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Hydrogenized Water Effects on Protection of Brain Cells from Oxidative Stress and Glutamate Toxicity

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Abstract Hydrogenized water is known to have protective effects on cells and tissues, mainly through its antioxidant activities. Here we examined the protective effects of a commercial source of hydrogenized water on cultured human brain cells. Hydrogenized water was able to protect brain cells from oxidative stress and glutamate toxicity. At H_2 concentrations above 0.01 mM the glutathione levels increased in cultured brain cells. The level of glutathione rose from approximately 500 to approximately 850 μM at the maximum dose of hydrogenized water with an EC_{50} of approximately 0.030 mM. Hydrogenized water was also able to enhance the signaling pathway for oxidative stress response mediated by Nrf2 (Nuclear factor erythroid 2 like factor). Treatment of cells with hydrogenized water at concentrations above 0.01 mM H_2 induced activation of Nrf2 (EC_{50} approximately 0.05 mM). Hydrogenized water was also able to protect brain cells against glutamate toxicity. Using a DNA damage response element, (γH2AX, to monitor the damage of glutamate toxicity we found that concentrations of H_2 above 0.01 mM protected cells from glutamate damage with an EC_{50} of approximately 0.05 mM H_2. These in vitro results demonstrated that hydrogenized water can protect brain cells against common types of damage from oxidative stress and glutamate toxicity.

Keywords: *hydrogenized water, bioassays, glutathione, oxidative stress, Nrf2, glutamate toxicity*

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1. Introduction

Cells within the central nervous system (CNS) are among the most metabolically active cells in our bodies, and they are also among the most sensitive to oxidative stress [1,2,3]. Oxidative stress occurs when there is an excess of reactive oxygen and nitrogen species (ROS, RNS) over natural and added antioxidant molecules [3,4,5,6,7]. Under normal circumstances reactive oxidants, ROS and RNS, are produced in low amounts where they can act as important signaling molecules in cytoplasmic events, such as growth factor signaling, mitochondrial autophagy, and nuclear cell division. ROS and RNS are also important in immune function, inflammation and other important cellular and tissue responses [3,4,5,6,7].

Excess oxidative stress in the brain is associated with and contributes to the etiology or progression of neurodegenerative diseases. This is due to the disproportionate amount of oxygen use in this tissue and the chance of excess production of ROS/RNS that can damage brain cell lipids, proteins and nucleic acids [7,8,9]. One of the systems that neutralizes excess oxidative stress due to ROS/RNS is the glutathione system [10]. Glutathione is a ubiquitous thiol that acts in concert with other antioxidants to reduce ROS/RNS and prevent damage to brain cells [9,10,11,12]. It is also a transfer molecule for cysteine and is involved in keeping sulfhydryl proteins in a reduced state, which is important in maintaining cellular redox potential as well as for detoxification [10,12,13].

Another cellular defense system against oxidative stress in the brain is mediated by the nuclear factor erythroid 2-related factor (Nrf2) [14,15]. Nrf2 is a member of the cap'n'collar family of leucine zipper transcription factors that in the nucleus heterodimerizes with small proteins (MAF, JUN proteins) and binds to DNA sites containing anti-oxidant response elements (ARE) and other sites to

initiate transcription of cytoprotective genes [16]. The Nrf2 system is also linked to activation of NFκB to mediate anti-inflammatory effects [17,18]. Insufficient Nrf2 activation in humans has been linked to neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis [17,19].

In addition to oxidative stress, there are several markers for toxicity in the brain, but one of the most widely studied is the neurotransmitter L-glutamate. L-glutamate is a major excitatory neurotransmitter in the brain, and it is involved in virtually all activities of the nervous system [20,21]. L-glutamate binds to and activates both ligand-gated ion channels (ionotropic glutamate receptors) and a class of G protein-coupled receptors (metabotropic glutamate receptors). The intracellular glutamate concentration in the brain is normally in the millimolar range; however, extracellular glutamate concentration is kept low (in the low micromolar range) by excitatory amino acid transporters that import this molecule into astrocytes and neurons. When extracellular glutamate is in excess, this can result in excitotoxicity as seen in ischemic stroke and neurodegenerative diseases [20,21].

