


Effects of Electrolyzed Water on the Growth of Oral Pathologic Bacteria Species and its Cytotoxic Effects on Fibroblast and Epithelial Cells at Different pH Values

Surena Vahabi¹, DDS, MSc; 
Mehdi Shokri², PhD;
Masih Lazar³, DDS

¹Dental Research Center, Research Institute of Dental Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran;

²Department of Dental Biomaterials, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran;

³School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Correspondence:

Surena Vahabi, DDS, MSc;
School of Dentistry, Daneshjoo Blvd., Evin, Shahid Chamran Highway, Postal code: 1983963113, Tehran, Iran

Tel: +98 21 22403075

Fax: +98 21 22403194

Email: ivsure1@gmail.com

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What's Known

- Microbial plaque-induced oral diseases are among the most common diseases worldwide. Nonetheless, there is still the need for an effective, safe, and ideal disinfectant.
- Some mechanisms have been postulated for the antibacterial activity of the electrolyzed water. However, some negative effects such as cytotoxicity have been reported.

What's New

- Electrolyzed water types with different pH ranges showed efficient antibacterial activities against four oral bacteria within 2 hours. Acidic electrolyzed water and basic electrolyzed water eradicated more bacteria compared to mildly basic electrolyzed water.
- The cytotoxicity assay indicated the adverse effect of these electrolyzed water types on L929 and HeLa cells, at least in the 30-second treatment period.

Abstract

Background: Microbial plaque-induced oral diseases are among the most common diseases worldwide. The present study aimed to compare the antimicrobial effect of electrolyzed water (EW), (acidic, mildly basic, and basic) on the growth of bacterial species producing dental plaque and to assess their cytotoxicity on fibroblasts and epithelial cells.

Methods: The study was performed at Shahid Beheshti University of Medical Sciences in 2019. Several bacterial species (*Streptococcus salivarius*, *Staphylococcus aureus*, *Lactobacillus casei*, and *Aggregatibacter actinomycetemcomitans*) were treated with different EW types at three pH values (3, 9, and 11) for 30 seconds and subsequently, the colonies were counted. The cytotoxic effect of these EW types was evaluated on HeLa and L929 cell lines at 30 seconds, 1 minute, and 5 minutes. GraphPad Prism 6.0 was used for statistical analysis. The Kruskal-Wallis test followed by Mann-Whitney U and one-way analysis of variance followed by Tukey's test were used to analyze bacterial activity and cell cytotoxicity, respectively. $P < 0.05$ was considered statistically significant.

Results: EW types significantly inhibited bacterial growth at all pH values. The strongest antibacterial activity of EW was against *A. actinomycetemcomitans* ($P < 0.001$) and the least significant antibacterial activity was against *S. aureus* ($P < 0.001$). The EW types showed increased cytotoxic activity against L929 cells as the treatment time increased. The most cytotoxic effect was seen at 5 minutes of treatment in all EW types compared with the negative control group ($P < 0.0001$). This negative cytotoxic effect on HeLa cells was shown just after 30 seconds and viable cell counts increased over time, reaching its highest value at 5 minutes of treatment with basic EW ($P < 0.0001$).

Conclusion: The contradictory effects of the EW types on both HeLa and fibroblasts, in addition to variable results at different exposure times, indicated that the effect of EW could vary depending on cell types and treatment periods.

