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Neutral pH Hydrogen-Enriched Electrolyzed Water Achieves Tumor-Preferential Clonal Growth Inhibition Over Normal Cells and Tumor Invasion Inhibition Concurrently With Intracellular Oxidant Repression

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The properties and effects of neutral pH hydrogen-enriched electrolyzed water (NHE water) on tumor cells were examined. NHE water diminished hydroxyl radicals as demonstrated by ESR in a cell-free system. Human tongue carcinoma cells HSC-4 were inhibited for either colony formation efficiencies or colony sizes by NHE water without significant inhibition to normal human tongue epithelial-like cells DOK. Furthermore, NHE water caused growth inhibition, cell degeneration, and inhibition of invasion through the reconstituted basement membrane to human fibrosarcoma cells HT-1080. Intracellular oxidants such as hydroperoxides and hydrogen peroxides were scavenged in HSC-4 or HT-1080 cells by NHE water. In the human oral cavity, a dissolved hydrogen concentrations (DH) of NHE water was drastically declined from 1.1 to 0.5 ppm, but settled to 0.3–0.4 ppm until 180 s, upon static holding without gargling. Thus, NHE water was shown to achieve tumor-preferential growth inhibition and tumor invasion together with scavenging of intracellular oxidants, and is expected as a preventive material against tumor progression and invasion.

Key words: Neutral pH hydrogen-enriched electrolyzed water; Tumor repression; Human tongue carcinoma; Reactive oxygen species

INTRODUCTION

It is known that human tumor cells produce reactive oxygen species (ROS) more abundantly than nontransformed cell lines (1), and an elevated oxidative stress has been found in many different types of cancer cells (2). Evidence suggests that ROS are related to diverse abilities of cancer cells: increased cell proliferation, DNA synthesis (3), survival, cellular migration (4), invasion (5,6), tumor metastasis (7,8), and angiogenesis (9). On the other hand, it is also well known that antioxidants can inhibit tumor cell proliferation, which indicates an important role of ROS in mediating the loss of growth control (10–12).

Electrolyzed reduced water (ERW) that is produced at cathode side by electrolysis of tap water exhibits alkaline pH (9.0–10.0), extremely negative oxidation reduction potential (ORP) values, lower dissolved oxygen (DO), and higher dissolved hydrogen concentrations (DH) than tap water. Recently, many reports have shown that ERW has anitioxidative effects and ERW is expected to reduce oxidative stress causing various diseases (13–16). ERW decreases superoxide anion radicals (17,18), hydrogen peroxide (19), and hydroxyl radical (19), and protects DNA from oxidative damages (17,20), an alloxan-derived ROS-induced pancreatic β-cell damage (13), and a hemodialysis-induced oxidative stress in end-stage renal disease patients (14,15). Although the antioxidative mechanisms of ERW have not been elucidated, some reports suggest that dissolved hydrogen in ERW is a key factor responsible for its antioxidative effects (17,18,20). Furthermore, Ohsawa et al. showed that dissolved molecular hydrogen (H_2) in solutions selectively reduced the hydroxyl radical, the most cytotoxic ROS, and effectively protected PC12 cells from antimycin A-induced death, and the inhalation of H_2 gas markedly suppressed focal ischemia and reperfusion-induced brain injury in rats (21). Therefore, the water

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containing abundant hydrogen, which is produced by electrolysis, has antioxidative potential and is expected to have anticancer effects.

In the present study, using a novel type of electrolysis device that can produce neutral pH hydrogen-enriched electrolyzed water (NHE water), we determined the antioxidant ability, anticancer effects, and stability of NHE water in the oral cavity.

MATERIALS AND METHODS

NHE Water

NHE water was produced by a water-electrolysis apparatus Active BIO (Takaoka Electric MFG. Inc., Tokyo), which electrolyzed tap water in a nondiaphragm-type cell with a super-flatness surface platinum-coated titanium electrode plate and once accumulated the generated hydrogen in a high-pressure compressed activated carbon block for producing NHE water (Fig. 1). Concentrations of dissolved molecular hydrogen (DH) in solution were measured using a DH meter (DH-35A, DKK-Toa Inc., Tokyo), and concentrations of dissolved oxygen (DO) were measured by a pH/DO meter (D-55, Horiba Inc., Kyoto), and oxido-reduced potential (ORP) was measured using an ORP meter (RM-20P, DKK-Toa) at 20–25°C. In all the experiments, we used NHE water, which indicated each parameter at 0.5–1.1 ppm of DH, −590 to −104 mV of ORP, 4.9–10.0 ppm of DO, and 6.6–7.8 of pH.