Hydrogenized water has been shown to act as a cellular antioxidant and gene regulator [22,23]. Previously we found that hydrogenized water was an effective cellular antioxidant and could decrease transport of glucose in a dose- and time-dependent manner and elevate the levels of phosphorylation of Akt, a protein kinase B biomarker for insulin signaling [24]. We also found that hydrogenized water could increase the levels of age-related SIRT1 gene expression and increase the expression of telomerase activity in a dose-dependent process [24]. Here we examined the ability of hydrogenized water in vitro to protect brain cells from oxidative damage by increasing glutathione levels and reducing glutamate toxicity. We also examined the ability of hydrogenized water to activate the Nrf2 pathway as a marker for the anti-oxidative and anti-inflammatory activities.

2. Materials and Methods

Hydrogenized water identified as "IZUMIO Hydrogenized Water" was acquired from Naturally Plus USA (NPUSA) Las Vegas, Nevada. At the time of initial packaging, the infused hydrogen within the test water was analyzed by both manometric and diaphragm polarographic electrode measurements at final concentration of 2.6 ppm (1.3 mM H_2) and pH 7.0. The Izumio hydrogenized water was used in all of the assays [24].

The human neuroblastoma SH-SY5Y cell line and HepG2 cell line were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Eagle's Minimum Essential Medium (EMEM), and F12 Medium were purchased from GIBCO™ (Grand Island, NY, USA). Fetal bovine serum (FBS), Minimum Essential Media (MEM) and Earle's Balanced Salt Solution were purchased from Hyclone (Pittsburg, PA, USA). Sodium pyruvate, penicillin, streptomycin, quercetin, formaldehyde solution, Triton X-100 and cell culture grade water were obtained from Sigma (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (DPBS), Monochlorobimane, Hoechst 33342 (DNA stain), primary antibody cocktail consisting of mouse-anti-phospho-histone H2A.X and Hoechst 333342 and secondary antibody Cy3-donkey-anti-rabbit-IgG were purchased from Thermo Fisher Scientific (Fremont, CA, USA). Reagents used in Nrf2 luciferase assay were purchased from Promega. Cell plates were purchased from Corning Costar Corporation (Cambridge, MA, USA).

2.1. Glutamate Toxicity Assay

The effects of hydrogenized water on brain cell protection was studied using glutamate toxicity as the cellular biomarker [25]. Human neuroblastoma SH-SY5Y cells were cultivated in a 1:1 mixture of Eagle's Minimum Essential Medium and F12 Medium with fetal bovine serum at a final concentration of 10%, and were seeded onto a 24-well cell culture plate and cultured for 24 hr before addition of the test samples in cell culture media. In situations of hydrogenized water treatment, culture medium as dry powder was suspended and dissolved in freshly opened hydrogenized water. Different final concentrations of the hydrogenized water in cell culture grade water were used or at v/v ratios of 1, 0.5, 0.25, 0.125, 0.062 and 0.03125 of hydrogenized water to cell culture grade water). After preparation, the culture medium was sterile-filtered and used to treat neuronal cells immediately. All experiments were performed in triplicate.

The SH-SY5Y cells were exposed to normal culture media or culture media made with various concentrations of hydrogenized water for 24 hr, and then challenged with 250 μM glutamate for 24 hr. After washing twice with culture media, the cells were fixed with 3.7% formaldehyde solution for 30 minutes. The fixed cells were then washed with DPBS twice on a Biotek plate washer and then treated with permeabilization solution composed of 0.5% (v/v) Triton X-100 for 10 min. After 10 min treatment, the cells were washed with DPBS once and blocked for 30 minutes with blocking solution (1% bovine serum albumin in DPBS) prior to antibody staining. After incubation in the blocking solution, cells were washed again with DPBS once and incubated with a primary antibody cocktail consisting of mouse-anti-phospho-histone H2A.X and Hoechst 333342 at room temperature for 1 hr. After washing twice with DPBS on a Biotek plate washer, secondary antibody (Cy3-donkey-anti-rabbit-IgG) was dispensed into each well and incubated continued at room temperature for another hr. After washing twice with DPBS on a Biotek plate washer, 200μL of DPBS was left in each well. The plate was then sealed and read on the ArrayScan VTi using a Compartmental Analysis Bioapplication. Data was processed and reported with the LIM system. Inhibition curves and EC_{50}s were generated via Graphpad's Prism.

