Durability of Bactericidal Activity in Electrolyzed Neutral Water by Storage

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Electrolyzed strong and weak acid waters have been widely used for sterilization in clinical dentistry because of their excellent bactericidal activities. Electrolyzed neutral water was recently developed with a new concept of long-term good durability in addition to the excellent bactericidal activity similar to acid waters. The present study, evaluated the storage life of this water compared with the acid waters in terms of the changes in pH, oxidation-reduction potential (ORP), residual chlorine and bactericidal activity under several conditions using Staphylococcus aureus 209P. The strong acid water showed a rapid deterioration of its bactericidal activity. The weak acid and neutral waters exhibited excellent durability. Although all the bacteria were annihilated by the contact with the waters even stored for 40 days in the uncapped bottle, the neutral water was superior in further long-term duration.

Key words: Electrolyzed neutral water, Bactericidal activity, Storage

INTRODUCTION

Electrolyzed acid water has been widely used for sterilization and other treatments in several scenes of clinical medicine and dentistry1-4). Especially, its excellent bactericidal and virucidal activities have attracted attention5-10) and application of this water has been investigated in operative11,12), prosthetic13-16) and other3) fields in clinical dentistry. We previously reported that the dental impressions17-19), instruments20) and acrylic denture base resin21) experimentally contaminated with bacteria could be successfully sterilized by 1-minute immersion or ultrasonic cleaning in the electrolyzed strong or weak acid water. These acid waters exhibit excellent immediate bactericidal effects3,5-9,14,15,17-21) and little risk of contamination of the environment through the drainage. Their disadvantages, on the other contrary, are the potential corrosion of the metallic materials maybe accelerated by them20,22) and their poor storage life, especially in the strong acid water23,24). Although it is desirable to deal with the fresh acid water shortly after its preparation, it is sometimes necessary to store it for a period.

Recently, the electrolyzed neutral water was developed as an improved type in the corrosion resistance and stability of the bactericidal efficacy. In the present study, the bactericidal validity of the electrolyzed neutral water was examined in terms of storage life using Staphylococcus aureus 209P, as compared with those of
the electrolyzed strong and weak acid waters.

MATERIALS AND METHODS

Preparation of electrolyzed waters

Three types of electrolyzed waters were used in this examination (Tables 1 and 2). The strong acid water (SW) was prepared by electrolyzing 0.05% sodium chloride aqueous solution with an exclusive electrolyzing apparatus (SUPER WATER mini, Hirata Corp., Osaka, Japan). The weak acid water (WW) was prepared by electrolyzing tap water adding specified electrolysis with an automatic apparatus (ACIDENT, J. Morita Tokyo MFG. Corp., Tokyo, Japan). The neutral water (NW) was also automatically prepared using tap water containing 5% sodium chloride (AP aqua21, Asahipretec Corp., Kobe, Japan). Just after electrolyzing, 500 ml of each fresh water was poured into polyethylene terephthalate (PET) bottle and stored under the conditions shown in Table 3 and Fig. 1.

Table 1 Properties of electrolyzed waters published by the manufacturers of the apparatus

<table>
<thead>
<tr>
<th>Electrolyzed water</th>
<th>Code</th>
<th>pH</th>
<th>ORP (mV)</th>
<th>Residual chlorine (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong acid water</td>
<td>SW</td>
<td>less than 2.7</td>
<td>more than +1,100</td>
<td>-</td>
</tr>
<tr>
<td>Weak acid water</td>
<td>WW</td>
<td>5.0-7.0</td>
<td>-</td>
<td>30 or 50</td>
</tr>
<tr>
<td>Neutral water</td>
<td>NW</td>
<td>neutrality (6-7)</td>
<td>+700-800</td>
<td>more than 30</td>
</tr>
</tbody>
</table>

1SUPER WATER mini, Hirata Corp., Osaka, Japan,
2ACIDENT, J. Morita Tokyo MFG. Corp., Tokyo, Japan,
3AP aqua21, Asahipretec Corp., Kobe, Japan,
-: not published

Table 2 Properties of fresh electrolyzed waters before storage

<table>
<thead>
<tr>
<th>Code</th>
<th>pH</th>
<th>ORP (mV)</th>
<th>Residual chlorine (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW</td>
<td>2.4±0.05</td>
<td>+1,159±3.2</td>
<td>49±1.7</td>
</tr>
<tr>
<td>WW</td>
<td>6.5±0.27</td>
<td>+877±12.2</td>
<td>51±2.0</td>
</tr>
<tr>
<td>NW</td>
<td>7.0±0.07</td>
<td>+849±4.5</td>
<td>38±0.0</td>
</tr>
</tbody>
</table>