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Introduction

Gum diseases and periodontitis are common dental problems,

mainly caused by dental plaque and the accumulation of oral colonizing bacteria. Microbial plaque-induced oral diseases are among the most common diseases worldwide, resulting in gum inflammation and periodontal diseases if plaque formation is not prevented.¹ Currently, mechanical debridement (brushing, flossing) and chemical treatment (mouthwash) are being used to reduce and control dental plaque.² However, because of their inefficiency and side effects, there is still the need for an effective, safe, and ideal disinfectant.³

Electrolyzed water (EW) has a variety of applications; from disinfection to improving digestive functions and enhancing the quality of agricultural products in the food industry.⁴⁻⁶ EW is prepared by electrolysis of tap water by ionizer machines. Depending on the electrolysis process conditions, five types of EW are produced, namely basic (pH: 10-12), mildly basic (pH: 8-10), neutral (pH: 6.5-7.5), slightly acidic (pH: 5-6.5), and acidic (pH: 3-5). Acidic electrolyzed water (AEW) is produced in the anode chamber of an ionizer machine and is mainly used in the medical field (wound cleaning and disinfection of instruments and surfaces). Whereas basic electrolyzed water (BEW) is produced in the cathode chamber and, because of its health benefits often applied to suppress oxidative stress-related diseases as well as for its anti-cancer and anti-diabetes properties.⁷⁻¹² It has been reported that these water types have the ability to destroy all types of anaerobic and aerobic bacteria that cause dental decay. Some mechanisms have been postulated for this antibacterial activity such as the effect of a high or low pH, oxidation-reduction potential, chlorine concentration, and EW's high concentrations of molecular hydrogen.^{13, 14} However, some researchers have also reported negative effects such as the cytotoxicity of acidic water and growth retardation in high basic water.¹³ Acidic water with low chlorine concentrations also has a lower rate of tissue destruction compared with water with higher chlorine concentrations at the same pH value.¹⁵

Despite various studies on EW, its full range of properties is still unknown and there is insufficient evidence on its applications and disinfectant use on microbial contamination. Hence, the present study aimed to compare the antimicrobial effects of EW (at three pH values) on the microorganisms of microbial plaque. In addition, the cytotoxic effect of these water types was assayed by exposure to epithelial and fibroblast cell lines at three time periods.

Materials and Methods

The study was conducted in 2017 in the

Biomaterial Department of Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The study protocol was approved by the institutional ethics committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Test Solutions

EW was prepared at three pH values (3, 9, and 11) using a 7-plate water ionizer (iWater-sharp, Korea) in accordance with the manufacturer's instruction. These EW types were used to treat different bacteria and cells. The time span between water preparation and cell treatment was about 2 hours. Chlorhexidine (CHX) 0.2% (Iran Najo, Iran) was used as the gold standard antibacterial mouthwash (positive control) and CHX 0.009% was used for treatments. The latter was prepared by diluting CHX 0.2% with a sodium phosphate buffer (PBS: pH 7) purchased from Sigma, USA.

Bacteria

The bacterial species, *Streptococcus salivarius* PTCC 1448 (*S. salivarius*), *Staphylococcus aureus* PTCC 1431 (*S. aureus*) and *Lactobacillus casei* PTCC 1608 (*L. casei*) were obtained from the Iranian Research Organization for Science and Technology (IROST, Iran). *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans* JP2) was purchased from the American Type Culture Collection (ATCC, USA).

Gas packs, brain heart infusion (BHI) broth, and "de Man, Rogosa, and Sharpe" (MRS) agar media were purchased from Merck, Germany. *S. salivarius* and *S. aureus* were aerobically incubated in a BHI broth at 37 °C. *A. actinomycetemcomitans* (BHI broth) and *L. casei* (MRS broth) were incubated anaerobically in an anaerobic jar with a gas pack (anaerocult® A and anaerocult® C, respectively) to absorb disposable O₂ and generate CO₂. All bacterial species were cultured to the logarithmic phase prior to the start of the experiments. The logarithmic phase was measured by plotting the cell growth (absorbance at 600 nm) versus the incubation time.