2.2. Glutathione (GSH) Level Assay

The effects of hydrogen water on brain cell protection were studied using the intracellular glutathione level as the cellular biomarker [26]. Human neuroblastoma SH-SY5Y cells were cultivated in a 1:1 mixture of Eagle's Minimum Essential Medium and F12 Medium with fetal bovine

serum at a final concentration of 10%, seeded into a 96-well cell culture plate and cultured for 24 hr before addition of the test compound in cell culture media. When media containing hydrogenized water was used, culture medium dry powder was suspended and dissolved in freshly opened hydrogenized water, and different final concentrations of the hydrogenized water in cell culture grade water were used at v/v 1.00, 0.50, 0.250, 0.0125, 0.00625 and 0.003125 of hydrogenized water to cell culture grade water, as previously used for other cell types [24]. Culture medium was then sterile-filtered and used to treat neuronal cells immediately. All experiments were performed in triplicate.

To test for response to oxidative stress cells were treated with the hydrogenized water media for 24 hr and then exposed to H_2O_2 (300 µM) for 2 h. Cells were then incubated in control or test hydrogenized water media at the concentrations stated above for 24 hr. After washing twice with culture media, the cells in each well were incubated with Monochlorobimane (final concentration of 50 µM) mixed with 30 µl of 10 mg/ml Hoechst 33342 for 15 min at 37°C. After washing twice with PBS, the plates were read on an arrayscan VTI using 2 channels and acquiring a 10X protocol for one field. Data were processed and reported with the LIM system. Inhibition curves and EC_{50}s were generated via Graphpad's Prism.

2.3. Cellular Nrf2 Activation Assay

The effects of hydrogen water on cellular Nrf2 pathway were also studied. [27]. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA) and antibiotics (50 U/mL of penicillin and 50 µg/mL streptomycin) under an atmosphere of 5% CO_2 95% air at 37°C. The cells were then seeded at a density of 35,000 cells per well into a clear-bottom 96-well plate containing 100 µl of growth medium (MEM/EBSS (Hyclone), 10% FBS, 1% non-essential amino acids, 1 mM Na-pyruvate, and 1% Pen/Strep, and then incubated overnight at 37°C in a CO_2 incubator. ARE reporter was transfected into cells the next day and incubated overnight at 37°C in the CO_2 incubator. Cells were then treated with the test media or control media on the following day. In situations where hydrogenized water in culture media were used for treatment, culture medium dry powder was suspended and dissolved in freshly opened hydrogenized water at different concentrations of the hydrogenized water in cell culture grade water at v/v of 1.00, 0.50, 0.250, 0.0125, 0.00625 and 0.003125 of hydrogenized water to cell culture grade water. Culture media were sterile-filtered and used to treat cells immediately. After incubation for 18 h, the cells were lysed with 20µL cell culture lysis buffer as before, and incubated with 100µL luciferase assay substrate. Luminescence was measured using a multimode plate reader

2.4. Statistical Analyses

Because of the small sample sizes a non-parametric test was used, the Kruskal-Wallis test. The Kruskal-Wallis test is the non-parametric alternative to a one-way ANOVA and does not assume normality of the residuals. The null hypothesis of this test is that all the medians are equal. If the Kruskal-Wallis test is significant, it indicates that at least two concentrations have significantly different medians. Statistical analysis was performed using JMP®, version 13 (SAS Institute, Inc., Cary, NC) by the Cornell University Statistical Consulting Unit, Ithaca, NY [28].

3. Results and Discussion

3.1. Glutamate Toxicity

Glutamate is a major excitatory neurotransmitter in the central nervous system that is involved in brain functions including cognition, memory and learning [29]. It articulates the signaling network that regulates brain development, differentiation and functioning of synapses [30]. Whereas an optimal dose of glutamate is essential for normal brain physiology, low and high doses can trigger neurotoxic or excitotoxic cascades [31]. Glutamate is mediated mainly via two types of ionotropic glutamate receptors, the NMDA receptor and non-NMDA receptor [32].