Table 3 Conditions of storage of electrolyzed waters in PET bottles

<table>
<thead>
<tr>
<th>No.</th>
<th>Cover of bottle</th>
<th>Cap of bottle</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>shaded</td>
<td>capped</td>
<td>4°C in refrigerator</td>
</tr>
<tr>
<td>II</td>
<td>shaded</td>
<td>capped</td>
<td>at room temperature (23±2°C)</td>
</tr>
<tr>
<td>III</td>
<td>transparent</td>
<td>capped</td>
<td>at room temperature (23±2°C)</td>
</tr>
<tr>
<td>IV</td>
<td>transparent</td>
<td>uncapped</td>
<td>at room temperature (23±2°C)</td>
</tr>
</tbody>
</table>
Measurement of the properties of electrolyzed waters
The pH value and oxidation-reduction potential (ORP) were examined with a pH meter (HM-14P, TOA Electronics Ltd., Tokyo, Japan). The concentration of residual chlorine was determined with a chlorine comparator (Portable Type II, Toyo ENGINEERING WORKS LTD., Chiba, Japan) and a photoelectric colorimeter (AE-30F, Erma INC., Tokyo, Japan) based on the O-tolidine method. Those values of the fresh waters just after preparation are listed in Table 2. The changes in these properties during 120-day storage under several conditions were examined for the two sampling methods in which the test water was taken from one bottle through the test period (code: M-1) and from every full bottle stored for each specified period (code: M-2). Fig. 2 shows the changes in the amount of the water in the bottle during 120-day storage for M-1 and M-2. All the properties of these waters were measured at room temperature (23±2°C).

Preparation of bacteria solution
The bacteria used were Staphylococcus aureus 209P which were commonly used for evaluating sterilization effects. The bacteria were incubated in a brain heart infusion (BHI, Lot 103476JC, DIFCO, Detroit, MI, USA) at 37°C for 24 hr. The bacteria suspensions were prepared to be 1.0×10^8/ml in BHI.

Bactericidal test
For the bactericidal test, 0.1 ml of bacteria solution containing 1.0×10^7 bacteria was mixed with 9.9 ml of the electrolyzed water for 3 min at room temperature. After treatment, 0.1 or 1 ml from the solution was added to the agar culturing media (Nutrient Agar, lot. No.057907, Nissui, Tokyo, Japan). The amount of the solution to be added was selected according to the estimated number of the bacteria by the
preliminary examination. The number of the colonies in the media was counted after incubation at 37°C for 24 hr and the total number of the surviving bacteria was calculated.

All the experiments were repeated 3 times and the results were statistically compared by ANOVA and Student’s t test.

RESULTS

The changes in the pH value during 120-day storage in both the cases of M-1 and M-2 are shown in Fig. 3. The strong acid water (SW) showed only a slight increase

![Graphs showing pH changes](image)

**Fig. 3** Changes in the pH value of electrolyzed water during 120-day storage in the cases of M-1 and M-2.

- a. M-1: with a decrease by sampling for bactericidal tests
- b. M-2: without a decrease by sampling for bactericidal tests
in pH in the condition of the uncapped bottle up to 10 weeks. There was no significant difference in the pH change through the testing period between M-1 and M-2. In the weak acid water (WW) and the neutral water (NW), on the other hand, the pH values significantly increased during the first 8 and 12 days, respectively, in M-1 when stored in the transparent and uncapped bottle in the room (condition IV). The values showed almost steady state thereafter, exceeding the nominal ranges 5 to 7 for WW and 6 to 7 for NW published by the manufacturers. The similar tendencies were also found in M-2 although the rapid increases appeared later than those in M-1, at 8 to 21 days in both WW and NW.

The changes in the ORP value during 120-day storage in both the cases of M-1

Fig. 4 Changes in the ORP value of electrolyzed water during 120-day storage in the case of M-1 and M-2.

a. M-1: with a decrease by sampling for bactericidal tests
b. M-2: without a decrease by sampling for bactericidal tests
and M-2 are shown in Fig. 4. The smallest changes were found under the condition I (storage in the refrigerator), and the largest under the condition IV in every type of water (p<0.01). Especially, SW showed a marked drop in ORP under the condition IV within the first 7 days. When it was stored in the capped bottle (conditions I, II and III), on the other hand, the ORP value still maintained the level of +1,000 mV in M-1 and +1,100 mV in M-2 after 120 days. In WW and NW, the changes in ORP were smaller. Little or no changes in ORP were found in every type or water under these conditions I, II and III in M-2.

Fig. 5 shows the changes in the concentration of the residual chlorine during 120-day storage. The concentration of the residual chlorine decreased with time.

