Brief Report

DOI: 10.5582/ddt.2022.01045

Electrolyzed water produced using carbon electrodes promotes the proliferation of normal cells while inhibiting cancer cells

Kyoko Nakamura*

Pharmaceutical Research and Technology Institute, Kindai University, Osaka, Japan.

SUMMARY We previously developed electrolyzed water (EW) using carbon electrodes and estimated its ability to inhibit the proliferation of human cervical carcinoma HeLa cells. In this study, we found that EW-containing media could not only inhibit HeLa cell proliferation, but were also capable of promoting the proliferation of normal human dermal fibroblasts (NHDF). In addition, the developed EW could reduce cytochrome *c*, as demonstrated by the cytochrome *c* reduction assay. Interestingly, EW with a greater pH, which was unable to inhibit HeLa cell proliferation, completely lost the ability to reduce cytochrome *c*. Our results indicate that EW has opposite effects on cancer and normal cell proliferation and has the ability to reduce cytochrome *c*. Based on our findings, we suggest the possibility that the reducing capacity of our developed EW may be involved in the significant inhibition of HeLa cell proliferation.

Keywords electrolyzed water, cell proliferation, reducing ability, normal cells, cancer cells

1. Introduction

Electrolyzed water (EW) is usually produced using an electrolytic cell with a separation baffle between the anode and cathode. The produced water from the anode side is called electrochemically oxidized water (EOW) or acidic water and is characterized by low pH values. On the other hand, electrochemically reduced water (ERW) is characterized by high pH values and is also known as alkaline water. The particular EOW generated by electrolysis of sodium chloride solution has a strong bactericidal effect and can disinfect viruses, such as hepatitis B virus (*1,2*). There have been many reports on ERW, which has been suggested to have many health benefits in humans (*3*). With regard to the inhibition of cell proliferation, ERW inhibited the growth of human lung adenocarcinoma A549 and HeLa cells as well as the growth of human normal fibroblast TIG-1 cells (*4,5*). A recent study showed that ERW treatment of MCF-7 and MDA-MB-453 human breast cancer cells or a TUBO cloned cell line derived from a mammary carcinoma of Her2-neu transgenic mice inhibited cell survival and induced apoptosis (*6*). This study also showed that ERW treatment delayed the development of mammary tumors in transgenic mice (*6*). Thus, although ERW inhibits the proliferation of cancer cells and shows anticancer potential, it also inhibits the proliferation of normal cells. Recently, we showed that EW produced without a separation baffle using carbon electrodes exhibited acidic pH (pH ≤ 3.5) and inhibited human HeLa cancer cell proliferation (*7*). Several researchers have reported that EW produced without a separation baffle is neutral (pH 6.6 to 7.8) (*8*) and slightly acidic (pH 5.0 to 6.5) (*9-10*). Therefore, it is interesting to note that our developed EW without a separation baffle exhibits an acidic pH. There have been very few studies on the effect of acidic EW on cell proliferation. Nakamura *et al*. examined the effect of slightly acidic EW, which inhibited adult human gingival fibroblasts and normal human dermal fibroblasts (NHDF) (*9*). However, there are no reports on the effects of acidic EW on cancer cell proliferation. Therefore, we first reported the potential ability of acidic EW produced using carbon electrodes to inhibit the proliferation of cancer cells (*7*). We have yet to examine the effect of our developed acidic EW on normal cell proliferation.

Reactive oxygen species (ROS) play an important role in cell proliferation, and cancer cells are characterized by elevated levels of ROS compared to normal cells (*11*). A previous study showed that increased cell number by mammary carcinogens is mediated through ROS generation (*12*). Hence, ROS have been implicated in anticancer drug mechanisms (*13*). Furthermore, ERW acts as a scavenger of ROS-induced oxidative stress and is useful for treating various diseases caused by ROS. Kim *et al*. showed that ERW consumption in diabetic *db/db* mice reduces blood glucose concentration and

prevents the loss of β-cell mass by oxidative stress (*14*). Moreover, ROS have been shown to be associated with cisplatin toxicity, including nephrotoxicity, and ERW has been reported to exert a nephroprotective effect against cisplatin-induced kidney toxicity and oxidative damage in mice (*15*). Shirahata *et al*. and Huang *et al*. reported that ERW can scavenge ROS with a high reducing ability (*16,17*). Thus, ERW is an antioxidant that scavenges ROS. However, we do not know whether our developed EW has a reducing ability.