Cells and Cultures

All cell culture materials were purchased from Gibco (UK), unless otherwise stated. The human immortal epithelial cell line, HeLa (NCBI-C115), and mouse fibroblastic cell line, L929 (NCBI-C161), were purchased from the Pasteur Institute Resources Bank (Tehran, Iran). The cells were grown on Dulbecco's modified Eagle's medium supplemented with

10% heat-inactivated fetal bovine serum and a complex of 100 U/mL penicillin G (Gibco, UK) and 100 mg/mL streptomycin (Gibco, UK) in 25 cm² tissue culture flasks (SPL, South Korea) at 37 °C in an atmosphere of 5% CO₂ and 95% air. The medium was replaced every 3 days and the cells were detached using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), purchased from Sigma (USA), and transferred to 75 cm² flasks until the cells reached about 80% confluency.

Antibacterial Activity of Electrolyzed Water

The above-mentioned organisms at the logarithmic phase were centrifuged at 3,000 ×g for 15 minutes and the supernatants discarded. The pellet was washed two to three times with phosphate buffered saline (Sigma, USA) and the cells were then harvested by centrifugation. Fresh sterilized buffer was added and cell suspensions (approximately 10⁸ cells/ml) were prepared. The bacterial species were divided into six groups and each group was vortexed for 30 seconds with 1.0 mL of PBS (group 1), CHX 0.2% (group 2), CHX 0.009% (group 3), AEW-pH3 (group 4), mildly basic electrolyzed water (MBEW)-pH9 (group 5), and BEW-pH11 (group 6). One loop of each sample was spread on an agar plate and incubated for 3-4 days on the based conditions as described above. The colony-forming unit (CFU/mL) was evaluated by counting the *S. salivarius*, *S. aureus*, and *A. actinomycetemcomitans* colonies formed on the BHI agar and *L. casei* formed on MRS agar as a bactericidal effect. All tests were performed in triplicate.

Cell Cytotoxicity Assay

The MTT assay was performed to analyze the potential cytotoxic effect of the EW types. The confluent cells in the 75 cm² flasks were detached by trypsin-EDTA (Sigma, USA) and centrifuged at 800 ×g for 5 minutes. The supernatant was discarded and replaced with a freshly prepared medium to prepare a cell suspension of approximately 1.5×10³ cells/mL. Two hundred μL of the cell suspension was plated out into each well of 96-well plates and incubated at 37 °C for 24 hours in a CO₂ incubator. The medium was removed and the cells were washed with PBS. The cells were divided into seven groups and each group was incubated with 100 μL of PBS (group 1), distilled water (group 2), CHX 0.2% (group 3), CHX 0.009% (group 4), AEW (group 5), MBEW (group 6), and BEW (group 7) for 30 seconds, 1 minute, and 5 minutes. The solutions were then discarded and the cells were washed twice with PBS and 100 μL of medium containing 10%

yellow MTT salt (0.5 mg/mL; Sigma-Aldrich, USA) was added to the cells in the dark. The cells were then returned to the CO₂ incubator until formazan crystals were observed using a microscope (~3 hours). Subsequently, the MTT was replaced with an equal volume of dimethyl sulfoxide solvent to solubilized precipitate purple crystals. One hundred μL of each well (six repetitions) was transferred into each well of 96-well plates and the optical density (OD) was measured at 570 nm and 620 nm (reference) wavelengths using a microtiter plate reader instrument (Anthos 2020, Austria). The percentage of the subtracted OD (570-620) of the treated cells to the subtracted OD of untreated cells (control) was used as a percentage of cell viability.

Statistical Analysis

The Kruskal-Wallis tests and Mann-Whitney U tests were used for intragroup comparisons. The results of the MTT assay were analyzed using the one-way analysis of variance (Tukey's multiple comparison *post hoc* test) with GraphPad Prism 6.0 for Windows (GraphPad Software Inc., USA). P<0.05 was considered statistically significant.