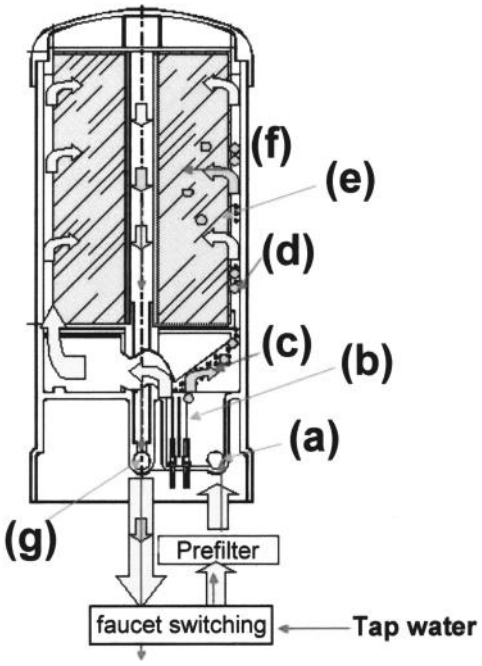

Figure 1. A structure of an NHE water production device. (a) Tap water inlet; (b) electrolysis tank equipped with three platinum-plating titanium electrodes; (c) electrolysis gas; (d) electrolytically produced hydrogen gas is collected; (e) high-pressure compressed activated carbon block, (f) the hydrogen gas that is dissolved in water is once stored up in a high-pressure compressed activated carbon block; (g) NHE water is taken out.

Measurements of Hydroxyl Radicals by Electron Spin Resonance (ESR)

The reactivity of NHE water with hydroxyl radicals that were generated by Fenton's reaction was measured with an ESR spectrometer. Sample solutions (60 µl) and 10 µM of $FeSO_4$ (10 µl) were mixed in a disposable plastic tube, followed by addition to 1.79 M of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; Labotech, Tokyo) (20 µl) and 100 µM of H_2O_2 (10 µl). The reaction mixtures were sucked into a quartz flat cell LLC04 (Labotech) and set in the ESR apparatus (type JES-ER30, JOEL, Tokyo). Measurements of hydroxyl radicals by ESR were carried out at 30 s after the addition of H_2O_2. The X-band spectrometer was equipped with an ESPRIT computer system at 100 kHz magnetic field modulations. Instrument setting was as follows: magnetic field 336 ± 5 mT; 4 mW microwave power; sweep time 1 min; modulation width 0.32 mT; amp 400; 0.3 s time constant; and 20 s scan time. Intensity of ESR signals was corrected against MnO used as an internal standard.

Cell Culture

Human tongue squamous cell carcinoma-derived cell line HSC-4 (RCB1902) was provided by the Health Science Research Resource Bank (Osaka). Cells were cultured in Eagle's minimum essential medium (MEM; Nissui Seiyaku, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen Corp., CA), and 2 mM L-glutamine (22). Normal human tongue epithelial-like cells DOK were supplied by DS Pharma Biomedical Co., Ltd. (Osaka). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku) supplemented with 10% heat-inactivated FBS, and 4 mM L-glutamine at 37°C (23). Human fibrosarcoma HT-1080 cells were obtained from Japanese Cancer Research Resource Bank (JCRB), and were cultured in MEM supplemented with 10% heat-inactivated FBS (24). All the cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Colony Formation Assay

Cells were seeded on 60-mm dishes and allowed to attach, then the spent medium was exchanged to fresh cell culture medium prepared with NHE water or Milli-Q ultrapure reversed-osmosis water. After 4 or 6 days of incubation, the colonies were stained, photographed,

and the total colony numbers per dish and cell numbers per colony were evaluated under a microscope.

Invasion Assay

Invasiveness into the reconstituted basement membrane Matrigel (BD Biosciences, MA) was determined as described previously (11). Cells were trypsinized to form single-cell suspension in culture medium produced by dissolution of the medium powder in NHE water or Milli-Q water, which was also added to the upper and lower compartments of a chamber. After invasion for 0.5, 1, 2, or 3 h, cells that invaded through the Matrigel-precoated membrane filter (BD Biosciences) were stained and counted under a microscope.

Measurement of Intracellular Reactive Oxygen Species (ROS)

Intracellular ROS production was determined based on oxidative conversion of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (CDCFH-DA) (Molecular Probes, OR) to CDCF, which is indicative of the amount of intracellular peroxide production such as hydroperoxides, hydrogen peroxides, and diverse peroxides. Cells were rinsed with phenol red (PR)-free DMEM and incubated for 30 min in PR-free DMEM containing 10 μM CDCFH-DA at 37°C. After they were rinsed with PR-free DMEM four times, the fluorescence intensity was measured with a fluorescence microplate reader (CytoFluor 2350, Millipore, MA) with excitation and emission wavelengths of 485 and 530 nm, respectively. The cells were also observed using a fluorescence microscope ECLIPSE E600 (Nikon, Tokyo) with excitation and emission wavelengths of 485 and 520 nm, respectively, and pseudocolor images were produced using AquaCosmos software (Hamamatsu Photonics, Shizuoka).