The goal of the present study was to evaluate the reducing ability of our developed EW and its effect on NHDF cell proliferation. We first examined whether the developed EW affected the cell proliferation of the normal cell line NHDF and then, using a cytochrome *c* reduction assay, investigated whether it had a reducing ability. Our results demonstrate that our developed EW promotes the proliferation of NHDF while inhibiting that of cancer cells is associated with its reducing ability.

2. Materials and Methods

2.1. Preparation of electrolyzed water (EW) -containing media

The EW production method used in our study has been described in detail elsewhere (*7*). To investigate the effects of EW on the proliferation of cancer and normal cells, media was prepared using EW or tap water (W) (control). The EW had a pH of 3-3.5. Dulbecco's modified Eagle medium (DMEM) powder (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) was dissolved in W or EW (①-W or ①-EW, respectively) according to the manufacturer's instructions. We observed differently sized black dots in the ①-EW medium under the microscope in a previous study (data not shown) (*7*). Then, these media were filtered through a 0.2-μm filter (cellulose acetate membrane) to remove relatively large fragments that had broken off a piece of carbon electrodes (②-W and ②-EW). W-medium and EW-medium were sterilized by autoclaving at 121°C and then supplemented with 10% fetal bovine serum (FBS) (Nichirei Biosciences Inc., Tokyo, Japan), 0.584 g/L glutamine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 100 μg/mL streptomycin (Nacalai Tesque, Kyoto, Japan), 100 U/mL penicillin (Nacalai Tesque), and 10% sodium hydrogen carbonate (Wako). Finally, the W-culture medium had a pH of about 8.3 and the EW-culture medium a pH of about 8.1.

2.2. Cell culture

In this study, a human cancer cell line (HeLa) and a normal cell line (NHDF) were used. HeLa and NHDF cells were maintained in low-glucose DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin. The cells were grown in culture dishes under standard conditions at 37°C and 5% CO_2. To study the effects of EW on the proliferation of HeLa and NHDF cells, these cells were cultured in EW or W (control) media.

2.3. Cell proliferation assay

Cell proliferation assays were performed on HeLa and NHDF cells as described previously (*7*). Briefly, cultured cells were trypsinized and collected by centrifugation at 800× g for 5 min. Subsequently, the cells were resuspended in EW or W control culture media, and 8 × 10^4 cells were seeded in culture flasks, followed by incubation for 3 d at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The effects of EW on cell proliferation were assessed by counting the number of viable cells. HeLa and NHDF cells were trypsinized, stained with trypan blue, and counted using a hemocytometer daily for 3 days.

2.4. Cytochrome *c* reduction assay

Cytochrome *c* reduction was monitored by measuring the increase in absorbance at 550 nm at 37°C using a spectrophotometer. Cytochrome *c* was soluble in 0.15 M NaCl 20 mM Tris-HCl, pH 7.4 (1 mM cytochrome *c* solution). The assay was carried out in a 1 cm cuvette, and the reaction mixture consisted of 50 μL Tris-HCl pH 7.5 (1 M), 20 μL cytochrome *c* solution (1 mM), and 830 μL distilled water. To initiate the reaction, 100 μL of EW test samples were added to the reaction mixture in a cuvette. The activity was expressed as the change in absorbance at 550 nm and was monitored for a period of 7200 sec (2 h).