Results

Bactericidal Effect of Electrolyzed Water Types

In comparison with other groups, the CHX 0.2% group had the highest effect with a 100% reduction of antibacterial activity and no viable bacteria after treatment with this solution (table 1). However, all EW types had a significant (P<0.001) and strong antibacterial efficiency against all species in the AEW, MBEW, and BEW groups compared with the negative control. The maximum antibacterial activity of the EW at pH 3, pH 9, and pH 11 among the selected bacterial species was against *A. actinomycetemcomitans* with a bacterial reduction of 100%, 99.3%, and 100%, respectively. The least antibacterial activity at those pH values was against *S. aureus* with a bacterial reduction of 98.04%, 89.16%, and 88.75%, respectively. At three EW types had an equal antibacterial potency against *L. casei* with 99.99% CFU/mL reduction. The CFU reduction of *S. salivarius* was 99.92%, 99.3%, and 99.94% for EW types with AEW, MBEW, and BEW, respectively. CLX 0.009% had a significant antibacterial effect (P<0.001) against *A. actinomycetemcomitans*, *S. salivarius*, and *L. casei*. However, this bacterial reduction was observed to be significant (but much weaker, P<0.002) against *S. aureus* compared with other bacterial species.

Cytotoxicity and Viability

The cytotoxic effect of EW types on the L929

Table 1: The antibacterial effect of different groups before and after treatments and the percentage of bacterial reductions

Treatment groups	Bacteria species	Mean value of CFUs		Reduction (%)	P value
		Before	After		
PBS	<i>A. actinomycetemcomitans</i>	8.50x10 ⁶	8.40x10 ⁶	1.17	0.5
	<i>S. salivarius</i>	5.80x10 ⁹	5.70x10 ⁹	1.72	0.5
	<i>L. casei</i>	9.10x10 ⁹	9.09x10 ⁹	0.1	0.87
	<i>S. aureus</i>	2.40x10 ⁹	2.39x10 ⁹	0	0.8
CLX 0.2%	<i>A. actinomycetemcomitans</i>	8.50x10 ⁶	0.00	100	0.0001
	<i>S. salivarius</i>	5.80x10 ⁹	0.00	100	0.0001
	<i>L. casei</i>	9.10x10 ⁹	0.00	100	0.0001
	<i>S. aureus</i>	2.40x10 ⁹	0.00	100	0.0001
CLX 0.009%	<i>A. actinomycetemcomitans</i>	8.50x10 ⁶	0.00	100	0.0001
	<i>S. salivarius</i>	5.80x10 ⁹	2.30x10 ⁶	99.96	0.0001
	<i>L. casei</i>	9.10x10 ⁹	2.30x10 ⁶	99.97	0.0001
	<i>S. aureus</i>	2.40x10 ⁹	1.20x10 ⁹	50	0.002
AEW	<i>A. actinomycetemcomitans</i>	8.50x10 ⁶	0.00	100	0.0001
	<i>S. salivarius</i>	5.80x10 ⁹	4.20x10 ⁶	99.92	0.0001
	<i>L. casei</i>	9.10x10 ⁹	7.40x10 ⁵	99.99	0.0001
	<i>S. aureus</i>	2.40x10 ⁹	4.70x10 ⁷	98.04	0.0001
MBEW	<i>A. actinomycetemcomitans</i>	8.50x10 ⁶	5.50x10 ⁴	99.3	0.0001
	<i>S. salivarius</i>	5.80x10 ⁹	3.40x10 ⁸	94.1	0.0001
	<i>L. casei</i>	9.10x10 ⁹	5.60x10 ⁴	99.99	0.0001
	<i>S. aureus</i>	2.40x10 ⁹	2.70x10 ⁸	88.75	0.0001
BEW	<i>A. actinomycetemcomitans</i>	8.50x10 ⁶	0.00	100	0.0001
	<i>S. salivarius</i>	5.80x10 ⁹	4.00x10 ⁸	93.1	0.0001
	<i>L. casei</i>	9.10x10 ⁹	6.00x10 ⁴	99.99	0.0001
	<i>S. aureus</i>	2.40x10 ⁹	2.60x10 ⁸	89.16	0.0001