Statistical Analysis

The unpaired Student's *t*-test was used to evaluate the significance of differences between groups, and the criterion of statistical significance was taken as $p < 0.05$.

RESULTS

Scavenging Effect Against Hydroxyl Radicals by NHE Water

To evaluate the potential activity of NHE water as a radical scavenger, ESR spin trapping method was used to assess hydroxyl radicals generated by Fenton reaction [the treatment of H_2O_2 with ferrous ions (Fe^{2+})]. When NHE water was added into hydroxyl radicals generation system, the signal intensity of DMPO-OH was recorded. In this measurement, a typical four-line ESR signal as DMPO-OH adduct (1:2:2:1 quartet) was detected when each of the samples was treated with H_2O_2 and Fe^{2+}. Figure 2 shows a scavenging effect of NHE water, which markedly diminished DMPO-OH signal intensities compared with Milli-Q water. Therefore, this result suggests that NHE water has a scavenging effect against hydroxyl radicals.

Repressive Effect of NHE Water on Colony Formation of HSC-4 or DOK Cells

We investigated whether NHE water could prevent a colony formation of human tongue carcinoma HSC-4 cells or normal human tongue epithelial-like cells DOK. As shown in Figure 3a and b, the colony number was decreased to 72% for HSC-4 cells cultured in cell culture media prepared with NHE water, but was hardly changed for DOK cells. Furthermore, when we focused on a cell number per colony, HSC-4 cells had a tendency to show a large cell number per colony compared with DOK cells. The cell number per colony was diminished to 66% by NHE water treatment in HSC-4 cells, whereas the number was not affected in DOK cells (Fig. 3c)). These results indicate that NHE water exerted repressive effects against both colony formation and cell proliferation in HSC-4 cells, but not DOK cells. Therefore, these results suggested that NHE water did not or scarcely affected normal cells, but preferentially repressed proliferation of carcinoma cells.

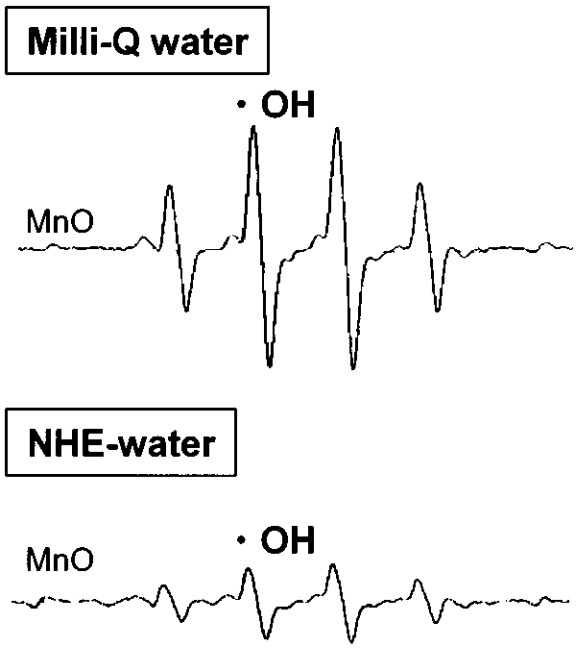

Figure 2. Scavenging effect of NHE water against hydroxyl radicals.