In this experiment, we monitored DNA damage response (γH2AX) to evaluate the effect of a test material (hydrogenized water) on protection of human brain cells from glutamate toxicity. We treated human SH-SY5Y neuroblastoma cells for 24 hr with a series of concentrations of hydrogenized water in culture media. Above H_2 concentrations of 0.01 mM H_2, the cellular content of γH2AX was reduced. The results indicated that γH2AX was lowered by 50% when cells were treated with a concentration equal to 52.8% (0.030 mM H_2) of hydrogenized water (significance in the Kruskal-Wallis test, χ=13.21, p=0.0215) (Figure 1). These results indicate that hydrogenized water may contribute to the recovery of DNA damage from glutamate toxicity in brain cells.

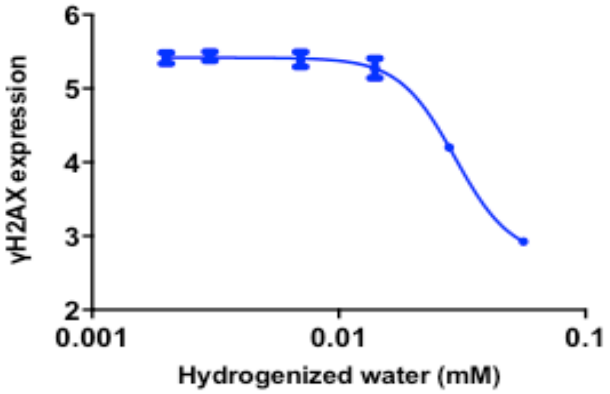

Figure 1. The effects of hydrogenized water on glutamate toxicity as monitored by DNA damage response (γH2AX). Human SH-SY5Y neuroblastoma cells were treated with a series of culture media containing various concentrations of hydrogenized water for 24 hr prior to exposure to glutamate. The protective effect of hydrogen against glutamate toxicity was determined by reductions in γH2AX as described in Materials and Methods. In this plot of the relative amounts of γH2AX (compared to an untreated control) versus hydrogen concentration the EC_{50} was calculated to be 0.030 mM H_2

When excess extracellular glutamate is present, it can trigger excitotoxicity in vitro and in vivo in acute situations like ischemic stroke via over-activation of ionotropic

glutamate receptors. In addition, chronic excitotoxicity has been found to play a role in several neurodegenerative diseases, including amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease [33]. Thus maintenance of appropriate levels of extracellular glutamate is essential to prevent excitotoxicity in the central nervous system.

3.2. Glutathione (GSH)

Glutathione (GSH) is a key intracellular tripeptide thiol antioxidant that protects cells from free radical damage by providing reducing equivalents for the reduction of lipid hydroperoxides [9]. GSH depletion has been implicated in the pathogenesis of neurological diseases, particularly in Parkinson's disease (PD) [34,35]. In this assay, we evaluated the effectiveness of hydrogenized water in increasing the concentration of GSH in brain cells as a marker for antioxidant levels in neuronal cells [36]. This was done by treating human neuroblastoma SH-SY5Y cells for 24 hr with a series of concentrations of hydrogenized water, then exposing the cells to oxidative stress and measuring the impact of hydrogenized water on GSH levels. The results indicate that GSH levels increased at H_2 concentrations above 0.01 mM H_2 or by 50% when cells were treated with 35% (0.0196 mM H_2) of hydrogenized water (significant difference in the Kruskal-Wallis test, χ=14.38, p=0.0134) (Figure 2). This indicates that hydrogenized water can contribute to the recovery of GSH levels during oxidative stress in brain cells.

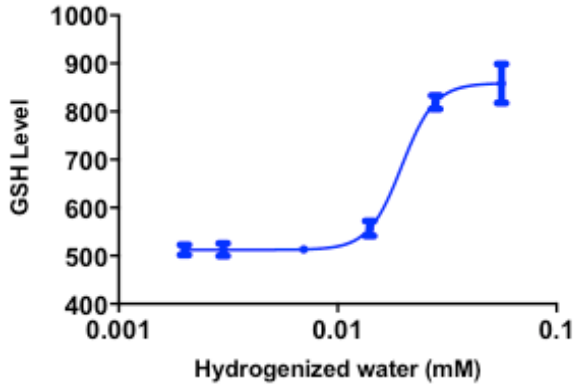

Figure 2. The effects of hydrogenized water on intracellular glutathione (GSH) levels in human SH-SY5Y neuroblastoma cells. The cells were treated with a series of culture media containing various concentrations of hydrogenized water for 24 hr prior to exposing to oxidative stress challenge, and GSH levels were determined as described in Materials and Methods. In this plot of GSH level (compared to an untreated control) versus hydrogen concentration the EC_{50} was calculated to be 0.0196 mM H_2