PBS: Phosphate buffered saline, AEW: Acidic Electrolyzed Water, MBEW: Mildly Basic Electrolyzed Water, BEW: Basic Electrolyzed Water, *A. actinomycetemcomitans*: Aggregatibacter actinomycetemcomitans, *S. salivarius*: Streptococcus salivarius, *L. casei*: Lactobacillus casei, *S. aureus*: Staphylococcus aureus

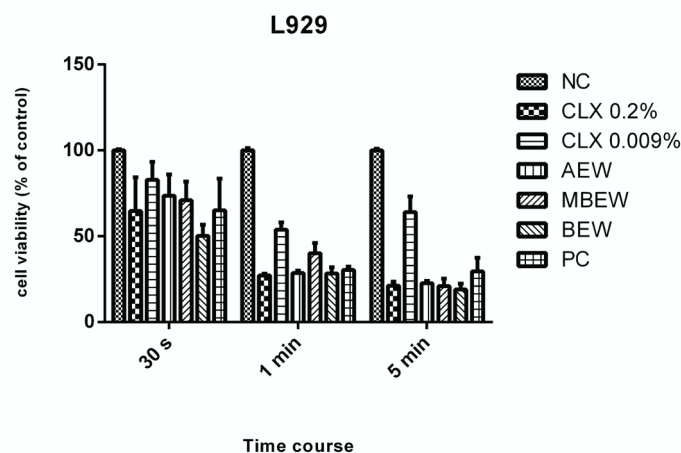


Figure 1: The figure shows the cytotoxicity/viability effect of electrolyzed water types on the L929 cell line in three periods (30 seconds, 1 minute, and 5 minutes). NC: Negative Control, AEW: Acidic Electrolyzed Water, MBEW: Mildly Basic Electrolyzed Water, BEW: Basic Electrolyzed Water, PC: Positive Control

cell line was intensified by increasing the time from 30 seconds up to 5 minutes (figure 1). The cytotoxicity of BEW was the highest at 30 seconds among the groups, even more than CLX 0.02% in the same time period. This cytotoxic effect was comparable to other groups except for CLX 0.009% in 1- and 5-minute treatment periods. CLX 0.009% could effectively keep most cells viable and had the least cytotoxic effect among different groups.

The exposure of L929 cell lines to AEW

for 30 seconds had a strong cytotoxic effect ($P < 0.0001$). The viable cells were continuously reduced after 1 minute of treatment ($P < 0.0001$); figure 2A. However, there were no significant differences in cell viability between the 1 minute and 5 minutes of treatment periods. Treatments with AEW showed equal cytotoxic potency on L929 cells. This cell line was also significantly reduced when treated with MBEW in the three time periods ($P < 0.0001$); figure 2B. The same cytotoxic and significant effect was also seen in