![Fig. 5](image)

**Fig. 5** Changes in the concentration of residual chlorine in electrolyzed water during 120-day storage.

a. M-1: with a decrease by sampling for bactericidal tests

b. M-2: without a decrease by sampling for bactericidal tests
(p<0.01) in any water and this deterioration of the concentration was accelerated by the storage conditions of the decreasing water in the storing bottle in M-1, the uncapped bottle, the transparent bottle and the room temperature. SW showed the fastest decrease in the concentration and the residual chlorine was no more detected after 12- and 14-day storage under the condition IV in M-1 and M-2, respectively. It remained at the level of 30 ppm after 120 days only when the water was stored in the refrigerator in M-2. The decrease in the concentration of the residual chlorine of WW and NW was significantly smaller than that of SW. Although the original concentration of WW was much higher than that of NW, WW showed the larger decrease and its final concentration after 120-day storage in the transparent and uncapped bottle at room temperature was rather lower than that of NW.

Fig. 6 Changes in the number of surviving bacteria after treatment by electrolyzed water sampled during 120-day storage in the case of M-1.
Fig. 6 shows the changes in the number of the surviving bacteria in the $1 \times 10^7$ bacteria-suspended solution after the treatment with the electrolyzed waters at each specified period of 120-day storage in M-1. None of the bacteria could survive in shortly stored SW. A small number of the surviving bacteria appeared in SW stored for 6 days under the condition IV and $5 \times 10^3$ bacteria were detected after 8-day storage. Then the bactericidal activity of SW rapidly deteriorated with the storage time under the condition. When the storing bottle was shaded, capped and/or placed in the refrigerator, the bactericidal activity of SW was markedly prolonged. SW had no bactericidal activity after 120 days even if it was placed in the refrigerator. On the other hand, no surviving bacteria were detected in WW and NW stored for 35 days under the condition IV. Although the bactericidal activity of NW was more rapidly reduced than that of WW under this condition, NW showed more prolonged storage life by about 10 days than WW under the other conditions. After 120-day storage, the number of the surviving bacteria in WW and NW was at the level of $10^3$ under the condition II and III, while it was only at the level of 10 under the condition I.

DISCUSSION

Electrolyzed acid water has been conventionally used for sterilization in dentistry\textsuperscript{1-5}). It involves strong and weak acid waters. They were generally defined by the pH value: 2.7 or less for the strong acid water and 5-7 for the weak acid water.

The strong acid water used in the present study, SW, is produced by electrolyzing the 0.05% sodium chloride aqueous solution with a diaphragm between cathode and anode. The actual values of pH and ORP and the concentration of the residual chlorine of the fresh strong acid water used in the present study, SW, were $2.4 \pm 0.1$, $1.159 \pm 3.2$ mV and $49 \pm 1.7$ ppm, respectively, as listed in Table 2. Those pH and ORP values exist within the range where the microbes cannot live, and they seem to play the important roles on the bactericidal activity of SW as well as the presence of the hypochlorous acid (HClO). The excellent bactericidal effects of SW were confirmed in our previous studies on the sterilization treatment of the dental impressions and instruments\textsuperscript{17-20}). It is well known, on the other hand, that the strong acid water is poor in durability of the bactericidal activity\textsuperscript{23}). When SW was stored in the 500 ml PET bottle, almost no changes in pH were found for 120 days under any storing condition. However, the bactericidal activity was drastically deteriorated with time. Such behaviors were in accord with other reports\textsuperscript{23,24,26,27}). It indicates that the pH value itself might not be the major factor affecting the bactericidal activity. Provided that a criterion of the number of the surviving bacteria for the effective bactericidal activity was regarded as the level of $10^2$, the storage life of SW was 7 days in the transparent and uncapped bottle at room temperature (condition IV) when the test water was taken from one bottle reducing the residual amount of water (M-1). By closing and shading the bottle at room temperature (conditions II and III), the storage life was prolonged to 40 days and further prolonged to 65 days in the refrigerator (condition I). The deterioration of the bactericidal activity in SW was well
correlated to the decreased ORP and concentration of the residual chlorine, and it reached the threshold when the ORP value dropped to 1,100 mV or less and/or the residual chlorine was decreased to 20 ppm. The residual chlorine was completely lost in 14 days in the transparent and uncapped bottle at room temperature and in 100 days in the refrigerator. Finally the bactericidal activity disappeared after 120-day storage under any condition. It is suggested from these results that the storage life may also be 7 days even if the water is stored with the bottle being filled as far as the bottle is opened.