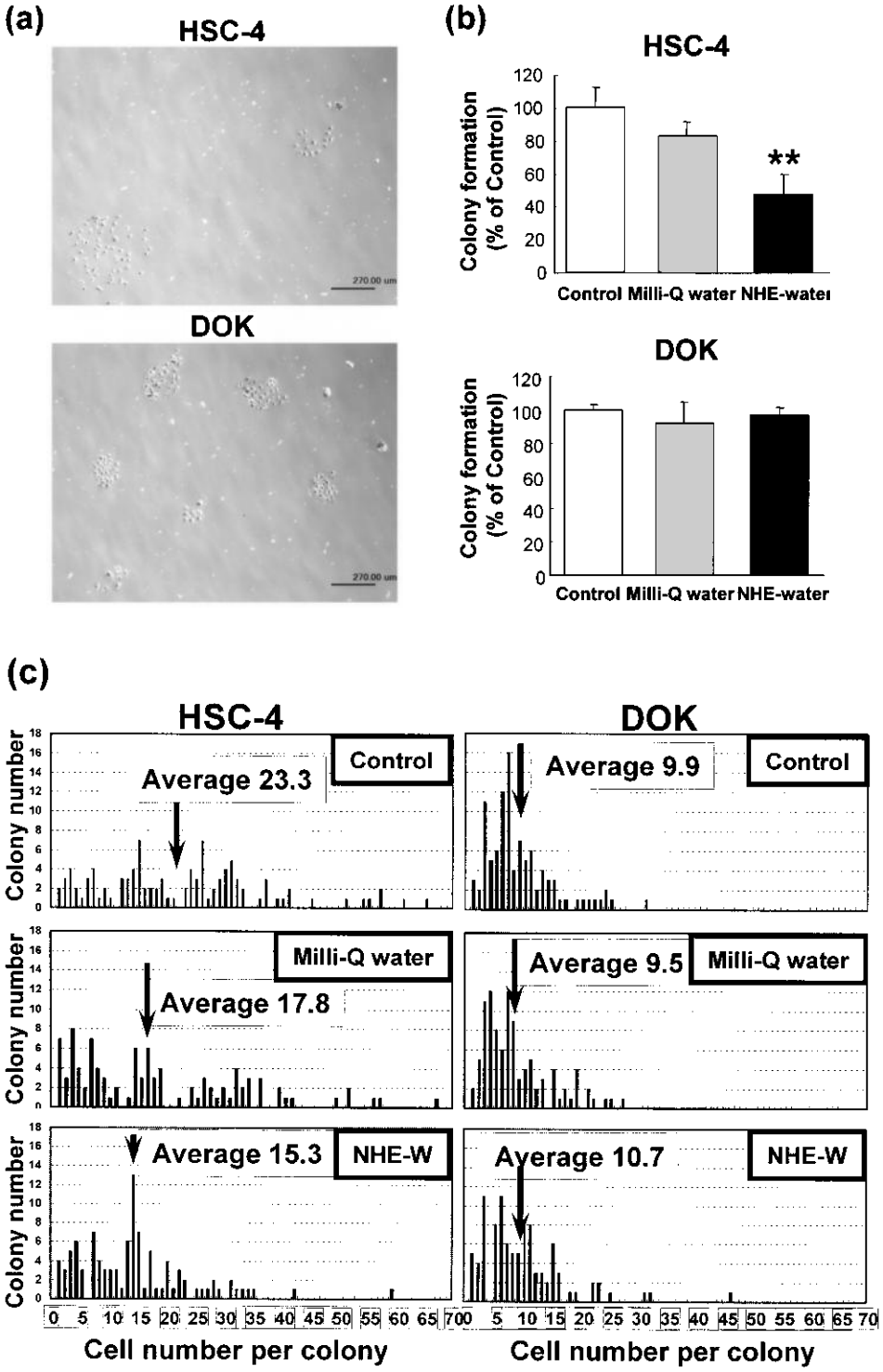

Figure 3. Repressive effect of NHE water on colony formation of human tongue carcinoma cells HSC-4 and normal human tongue epithelial-like cells DOK. Cells were seeded at a density of 2.0×10^3 cells in a 60-mm dish in cell culture medium. After 24-, 26-, and 28-h incubation, the medium was exchanged to new cell culture medium prepared with NHE water or Milli-Q water, whereas the control dishes received no medium exchange. After 4 days of incubation, the colonies were stained and photographed (a). And total colony numbers were evaluated by counting the colonies that consisted of more than a half of the average cell number per colony of nontreated HSC-4 or DOK cells, respectively (b). The distribution of cell numbers per colony was evaluated for 100 colonies under a microscope (c). Scale bar: 270 μm. Significantly different from Milli-Q water: $^{**}p < 0.01$. NHE-W: NHE water; average: an average of cell numbers per colony.

Repressive Effect of NHE Water on Intracellular Reactive Oxygen Species (ROS) Production in HSC-4 Cells

To examine whether NHE water could prevent intracellular ROS production in human tongue squamous carcinoma-derived cell line HSC-4, we quantified the intracellular ROS levels by fluorometry using the fluorescein derivative CDCFH-DA as a redox indicator. Our results showed that intracellular ROS levels markedly decreased by exchange to NHE water-containing medium (Fig. 4). This finding suggests that NHE water can suppress intracellular ROS such as hydroperoxides and hydrogen peroxides that might be routinely generated in HSC-4 cells.

Effect of NHE Water on Growth of HT-1080 Cells

We investigated the inhibitory effect of NHE water on tumor cell growth in human fibrosarcoma HT-1080 cells. The cell growth was markedly inhibited by incubation with cell culture media containing NHE water compared with Milli-Q water (Fig. 5). Besides the cartinostatic effects as mentioned above, carcinocidal effects such as extensive cell shrinkage and fragmentation were observed upon treatment with NHE water (Fig. 5a).