3. Results and Discussion

Our previous experiments showed that EW generated using a special carbon electrode and exhibited acidic pH (pH ≤ 3.5) inhibits the proliferation of human cervical carcinoma HeLa cells (*7*). The EW-containing medium used in this study inhibited HeLa cell proliferation (Figure 1A). Although it is known that EW-containing medium affects the proliferation of cancer cells, the effect of EW-containing medium on the proliferation of normal cells remains unknown. To investigate the effect of the EW-containing medium on the proliferation of normal cells, we utilized the NHDF cell line. Surprisingly, the proliferation of NHDF was remarkably upregulated by the EW-containing medium, whereas the proliferation of NHDF cultured in the W control medium exhibited slower proliferation for 3 days (Figure 1B). The growth of NHDF cells in the W control or the EW-containing medium was also observed as shown in Figure 1C. Our developed EW was containing carbon compounds observed very small black dots (Figure 1C) and we found that these carbon compounds are associated with

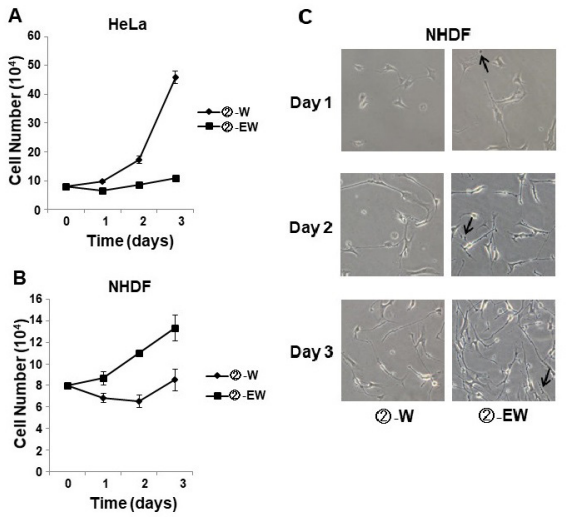

Figure 1. The effect of W and EW media on the proliferation of human cancer cell line HeLa and human normal cell line NHDF. Each ②-W and ②-EW was prepared by filtration (0.2 μm) of ①-W or ①-EW, respectively. Cells were seeded at a density of 8×10^4 cells in flasks containing each culture medium. Cells were counted daily for 3 d (**A**; HeLa cells, **B**; NHDF cells). NHDF cells were photographed for 3 days at ×10 (**C**). The arrow indicates very small black dots. Data are expressed as the mean ± SD of three independent experiments.

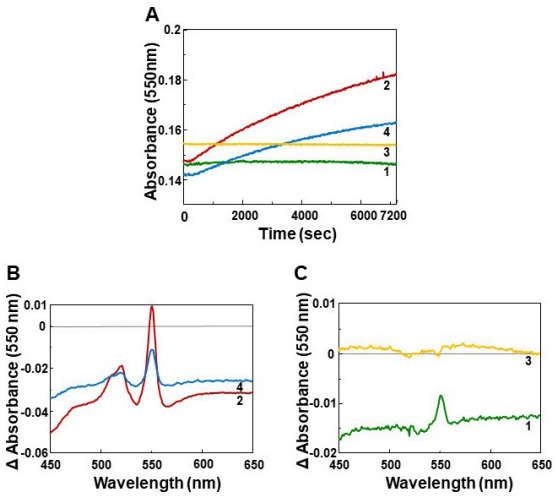

Figure 2. Reduction of cytochrome *c* by EW. Cytochrome *c* reduction by EW 17 h (1), EW 72 h (2), EW high pH (3), and frozen EW (4) (**A**). Each EW 17 h and EW 17 h was prepared by electrolysis of tap water for 17 h and 72 h, respectively. EW high pH meant that EW had a pH of 6.21. Number 4 denotes that EW was stored at − 20°C. Time difference absorption spectra (2 h aged spectra minus the corresponding 1 min aged ones) of four EW samples (**B**) and (**C**). Up to 2 h of incubation, EW 72 h evidenced increases in absorbance at 550 nm.