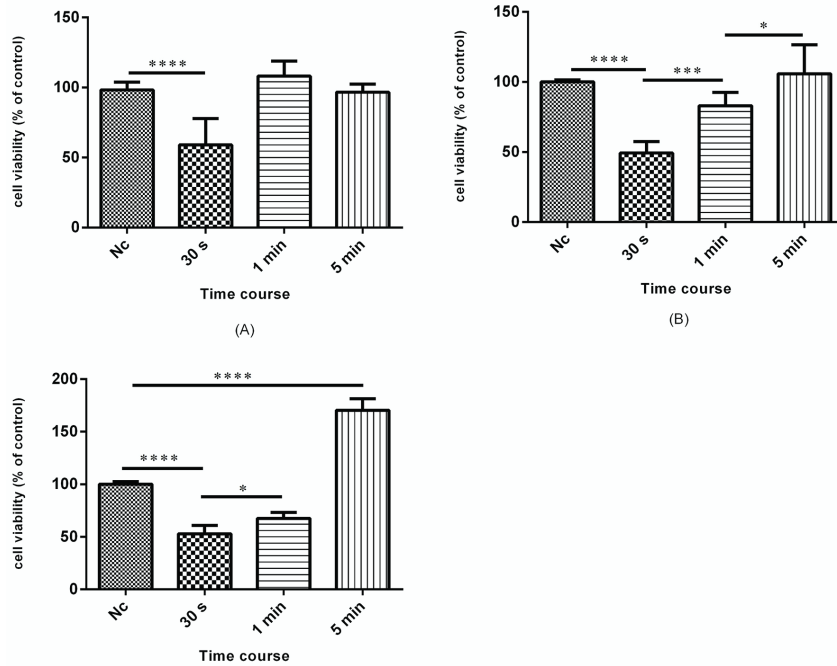


Figure 2: The effect of three electrolyzed water types on L929 cells in three periods; acidic electrolyzed water (a), mildly basic electrolyzed water (b), and basic electrolyzed water (c). All tests were repeated six times. Data expressed as mean±SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

the treatment of L929 cells with BEW and less viable cells were observed with exposure from 30 seconds to 5 minutes (figure 2C).

The treatment of HeLa cells with the EW types had an opposite effect compared with L929 cells. The viable HeLa cell numbers increased within the time periods in all EW types. Although, compared with the control group, the viable HeLa cells decreased at the 30 seconds of treatment with the EW types, however, the cells increased and were comparable to the control group in the 1- and 5-minute treatment periods with MBEW and BEW (figure 3). CLX 0.02% had the most

cytotoxic effect on HeLa cells in all three-time periods followed by de-electrolyzed water (PC: the positive control group). Compared with the control, AEW had a strong cytotoxic effect when treated with HeLa cells for 30 seconds; however, the viable cells significantly ($P<0.0001$) increased in the 1-minute and then insignificantly decreased in the 5-minute treatment periods (figure 4A). Both the MBEW and BEW had significant cytotoxic effects in the 30-second treatment with HeLa cells ($P<0.0001$). However, there was less cytotoxic activity in the 1- and 5-minute treatment periods and the viable HeLa

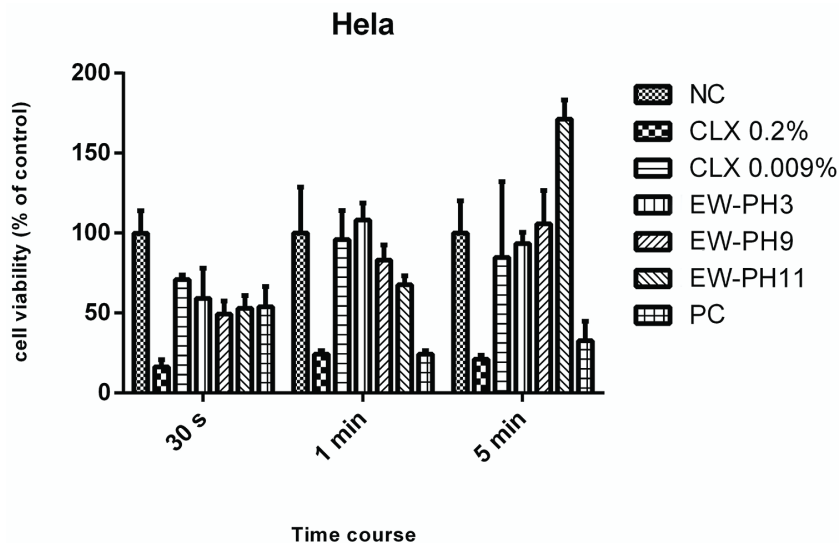


Figure 3: The figure shows the cytotoxicity and viability effects of electrolyzed water types on the HeLa cell line in three periods (30 seconds, 1 minute, and 5 minutes). NC: Negative Control, PC: Positive Control