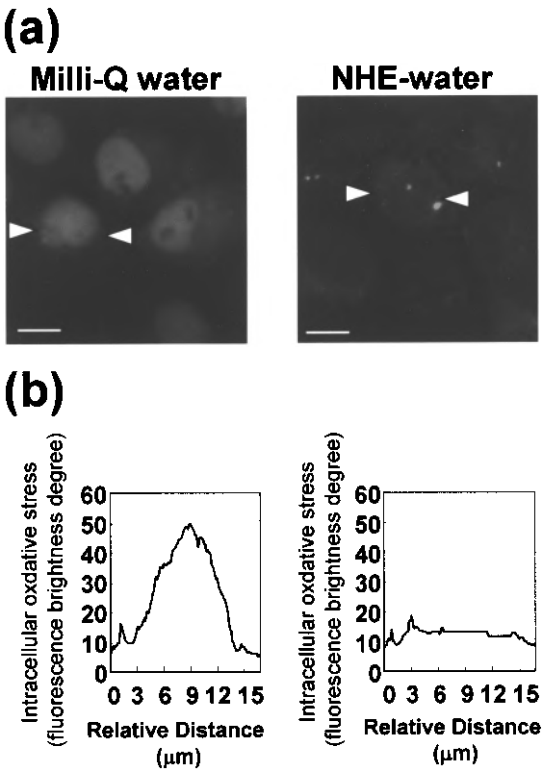

Figure 4. Preventive effect of NHE-water on intracellular ROS production in HSC-4 cells. (a) Cells were seeded at a density of 1.0×10^4 cells in four-well chamber slides and preincubated for 24 h. The cells were incubated for 0.5 h with medium produced with NHE water or Milli-Q water, and then treated with the redox indicator CDCFH-DA for 30 min. Thereafter, intracellular fluorescence was detected by fluorescence microscopy (ex: 485 nm; em: 530 nm). Scale bar: 10 μm. (b) The histograms represent the fluorescence distribution by the end-to-end linear scanning for the minor axis containing the nucleus in a typical single cell, which reflects the degree of the ROS and their intensities.

Repressive Effect of NHE Water on Invasion of HT-1080 Cells

Direct major mortal causes of cancer patients were occupied by tumor invasion and metastasis, which should be inhibited for cancer recurrence prevention and cancer therapy (25). We investigated the inhibitory effect of NHE water on tumor invasion through the reconstituted basement membrane Matrigel in human fibrosarcoma HT-1080 cells. The number of invasive cells was markedly lowered by incubation for 1–3 h with cell culture media containing NHE water compared with Milli-Q water (Fig. 6).

Repressive Effect of NHE Water on Intracellular ROS Production in HT-1080 Cells

Intracellular ROS levels in HT-1080 cells markedly decreased by incubation with cell culture media containing NHE water compared with Milli-Q water (Fig. 7). This finding suggests NHE water can suppress intracellular ROS that might be routinely generated in HT-1080 cells as in HSC-4 cells.

Temporal Changes of Dissolved Hydrogen Concentrations (DH) in NHE Water After Contact With Human Oral Cavity

When we take in water into the body, the oral cavity is the first place that the water contacts. Therefore, we examined the temporal changes of DH, oxido-reduced potential (ORP), and dissolved oxygen concentration (DO) of NHE water in the human oral cavity. Four healthy volunteers (three males, one female), who avoided eating for 2 h before an examination, put 45 ml of NHE water into their oral cavities for indicated times, and then spat out. Subsequently, each parameter was measured. We examined two patterns of "static holding" and "gargling." Static holding means keeping of water in the oral cavity for indicated times without moving of tongue and cheeks, whereas gargling means gargling of water in the oral cavity for indicated times with moving of tongue and cheeks. DH of NHE water was immediately decreased to 42% by putting it into an oral cavity (Fig. 8). Thereafter, the DH was maintained (>0.3 ppm) at least for 180 s in the case of static holding, whereas it was maintained (>0.2 ppm) at least for 30 s in the case of gargling. These values (0.2–0.3 ppm) were apprecia-