the inhibition of HeLa cell proliferation in our previous study (*7*). These results indicate that EW inhibits cancer cell proliferation and promotes normal cell proliferation. Although few studies on the effect of EW on normal cell proliferation have been reported, Shirahata *et al*. reported the anti-proliferative effects of ERW on normal cells (*4,5*). On the other hand, Satoh *et al*. reported that neutral pH hydrogen-enriched EW exerted repressive effects against both colony formation and cell proliferation in human tongue carcinoma HSC-4 cells, but not in normal human tongue epithelial-like cells DOK (*8*). Moreover, Tsai *et al*. reported that treatment with a combination of ERW and glutathione had no inhibitory effect on the viability of normal peripheral blood mononuclear cells, whereas cytotoxic effects were observed in leukemia cells (HL-60) (*18*). This phenomenon of promotion of normal cell proliferation in EW-containing culture medium has never been reported before. Thus, it is noteworthy that our developed EW stimulates normal cell proliferation while inhibiting that of cancer cells.

While ERW is considered to play a potentially beneficial role in inhibiting cancer cell proliferation, several findings have also indicated that ERW has the ability to scavenge ROS such as hydrogen peroxide (H_2O_2), with has a high reducing ability (*16,17*). ROS scavengers are a group of antioxidant substances that can be considered reducing agents (*19*). Furthermore, Komatsu *et al*. reported that scavenging ROS by ERW is associated with the suppressive effect of ERW on the growth of cancer cells (*20*). Satoh *et al*. reported that neutral pH hydrogen-enriched EW caused growth inhibition of cancer cells, together with scavenging of intracellular oxidants (*8*). Thus, the reducing ability may contribute to the inhibitory effect of ERW on cancer cell proliferation. Since our developed EW could inhibit the proliferation of cancer cells, we investigated whether it possessed a reducing ability. To evaluate the potential activity of EW as a reducing agent, a cytochrome *c* reduction assay was performed with EW electrolyzed for 17 h (EW 17 h) and 72 h (EW 72 h). EW used in our study of cell proliferation was electrolyzed for 72 h and has a pH of 3-3.5 (*7*). EW 17 h has a pH of less than 4; however, it remains unknown whether EW 17 h inhibits the proliferation of HeLa cells. A reduction in cytochrome *c* level was observed at 550 nm (*21*). As shown in Figure 2A and 2B, EW 72 h was able to reduce cytochrome *c*. On the other hand, EW 17 h barely reduced cytochrome *c* (Figure 2A), and a slight reducing ability of EW was observed (Figure 2C). These results suggest that the EW developed in this study has reducing ability and that 72 h of electrolysis may be required to gain this reducing ability. Furthermore, we evaluated the reducing ability of EW at a pH greater than 3.5. In a previous study, we found that EW with at a pH greater than 3.5 was unable to inhibit HeLa cell proliferation (*7*). EW with a pH of 6.21 had no reducing ability (Figure 2A and 2C). Thus, our findings indicate that the reducing ability of EW contributes to the inhibition of HeLa cell proliferation.

ROS also plays an important role in the control of cellular senescence. Cellular senescence was first reported by Hayflick and Moorhead (1961). They discovered that cultured normal human fibroblasts had a slow proliferative rate and a finite proliferative capacity,

now termed cellular senescence (*22*). Increased ROS levels in normal cells cause oxidative stress, resulting in cellular senescence (*23*). Thus, cultured normal cells are limited in the number of times they can divide and exhibit a process termed cellular senescence or cellular aging (*24*). However, cancer cells generally have higher levels of ROS than normal cells, and the increase in ROS levels contributes to the promoting of cancer cell proliferation (*25,26*). Hence, as shown in Figure 1, it could be speculated that ROS levels are associated with the proliferative rate of normal (NHDF) or cancer (HeLa) cells cultured in a W control medium. Conversely, normal (NHDF) or cancer (HeLa) cell proliferation was enhanced or inhibited in the EW-containing medium when compared with that in the W control medium (Figure 1). It is thought that one of the reasons for these results may be the reduced ability of the EW-containing medium to lower cellular ROS levels.