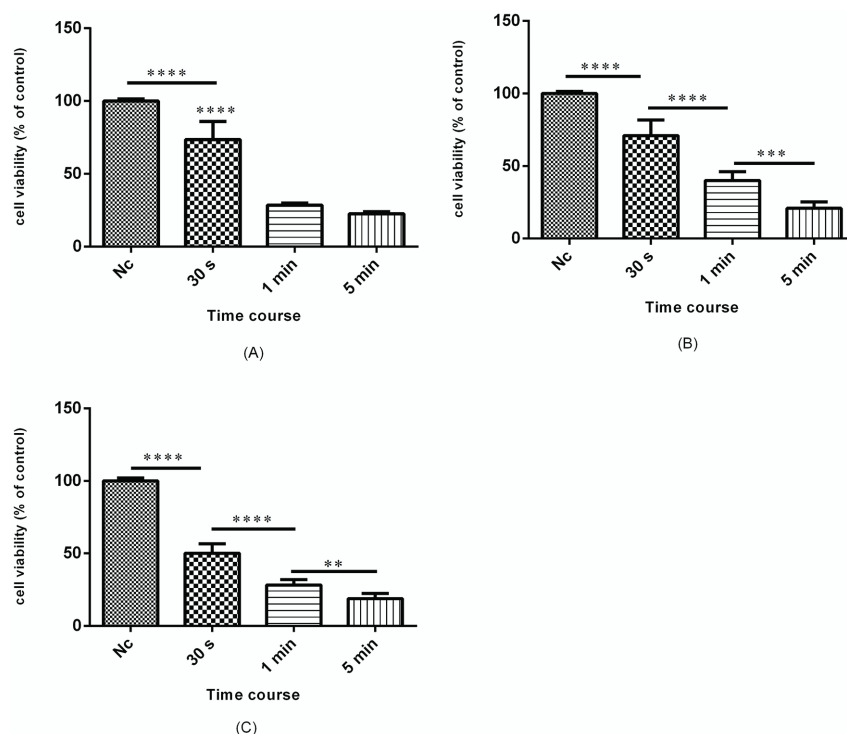


Figure 4: The figure shows the effects of three electrolyzed water types on HeLa cells in three periods; acidic electrolyzed water (a), mildly basic electrolyzed water (b), and basic electrolyzed water (c). All tests were repeated six times. Data are expressed as mean±SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

cells were highest in the 5-minute treatment period with MBEW and BEW (figure 4B, 4C).

Discussion

Electrolyzed water had efficient antibacterial action at all three pH values. In contrast with other studies, this antibacterial property developed 2 hours after preparation and was significantly stronger than the negative control group (NC). Previous studies have shown strong and efficient antibacterial activities of EW at different pH values against both aerobic and anaerobic bacterial species, such as cariogenic and periodonto-pathogenic bacteria.^{10, 13-17} Another study reported similar bactericidal activity at different pressures, temperatures, and pH values which was intensified by the combination of acidic and basic EW types to improve the microbiological quality of pork meat.¹⁸ However, some of these studies used EW immediately or shortly after preparation¹⁷ and claimed that EW had a bactericidal activity in just under 2 hours after production.¹⁸

The most antibacterial activity was observed against *A. actinomycetemcomitans* when exposed to EW at the three pH values for 30 seconds. This could be due to the bacterial cell wall properties since *A. actinomycetemcomitans* was the only gram-negative bacterium and was completely eradicated by AEW and BEW,

but not by MBEW (99.3% reduction). The EW at these pH values had an equal antibacterial effect against *L. casei* with 99.99% CFU/mL reduction, but the antibacterial activities of the EW types were different against the other three bacterial species. AEW and BEW had an equal antibacterial efficiency against both *A. actinomycetemcomitans* and *L. casei* with 100% and 99.99% CFU reduction, respectively. AEW had the strongest activity against *S. aureus*, whereas BEW had the strongest activity against *S. salivarius*. MBEW had the lowest antibacterial potency against bacteria except for *L. casei*, with an equal bactericidal effect, indicating the role of pH in bactericidal activity.