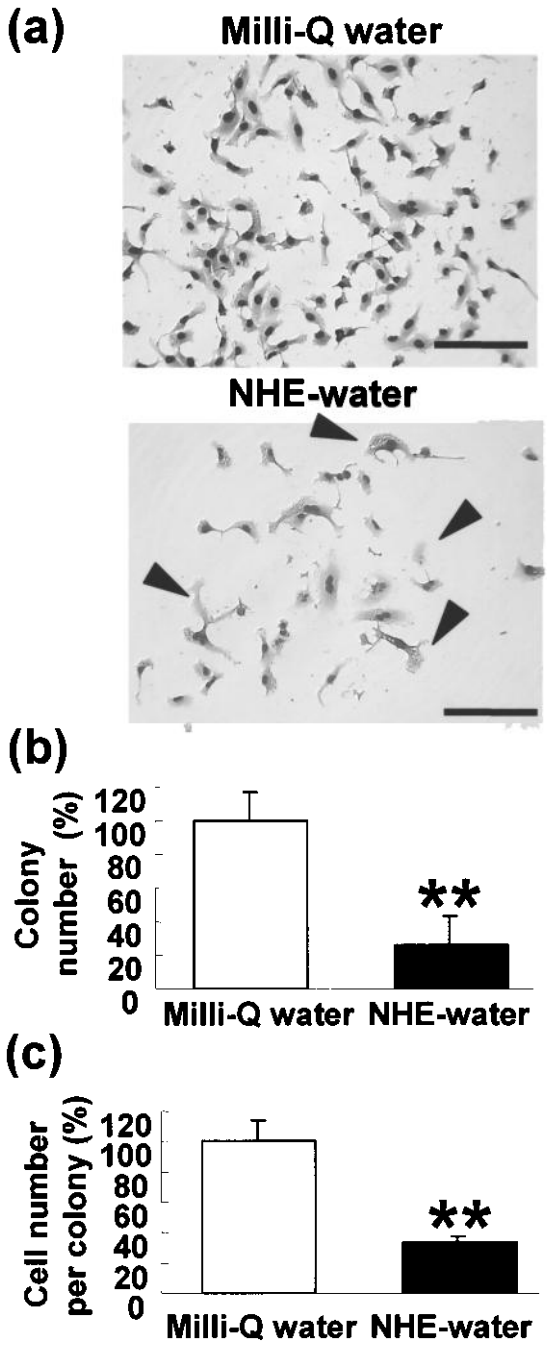

Figure 5. Repressive effect of NHE water on growth of HT-1080 cells. Cells were seeded at a density of 2.0×10^2 cells in a 60-mm dish containing cell culture medium. After 24-h incubation, the medium was exchanged to new cell culture media produced by NHE water or Milli-Q water. After incubation for 6 days, cells were stained, photographed (a), and counted as colony number per dish (b) and cell number per colony (c) under a microscope. Arrowheads indicate the degenerated cells. Scale bar: 100 μm. Significant difference from Milli-Q water: $^{**}p < 0.01$.