Although EW was stored at 4°C in our previous study, it was dispensed into 50 mL tubes and stored at −20°C in this study. We also assessed the reducing ability of the frozen EW. Surprisingly, the reducing ability of EW was decreased upon freezing (Figure 2A and 2B). In a previous study, we found that carbon compounds present in EW were associated with inhibition of HeLa cell proliferation (*7*). Therefore, it is possible that carbon compounds have a reducing ability, which is affected by frozen storage, whereas storage at 4°C has no effect. We hope to investigate these possibilities in future studies.

In conclusion, we found that the developed EW not only inhibited tumor cell proliferation, but also stimulated normal cell proliferation. We also show that EW has the ability to reduce cytochrome *c*, suggesting that the reducing ability of EW may contribute to the inhibition of cancer cell proliferation in an EW-containing medium. Thus, our results indicate that the developed EW has dual functions in inhibiting cancer cell proliferation and promoting normal cell proliferation. Previous studies have shown that the inhibitory effect of carbon compounds in EW on tumor cell proliferation can be removed by washing cultured cells with PBS. We then suggested that carbon compounds predicted to be associated with cell growth inhibition are reversibly attached to the cell surface. Therefore, as a treatment for cancer, particularly gastric cancer, carbon compounds in EW may be better administered orally than injected into patients. We hope that EW with reducing ability will be useful for developing anti-cancer agents.

Acknowledgements

The authors thank Mr. Yoshihiro Kojima and Mr. Masashi Zenki (Toyo Tanso Co., Ltd., Osaka, Japan) for providing special carbon electrodes and for performing the cytochrome *c* reduction assay and data analysis.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Okuda R, Sasazaki H, Kanehira M, Okabe T, Abe S, Tagami A, Iwamatsu Y, Miya Y, Shimizu Y. Bactericidal effect of high oxidation potential water viewed from a morphological change. Jpn J Conserv Dent. 1994; 37:755-765. (in Japanese)
2. Hati S, Mandal S, Minz PS, Vij S, Khetra Y, Singh BP. Electrolyzed oxidized water (EOW): Non-thermal approach for decontamination of food borne microorganisms in food industry. Food Nutr Sci. 2012; 3:760-768.
3. Shirahata S, Hamasaki T, Teruya K. Advanced research on the health benefit of reduced water. Trends Food Sci Technol. 2012; 23:124-131.
4. Shirahata S, Kabayama S, Kusumoto K, Goto M, Teruya K, Otsubo K, Morisawa S, Hayashi H, Katakura K. Electrolyzed reduced water which can scavenge active oxygen species suppresses cell growth and regulates gene expression of animal calls. In: New Development and New Applications in Animal Cell Technology (Merten OW *et al*., eds.). Kluwer Academic Publishers, the Netherlands, 1998; pp. 93-96.
5. Shirahata S, Murakami E, Kusumoto K-I, amashita M, Oda M, Teruya K, Kabayama S, Otubo K, Morisawa S, Hayashi H, Katakura Y. Telomere shortening in cancer cells by electrolyzed-reduced water. In: Animal Cell Technology: Challenges for the 21st Century (Ikura K, ed.). Kluwer Academic Publishers, Dordecht, 1999; pp. 355-359.
6. Frajese GV, Benvenuto M, Mattera R, Giampaoli S, Ambrosin E, Bernardini R, Giganti MG, Albonici L, Dus I, Manzari V, Modesti A, Mattei M, Bei R. Electrochemically reduced water delays mammary tumors growth in mice and inhibits breast cancer cells survival *in vitro*. Evid Based Complement Alternat Med. 2018; 2018, 4753507.
7. Nakamura K, Muraoka O. Effect of electrolyzed water produced using carbon electrodes on HeLa cell proliferation. Biosci Trends. 2018; 11:688-693.
8. Saitoh Y, Okayasu H, Xiao L, Harata Y, Niwa N. Neutral pH hydrogen-enriched electrolyzed water achieves tumor-preferential clonal growth inhibition over normal cells and tumor invasion inhibition concurrently with intracellular oxidant repression. Oncol Res. 2008; 17:247-255.
9. Nakamura T, Oda H, Sato S. Effects of slightly acidic electrolyzed water on oral pathogens and human gingival fibroblasts. Jpn J Conserv Dent. 2010; 53:570-578. (in Japanese)
10. Ye Z, Wang S, Chen T, Gao W, Zhu S, He J, Han Z. Inactivation mechanism of Escherichia coli induced by slightly acidic electrolyzed water. Sci Rep. 2017; 7:6279.
11. Stzatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. 1991; 51:794-798.
12. Burdick AD, Davis JW 2nd, Liu KJ, Hudson LG, Shi H, Monske ML, Burchiel SW. Benzo(a)pyrene quinones increase cell proliferation, generate reactive oxygen species, and transactivate the epidermal growth factor