As expected, CLX 0.2% had the strongest activity against all microorganisms and completely eliminated all four bacterial species. CLX 0.2% is the standard solution for mouthwash disinfectant, however, dentists seek other solutions considering issues related to oral irritation, toothache, tooth/tongue staining, tartar build-up, and reduced taste sensation.¹⁹ In the current study, the antibacterial activity of CLX 0.009% was equal to that of CLX 0.2% against *A. actinomycetemcomitans*, but weaker against *S. salivarius*, *L. casei*, and *S. aureus*. This solution significantly eradicated all bacterial species (P<0.001 for *A. actinomycetemcomitans*, *S. salivarius*, and *L. casei*; P=0.002 for *S. aureus*), indicating that it can be used as an oral

mouthwash. However, further studies should be performed against other bacterial species to confirm its effectiveness as a standard antibacterial solution.

In the present study, two different cells were treated with EW in three incubation periods to demonstrate the safe use of EW on mammalian cell lines. Several studies have examined the cytotoxic effect of EW on human and animal organs and cells.^{20, 21} The majority reported that these water solutions caused no problem to normal cells and, in fact, could efficiently inhibit the growth of cancer cells^{4, 22} as well as other beneficial properties such as anti-diabetic effects²³ and wound healing.²⁴ In contrast, a previous study reported the cytotoxic effects of AEW.²⁵ Mokudai and colleagues reported that the *in vitro* cytotoxicity of AEW on rat fibroblast cells was related to a high concentration of reactive oxygen species in the rat fibroblast cell line.²⁶ The cytotoxic effect of AEW was also observed against pulp cells, however, this response was weaker than the hypochlorite sodium treatment.²⁷

Some other studies have shown the basic advantages of EW beyond bactericidal activities and demonstrated its application in medicine,^{13, 28} translational medicine,²⁹ and the food industry.¹² However, there are reports of unknown systemic effects and growth retardation in rats due to the basic drinking water.³⁰ In addition, recent reports have claimed that the Hydrogen molecule, rather than pH, is the main cause of beneficial and therapeutic effects of EW.^{13, 31}

Interestingly, and consistent with previous studies, we showed that EW at all pH values amplified cytotoxic activity on the L929 cell line. This negative activity increased with higher treatment time; indicating an additional negative effect of EW on L929 within a time period. In contrast, although HeLa cells significantly decreased when treated with EW for 30 seconds, more viable cells were observed in the 1- and 5-minute treatment periods compared with the control group. This contradictory result between fibroblast L929 and epithelial HeLa treated cell lines are thought to be due to cell-to-cell variability and cell origin (cancer or normal cells). Shirahata and colleagues showed that EW could efficiently suppress HeLa cell growth but not normal fibroblast TIG-1 cells, even after 3 months cultivation.⁸ In comparison, our results were similar, but HeLa cells decreased after 30 seconds exposure to EW. However, more viable cells were observed with an increase in treatment duration and the cell viability was comparable to the control in the 5-minute treatment period. This indicated that EW had no cytotoxic effect

on HeLa cells, at least in the 5-minute treatment period. These differences might be due to the source of the tap water, the type and model of the ionizer machine, and the time span between sampling and testing.

Conclusion

The present study demonstrated efficient antibacterial activities of EW at different pH values against four oral bacterial species within a 2-hour time span between water preparation and the test procedure. However, AEW and BEW eradicated more bacteria in some species compared with MBEW. The cytotoxicity assay indicated the adverse effect of these EW types on L929 and HeLa cells, at least in the 30-second treatment period. Further studies are recommended to assess the effects of EW on different cell lines to confirm their safety for human use. To the best of our knowledge, there are no reported negative results on the effect of basic EW on mammalian cell lines. We found that all acidic, mildly-basic, and even basic EW types had cytotoxic activities on cells up to 5 minutes of treatment. In the case of HeLa cells, these were observed only in the 30-second treatment period. Therefore, there is a need for more caution and further animal experimental studies are required prior to its clinical use.

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Conflict of Interest: None declared.

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