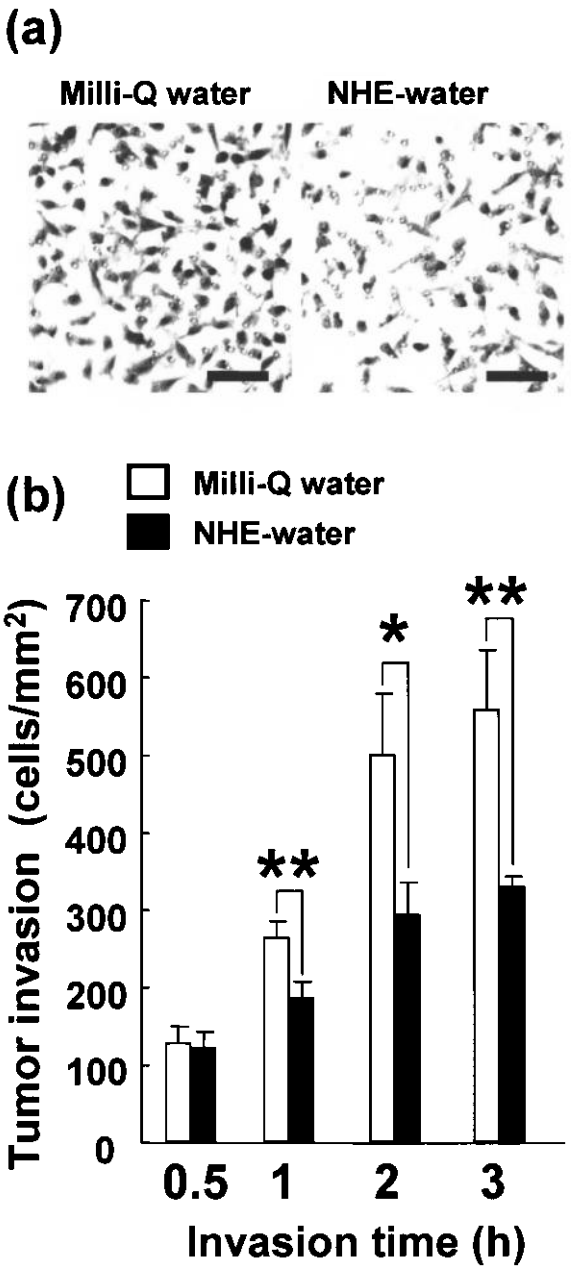

Figure 6. Repressive effect of NHE water on invasion of human fibrosarcoma HT-1080 cells. Cells were trypsinized to form single-cell suspension in culture medium prepared with NHE water or Milli-Q water, each of which was also added to the upper and lower compartments of a chamber. After in-vasion for 0.5–3 h, cells that invaded through the Matrigel-precoated membrane filter were stained, photographed (a), and counted under a microscope (b). The photographs show cells that completed the invasion through the Matrigel-containing membrane after 3-h incubation. Scale bar: 50 μm. Significantly different from Milli-Q water: $^{*}p < 0.05$, $^{**}p < 0.01$.

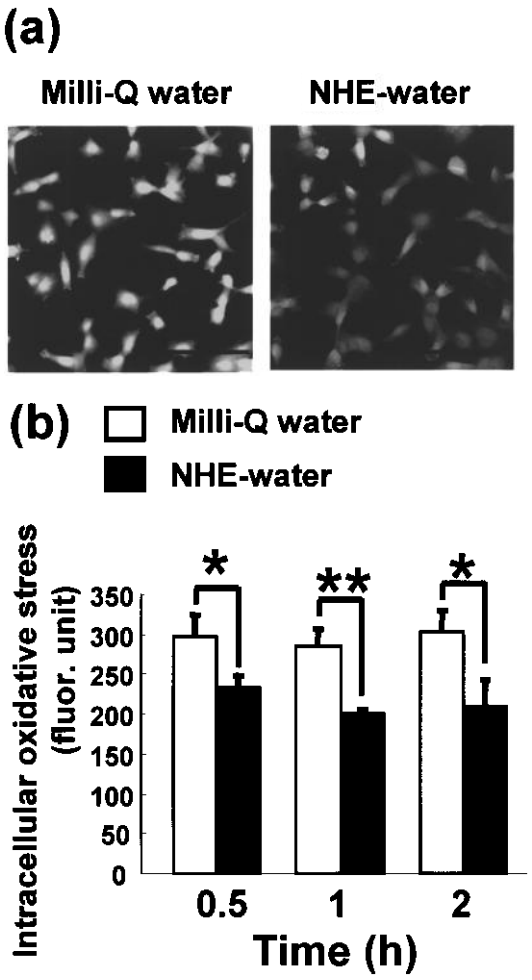

Figure 7. Repressive effect of NHE water on ROS production in HT-1080 cells. (a) Cells were seeded at a density of 3×10^4 cells/cm^2 in four-well chamber slides, and preincubated for 3 days. The cells were incubated for 2 h with medium produced with NHE water or Milli-Q water, and then treated with CDCFH-DA for 30 min. Thereafter, intracellular fluorescence was detected by fluorescence microscopy (ex: 485 nm; em: 530 nm). Scale bar: 100 μm. (b) Intracellular fluorescence was measured with a fluorescence microplate reader (ex: 485 nm; em: 530 nm). The bar represents the SD of triplicate wells. Significantly different from Milli-Q water: $*p < 0.05$; $**p < 0.01$.

bly high because the DH of purified water or tap water is less than 0.01 ppm. Therefore, it is thought that static holding is effective to keep higher DH in an oral cavity aiming at prevention against oral cavity cancers.

DISCUSSION

In the present study, NHE water was produced by a novel type of electrolysis device characteristic of inclusion of a hydrogen-trapping compressed-active carbon cartridge and a mode for nonmembrane separation of electrode compartments, and was evaluated for its antioxidant ability, anticancer effects, and stability in the oral cavity.

The ESR estimation on the ROS scavenging ability showed that hydrogen-dissolved water instead of Milli-Q pure water markedly reduced a signal intensity for hydroxyl radicals, indicating its ROS-scavenging ability (Fig. 2). This result is consistent with the previous reports that ERW possesses the scavenging effects on ROS such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical (19). The antioxidative effect