receptor in breast epithelial cells. Cancer Res. 2003; 63:7825-7833.
13. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009; 8:579-591.
14. Kim MJ, Jung KH, Uhm YK, Leem KH, Kim HK. Preservative effect of electrolyzed reduced water on pancreatic beta-cell mass in diabetic *db/db* mice. Biol Pharm Bull. 2007; 30:234-236.
15. Cheng TC, Hsu YW, Lu FJ, Chen YY, Tsai NM, Chen WK, Tsai CF. Nephroprotective effect of electrolyzed reduced water against cisplatin-induced kidney toxicity and oxidative damage in mice. J Chin Med Assoc. 2018; 81:119-126.
16. Shirahata S, Kabayama S, Nakano M, Miura T, Kusumoto K, Gotoh M, Hayashi H, Otsubo K, Morisawa S, Katakura Y. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochem Biophys Res Commun. 1997; 234:269-274.
17. Huang KC, Yang CC, Lee KT, Chien CT. Reduced hemodialysis-induced oxidative stress in end-stage renal disease patients by electrolyzed reduced water. Kidney Int. 2003; 64:704-714.
18. Tsai CF, Hsu YW, Chen WK, Ho YC, Lu FJ. Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells due to electrolyzed-reduced water and glutathione. Biosci Biotechnol Biochem. 2009; 73:280-287.
19. V Rajeswer Rao. Advances in structure and activity relationship of coumarin derivatives. Academic Press-Elsevier, Amsterdam, the Netherlands, 2016; pp. 47-76.
20. Komatsu T, Kabayama S, Hayashida A, Nogami H, Teruya K, Katakura Y, Otsubo K, Morisawa S, Shirahata S. Suppressive effect of electrolyzed-reduced water on the growth of cancer cells and microorganisms. In: Animal Cell Technology: From target to market. Kluwer Academic Publishers, Dordrecht, 2001; pp.220-223.
21. Massey V. The microestimation of succinate and the extinction coefficient of cytochrome *c*. Biochim Biophys Acta. 1959; 34:255-256.
22. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961; 25:585-621.
23. Kondoh H, Lleonart ME, Bernard D, Gil J. Histol Histopathol. 2007; 22:85-90.
24. Hayflick L. The cell biology of human aging. N Engl J Med. 1976; 295:1302-1308.
25. Snezhkina AV, Kudryavtseva AV, Kardymon OL, Savvateeva MV, Melnikova NV, Krasnov GS, Dmitriev AA. ROS generation and antioxidant defense systems in normal and malignant cells. Oxid Med Cell Longev. 2019; 2019:6175804.
26. Perillo B, Donato MD, Pezone A, Zazzo ED, Giovannelli P, Galasso G, Castoria G, Migliaccio A. ROS in cancer therapy: the bright side of the moon. Exp Mol Med. 2020; 52:192-203.

Received June 10, 2022; Revised July 22, 2022; Accepted August 16, 2022.

**Address correspondence to:*
Kyoko Nakamura, Pharmaceutical Research and Technology Institute, Kindai University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502, Japan.
E-mail: kyoko@phar.kindai.ac.jp

Released online in J-STAGE as advance publication August 21, 2022.