine, superoxide dismutase, catalase, glutathione S-transferases, UDP-glucuronosy transferase, hemooxigenase-1, hydrolysis, iron transport, detoxication of heavy metals, transport, and 26S protesome (Jung & Kwak, 2010). Nrf2 activity is modulated by Kelch-like ECH-associated protein 1 (Keap1), an intracellular redox sensor protein for oxidative stress. Keap1 binds to Nrf2 and inhibits the translocation of Nrf2 into nucleus. Keap1 is a cysteine-rich protein and modifications in sulfhydryl residues of Keap 1 protein, especially in the Cys273 and Cys288 residues, results in protein conformational changes. Oxidative stress induces the conformational changes in Keap1 to release Nrf2. Nrf2 translocate into nucleus and binds to the antioxidant responsible element to activate many antioxidant stress genes described above. Keap1 is a sensor protein responding to oxidative stress and environmental stresses through dynamic changes in cysteine reducing status.

In addition to PHDs and Keap1, there are many redox-sensitive proteins such as nuclear factor-κB and protein tyrosine phosphatase in cells. The functions of these proteins are regulated by conformational changes depending upon the ratio of intramolecular disulfide bonds. Disulfide bond formation is probably involved in the biogenesis of ~1/3 of human proteins. A central player in this essential process is protein disulfide isomerase (PDI)(Fatahet et al., 2009). Cellular mechanisms governing redox homeostasis likely involve their integration with other stresses. Endoplasmic reticulum (ER) stress triggers complex adaptive or proapoptotic signaling defined as the undfolded protein response, involved in several pathophysiological processes. Since protein folding is highly redox-dependent, convergence between ER stress and oxidative stress has attracted interest (Santos et al., 2009). Formation of disulfide bonds in the ER is catalyzed by the ER oxidoreduction (Ero1) family of sulfhydryl oxidases. Ero1 oxidizes PDI, which in turn introduces disulfides into ER client proteins. To maintain an oxidized state, Rro1 couples disulfide transfer to PDI with reduction of molecular oxygen, forming hydrogen peroxide. Thus, Ero1 activity constitutes a

potential source of ER-derived oxidative stress. Intricate feed back mechanisms have evolved to prevent Ero1 hyperactivity. Central to these mechanisms are noncatalytic cysteines, which form regulatory disulfides and influence catalytic activity of Ero1 in relation to local redox conditions (Tavender et al., 2010).

Oxidative protein folding is mediated by a proteinaceous electron relay system, in which the concerted action of PDI and Ero1 delivers the electrons from thiol groups to the final acceptor. Oxygen appears to be the final oxidant in aerobic living organisms; although, the existence of alternative electron acceptors, e.g. fumarate or nitrate, cannot be excluded. Interestingly, in vitro and in vivo evidence suggests that low molecular weight electron carriers such as ascorbate, tocopherol and vitamin K can contribute to the functioning of oxidative folding (Margittai et al., 2009). N-acetyl cysteine also cleaves the intramolecular disulfide bonds and alters IL-4 signaling (Curbo et al., 2009).

The oxidizing nature of the extracellular environment is vastly different from the highly reducing nature of the intracellular compartment. The redox potential of the cytosolic compartment of the intracellular environment limits disulfide bond formation, whereas the oxidizing extracellular environment contains proteins rich in disulfide bonds. If not for an extracellular antioxidant system to eliminate reactive oxygen and nitrogen species, lipid peroxidation and protein oxidation would become excessive, resulting in cellular damage. The coordinated action of both intracellular and extracellular antioxidants in limiting cellular oxidant stress will be important. (Filomena et al., 2008).

To investigate the physiological functions of hydrogen molecules in detail, we developed a new gas incubator that enabled long-term culture of animal cells under a mixed gas atmosphere of $H_2/O_2/CO_2$. We found that hydrogen molecules induce the gene expression of antioxidant enzymes such as SOD, catalase, glutathione peroxidase and hemoxigenease-1 via activation of the Nrf2 gene (Shirahata et al., in press).

There are several possibilities on the mechanisms of action of hydrogen molecules as ROS scavengers or redox regulators. (1) Are there specific receptors for hydrogen molecules which can convey the redox regulation signals? (2) Are there any oxidoreductases which use hydrogen molecules as substrates as well as NADH, NAPDH, FAD etc., (3) Can active hydrogen (hydrogen atoms) be produced by the catalysis action of metal nanoparticles and work as a signal transduction factor in vivo? (4) Can hydrogen molecules regulate the cellular redox balance via the regulation of the ratio of reduced glutathione and oxidized glutathione or other antioxidants, (5) Can hydrogen regulate the electron relay in the ER lumen and the conformational changes of redox-sensitive proteins like Ero1, DPI, Nrf2 and Keap1?

Based on all these findings, we propose an active hydrogen mineral nanoparticle reduced water hypothesis (Fig. 4), which may explain the common mechanism of action of reduced waters (Shirahata et al., 1997; Shirahata, 2002; Shirahata, 2004; Ye et al., 2008). Safe drinking water will be changed to ERW by electrolysis. ERW contains a lot of hydrogen molecules and mineral nanoparticles, which can transiently sustain reduction energy produced by electric energy. ERW will return to original safe drinking water after releasing reduction energy, which may be a reason that ERW do not exhibit side effects. NRW will sustain rock energy underground in the form of hydrogen molecules, mineral nanoparticels or mineral nanoparticle hydrides. Hydrogen-rich water and reductive minerals-containing waters are also categorized into reduced waters. Reduced waters exhibiting reducing activity only may overcome the paradoxical effects

of conventional organic antioxidants, which work as both antioxidants and prooxidants, opening a new research field of antioxidants.