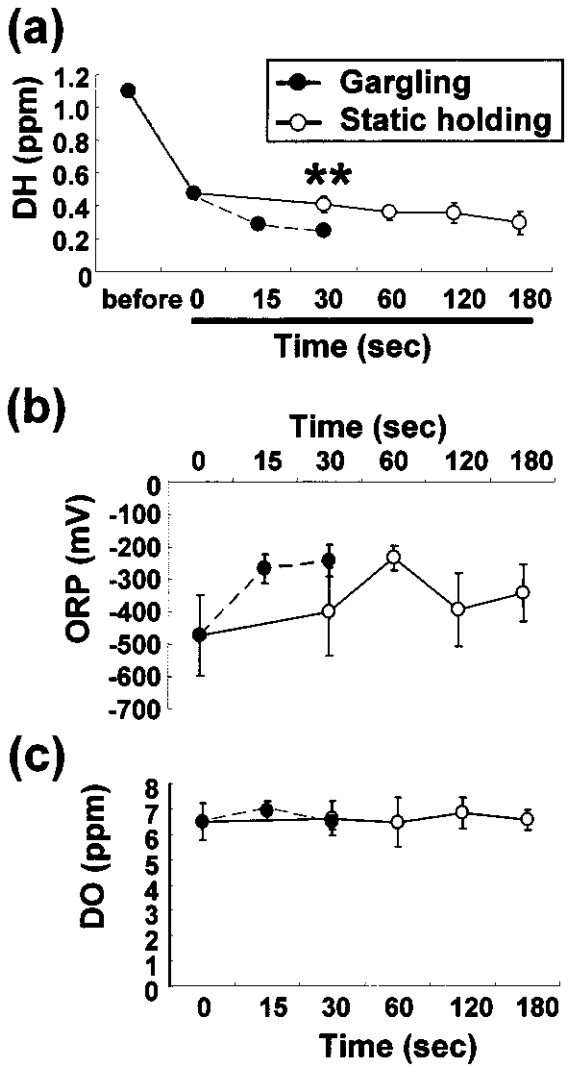

Figure 8. (a) Temporal changes of dissolved hydrogen concentrations (DH) in NHE water after contact with human oral cavity. Four healthy volunteers took 45 ml of NHE water in their oral cavities for indicated times, then spat out the water. Subsequently, each parameter of the water was measured. (b) ORP represents an oxido-reduced potential. (c) DO represents a dissolved oxygen concentration. Significantly different from gargling: $**p < 0.01$.

of NHE water is expected to reduce oxidative stress of cancer cells and exert anticancer effects, because features specific for cancer cells (e.g., increased cell proliferation, DNA synthesis, survival, cellular migration, invasion, tumor metastasis, and angiogenesis) were closely related to ROS (4–9).

Preferred prevention of NHE water against colony formation and cell proliferation of carcinoma cells over normal cells derived in common from human tongue, as shown in Figure 3, is considered to be due to the putative abundant intracellular influx of dissolved hydrogen molecules into carcinoma cells through the cell surface channel aquaporin, which remains to be analyzed in the future. In addition to inhibitory effects of NHE water on colony formation and cell proliferation of carcinoma cells, which correspond to "clonal growth" immediately after formation of a single cancer or mutated cell, NHE water was furthermore demonstrated to also inhibit "massive growth" as a cancer cell population, as shown in Figure 5, which corresponds to an early stage of cancer progression. And we also demonstrated that NHE water prevented invasion of fibrosarcoma cells HT-1080 through the reconstituted basement membrane (Fig. 6). Thus, NHE water can exert cytotoxic effects to tumor cells differentially over normal cells. Furthermore, the intracellular ROS levels were shown to be markedly decreased by NHE water (Figs. 4 and 7). These findings suggest that NHE water can scavenge ROS and maintain both intracellular and extracellular antioxidative potential, which were suggested to be responsible for its preventive effects on colony formation. Inhibitory effects on tumor angiogenesis, a prerequisite for tumor invasion, were recently reported for ERW (26), and are considered similarly for NHE water, which is therefore expected to be effective against metastasis or cancer progression.

On the other hand, it is thought that features characteristic for NHE water such as abundant hydrogen and neutral pH, unlike conventional alkaline ERW, can heighten an antioxidative potential because of saving consumptions of other antioxidants such as ascorbic acid. Moreover, we confirmed the excellent stability of NHE water in the human oral cavity. Although the DH (a dissolved hydrogen concentration) of NHE water was notably decreased immediately after taking water into the oral cavity, the decrease was thereafter drastically slowed down followed by retention at a DH level higher than in a case for tap water. Thus, NHE water is retained for a higher DH level, and may exert antioxidative and anticancer effects in human oral cavity. And we guess that the oral cavity may be a convincing one of target locus candidates suitable for NHE water, because the hydrogen is not easily lost at the first contact to the mouth from drinking NHE water.

The present study demonstrates that NHE water preferentially inhibited clonal growth of human tongue carcinoma cells over that of the corresponding normal cells and also inhibited tumor invasion of human fibrosarcoma cells concurrently with intracellular oxidant repression. An aim and scope in the present study might be restricted to the in vitro study because of placement of an important point on thorough establishment of the detailed in vitro data for preparation to the next in vivo study. Therefore, although NHE water is expected to become a useful tool for clinical application to anticancer therapy, further in vivo studies are necessary for clinical applications.

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