The electrolyzed weak acid water, WW, is obtained by electrolyzing the tap water automatically added the specified electrolyte which consists of dilute hydrochloric acid without a diaphragm. It showed quite different changes from SW by storage. The original pH of this water was near the neutral gender and the ORP value was much lower than that of SW. The pH value increased and the ORP decreased rapidly at the early stage of the storage followed by a slight change after that under the condition IV, while lesser changes were found under the other conditions. However, their values still lay within the range where it might not be supposed that they were hazardous against the microbes, and they were not correlated with the changes in the number of the surviving bacteria. The bacteria were completely annihilated by the contact with WW stored up to 40 days under any condition. Such an excellent bactericidal activity remained for 80 days in the refrigerator. These storage times were corresponding to those when the residual chlorine dropped from 50 to 40 ppm. As the residual chlorine decreased to less than 35 ppm, the water could no more maintain its sufficient bactericidal activity showing the level of $10^3$ surviving bacteria. Thus, it was supposed that the duration of WW might be about 50 days in the transparent bottle (conditions III and IV) and about 90 days in the shaded and capped bottle (condition II). When it was stored in the refrigerator, it showed good durability even after 120 days. It is suggested from the changes in the residual chlorine that the storage as the bottle being filled may be able to prolong the durability by about 10 days.

The electrolyzed neutral water, NW, was recently developed. It is obtained through two steps of electrolyzation using 5% sodium chloride aqueous solution, first without and second with a diaphragm. The resultant water shows neutrality. The manufacturer of this water claims some advantages over the conventional acid waters. The first, it shows excellent bactericidal effect at the low concentration of the residual chlorine because most of the chlorine consists of hypochlorous acid (HClO) having the strongest bactericidal effect. The second, it can maintain its bactericidal activity for more than 3 months in the capped bottle because the hypochlorous acid is stable in it and not easily converted into gas due to the specified double electrolyzing processes. The third, it is less affective on the human body and environment because it is neutralized to make the hypochlorous acid most effective. The fourth, it is produced safely with low running cost because it necessitates only sodium chloride, water and electric power.

The changes in the characteristics of NW by storage were similar to those of
According to the manufacturer of NW, the Cl₂ gas is fully solved to form stable HClO at the second stage of electrolyzation and equilibrium is established as follows:

\[
\text{Cl}_2 + \text{OH}^- \rightleftharpoons \text{HClO} + \text{H}^+ + \text{Cl}^- 
\]

When NW was stored in the present study, its pH increased especially in the uncapped bottle. It is assumed from this fact that OH⁻ might increase in the water, which means that the reaction from the right toward the left might occur in the above equation. The reaction might probably be derived from the loss of a small amount of Cl₂ getting out of the water. This may be the possible mechanism why the pH value of NW increased by storage.

Although the changing pattern of the pH and ORP values was not directly reflected in the deterioration of the bactericidal activity, the changes in pH would affect the amount of HClO in the water. The following equilibrium is controlled by pH as is well known (Fig. 7)²⁵).

\[
\text{Cl}_2 \rightleftharpoons \text{HClO} \rightleftharpoons \text{ClO}^- 
\]

The fraction of HClO is the maximum at the neutral gender. As the pH is shifted to the alkalinity, the fraction of HClO decreases and that of ClO⁻ increases. ClO⁻ from the dilute solution of sodium hypochlorite is generally used as a disinfectant with the concentration of 3-6 or 10% for dental materials and instruments and 50-100 ppm for mucous membrane operated in dentistry²⁸). Since the bactericidal activity of ClO⁻ is one-tenth of that of HClO at the most, it is preferable for the electrolyzed water to contain HClO as much as possible. Estimating from Fig. 7, the fresh NW with pH being 7 contains about 70% of HClO in the residual chlorine. When the pH changed to 8 in the transparent and uncapped bottle for about 10 days in the present study, the HClO content would be reduced to 20-30% with the rest being ClO⁻. Although this may be an unfavorable factor affecting the bactericidal activity, no
actual effect appeared on the annihilation on the bacteria until the residual chlorine dropped to less than 30 ppm in 40 days. When NW was stored for 40 days in the transparent and uncapped bottle (condition IV), the $10^9$ bacteria out of $10^9$ remained surviving in the water. Then the bactericidal effect gradually decreased with storage time. In NW stored in the capped bottle at room temperature (conditions II and III), the surviving bacteria was first detected at the storage time of 50 days and the number of them did not reach the level of $10^9$ even after 120-day storage. When NW was stored in the refrigerator, the sufficient bactericidal activity was kept after 120 days leaving only the level of 10 bacteria surviving. If this water is stored in the PET bottle as it is filled with the water (condition M-2), it is expected from the slight changes in the residual chlorine that it will show the excellent durability of its bactericidal activity after 120 days.

There were many resemblances in the pH and the changing pattern of the characteristics between WW and NW although their production processes are quite different. Thus, the mechanism of the deterioration in WW might be similar to that of NW. It is suggested from the present study, however, that the long-term duration of the bactericidal activity of NW may be better than that of WW. Such an elongated storage life of NW may contribute to further development of the electrolyzed water for dental use.

REFERENCES