GSH participates in numerous cellular functions, and thus it is a critical cellular molecule [35,36]. GSH levels are correlated with increased oxidative damage and are known to diminish with increased age and in certain disease states [37,38]. Thus elevation in GSH levels protects cells from oxidative damage as well as damage due to toxic chemicals [38,39].

3.3. Cellular Nrf2 Activation

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that binds to antioxidant response elements (ARE) to regulate the expression of antioxidant enzymes that protect against oxidative damage triggered by injury and inflammation [40]. Activation of the Nrf2 pathway has been found to prevent and has been used to treat a large number of chronic inflammatory diseases [41]. A number of natural occurring phytonutrients, such as resveratrol, sulforaphane, and curcumin have been reported to activate Nrf2, with additional phytonutrient Nrf2 activators being the focus of new investigations [42]. Nrf2 has also been investigated as a biomarker for regulating in vivo anti-oxidation and anti-inflammation responses [43].

In this experiment, we treated human cells with or without hydrogenized water, and the impact of the hydrogenized water on Nrf2 activation was determined. When hydrogenized water was added to the culture media at concentrations above 0.01 mM H_2, the Nrf2 activation response was increased. We found that the level of Nrf2 activation increased by 50% when cells were treated with approximately 0.05 mM H_2 (significance in the Kruskal-Wallis test, χ=11.99, p=0.035) (Figure 3).

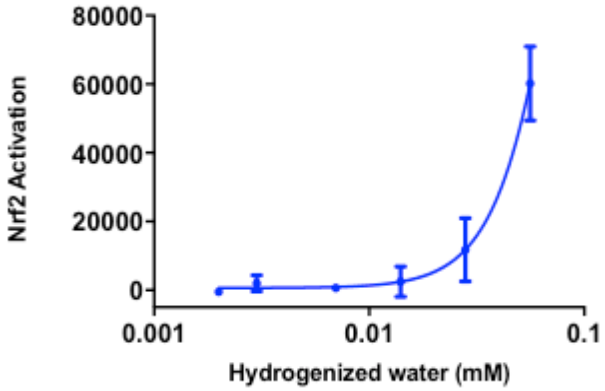

Figure 3. The effects of hydrogenized water on activation of transcription factor Nrf2 in human HepG2 cells. The cells were treated with a series of culture media containing various concentrations of hydrogenized water over night, and Nrf2 activation levels were determined as described in Materials and Methods. In this plot of relative Nrf2 activation level (compared to an untreated control) versus hydrogen concentration the EC_{50} was calculated to be 0.097 mM H_2

Nrf2 is considered a multifunctional nuclear regulation factor [14,16,40,41]. As described above, it plays an important role as a cytoprotective factor regulating the expression of genes involved in anti-oxidant, anti-inflammatory and detoxifying proteins, and it is also considered an important modulator of species longevity [44,45]. It is involved in the pathogenesis of some age-dependent disorders, including neurodegenerative diseases. Thus substances like hydrogenized water that act to increase Nrf2 can play a stress-protective and anti-aging role and should be considered protective molecules against cellular damage and aging [45].

4. Conclusion

Molecular hydrogen has been shown to be useful in the treatment of various medical conditions and as an anti-aging supplement [22,23]. Here we examined the effects of a commercial source of hydrogenized water on cultured human brain cells. Hydrogenized water protected brain cells from oxidative stress and glutamate toxicity. The commercial hydrogenized water also enhanced the signaling

pathway for oxidative stress response mediated by Nrf2. These results demonstrated that hydrogenized water can protect brain cells against common types of cellular damage from oxidative stress and glutamate toxicity, important events in stroke and neurodegenerative diseases.

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