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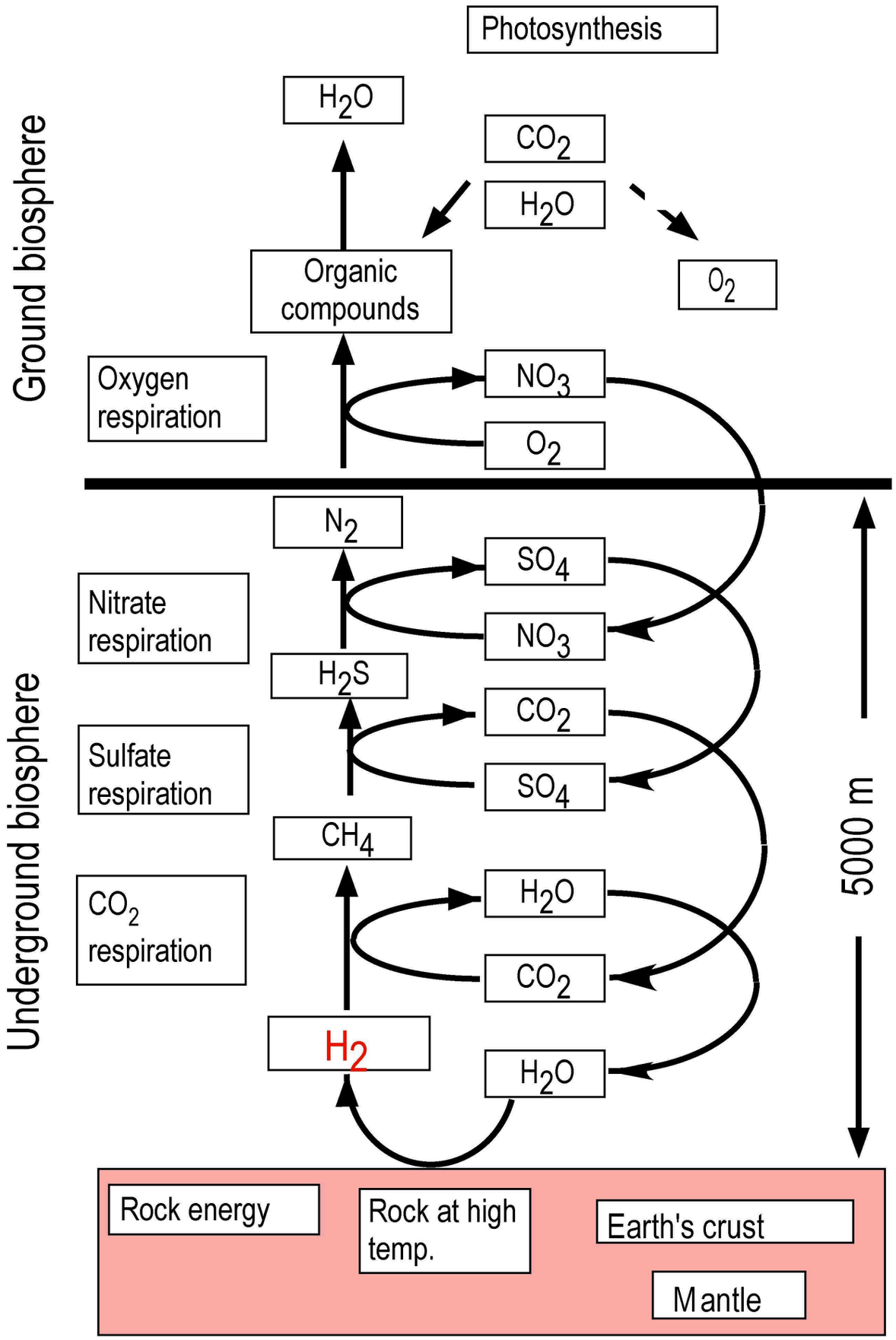
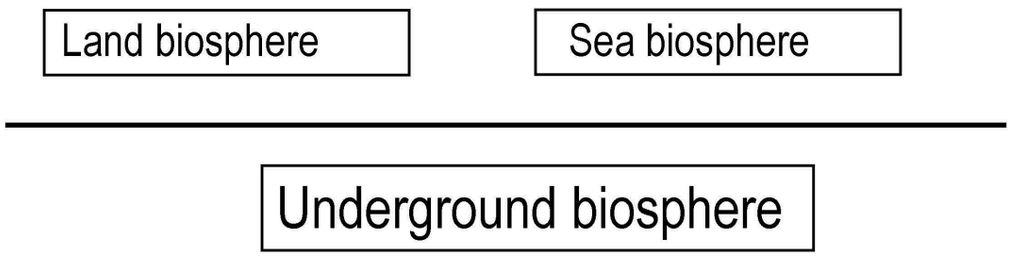
Supplementary Fig. 1. Generation of hydrogen in the underground biosphere. Rock like basalt underground reduces water in the aquifer, producing hydrogen molecules. Many lithotrophic organisms living up to 5000 m underground oxidize hydrogen molecule to methane, hydrogen sulfide, and nitrogen by chemical respiration systems. Ground water contains many mineral nanoparticles and possibly mineral nanoparticle hydrides. The figure was modified a figure in an article in the journal Newton (Newton editorial, 2001).



The biggest underground biosphere

Hydrogen produced by the reaction of rock (mineral) with water is oxidized by various oxidizers such as carbon dioxide, sulfuric acid, nitric acid, iron, manganese for chemical respiration of microorganisms in underground biosphere.

Lithotrophic organisms eating the earth



Natural reduced water may contains mineral nanoparticles and mineral hydrides sustaining hydrogen energy underground.