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Antifungal effect of electrolyzed hydrogen water on *Candida albicans* biofilm

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Purpose: *Candida albicans* can cause mucosal disease in many vulnerable patients. Also they are associated with denture-related stomatitis. Electrolyzed water is generated by electric current passed via water using various metal electrodes and has antimicrobial activity. The aim of this study was to investigate antifungal activity of electrolyzed water on *C. albicans* biofilm. **Materials and Methods:** *C. albicans* was cultured by sabouraud dextrose broth and F-12 nutrient medium in aerobic and 5% CO_2 condition to form blastoconidia (yeast) and hyphae type, respectively. For formation of *C. albicans* biofilm, *C. albicans* was cultivated on rough surface 6-well plate by using F-12 nutrient medium in CO_2 incubator for 48 hr. After electrolyzing tap water using various metal electrodes, the blastoconidia and hyphal type of *C. albicans* were treated with electrolyzed water. *C. albicans* formed blastoconidia and hyphae type when they were cultured by sabouraud dextrose broth and F-12 nutrient medium, respectively. **Results:** The electrolyzed water using palladium electrode (EWP) exhibited antifungal effect on blastoconidia of *C. albicans*. Also, the EWP significantly has antifungal activity against *C. albicans* biofilm and hyphae. In the electrolyzed water using various metal electrodes, only the EWP have antifungal activity. **Conclusion:** The EWP may use a gargle solution and a soaking solution for prevention of oral candidiasis and denture-related stomatitis due to antifungal activity. **(J Dent Rehabil Appl Sci 2015;31(3):212-20)**

Key words: electrolyzed water; antifungal activity; *Candida albicans*; Candidal biofilm

Introduction

Candida is found in the oral cavity about 50% of general population of commensal microorganism. Also, 80% of the isolates are detected *Candida albicans*.[1] *C. albicans* is normally a harmless commensal fungus in oral cavity. However, they can cause mucosal disease in most vulnerable patients such as some immunologically weak patients, xerostomia patients and older people.[2] The feature of *C. albicans* is ability to change morphology that grows either as budding yeast (blastoconidia) or as hyphal form according to growth conditions.[3,4] One of the major factors to the virulence of *C. albicans* is their morphological flexibility that results in the adaptation to environments, attachment to the surface and the communication between the species.[5] Thus, they easily have resistant ability for antibiotics and antifungal agents.

C. albicans forms biofilm in oral cavity. Also, their biofilm is frequently found on denture materials.[6] *C. albicans* attaches on denture wearers and forms biofilm. Next, they continuously swallow and penetrate

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into pore of denture wearers.[7] Finally, these conditions may constantly give opportunity infection to host. To defense their infection, mechanical cleaning as scrubbing with brush and chemical cleansing as mouth gargle and denture cleanser were used to remove for *C. albicans* biofilm on denture wearer.[8] Although the chemical cleansing is considered to be the most effective method for removing *C. albicans*, the chemicals are possible to change physical characteristics of denture wearers such as surface roughness, porosity and solubility.[9] Also, denture cleansers containing alkaline peroxide impair and enzymes resilient liners and have toxic effect on the patients.[8]

Electrolyzed water is generated by electric current passed via water using metal electrodes. Although the detail mechanisms of electrolyzed water has not been clearly known, the electrolyzed water has varies characteristics by what use metal electrodes.[10,11] The electrolyzed water has various effects on reducing cancer and oxidative stress and antibacterial activity.[12-14] Briefly introducing, acidic electrolyzed water has bactericidal effect on *Staphylococcus aureus* and *Escherichia coli*.[12] Also basic electrolyzed water affects oral hygiene including bactericidal activity, removing biofilm and bacterial growth inhibition.[15] Furthermore, slightly acidic electrolyzed water removes bacterial biofilm on dental unit water systems.[16] However, antifungal effect on *C. albicans* has not been reported so far. As these reasons, this study investigated antifungal activity of hydrogen enriched-electrolyzed water on *C. albicans* biofilm.

Materials and Methods

Fungal species and cultivation

Candida albicans ATCC 10231 was purchased from American type culture collection and used in this study. *C. albicans* was cultured by sabouraud dextrose broth (BD bioscience, Fanklin Lakes, NJ, USA) at 37°C in aerobic condition. For cultivating hyphal type of *C. albicans*, the fungus was cultivated by using F-12 nutrient media (Hyclone, Logan, UT, USA) at 37°C in 5% CO_2 condition.

Production of various electrolyzed water

Tap water was subjected to electrolysis for 5 min with 24 V of DC 350 mA using copper, silver or palladium electrode (cylinder of 2 mm × 10 cm) in undivided chamber. DC power supply was used powerPac basic (Bio-rad, Hercules, CA, USA). The electrolyzed water used for antifungal assay in 10 min after electrolysis.

Antifungal activity of the electrolyzed water against *C. albicans*

C. albicans was cultured by sabouraud dextrose broth at 37°C overnight and observed contamination and blastoconidia using phase contrast microscope. The fungus was counted with haemocytometer and adjusted the density at 1×10^5 cells/mL with sabouraud dextrose broth. The fungus was centrifuged at 1,200 × g for 10 min at 4°C and then discard the supernatants. After washing twice with phosphate buffered saline (PBS, pH 7.2), *C. albicans* was harvested by centrifugation at 1,500 × g for 10 min at 4°C. The fungus was treated with 1 mL of tap water, the electrolyzed water and listerine for various times and immediately added with 1 mL of sabouraud dextrose broth. The fungal suspensions were serially diluted from 10 to 10^8. 10 μL of diluted the fungal suspensions was inoculated on sabouraud dextrose agar and incubated at 37°C. The colony forming unit of *C. albicans* was counted.

Preparation of plate for candidal biofilm

The rough surface of 6-well plate was created by sandblasting. After washing with sterile distilled water, the plate was sterilized by ultra violet expose. Fresh F-12 nutrient media was dispensed into the well of UV exposed 6-well plate, and *C. albicans* was inoculated into the well. The fungus was incubated at 37°C in CO_2 incubation for 48 hours. The media was changed every 24 hours. The biofilm formation of *C. albicans* was observed by phase contrast microscope.

Antifungal activity of the electrolyzed water against *C. albicans* biofilm

C. albicans biofilm was treated with 1 mL of tap water, the electrolyzed water and listerine for various times and then immediately aspirated the solutions by vacuum pump. *C. albicans* biofilms were added with 1 mL of sabouraud dextrose broth and resuspended by mechanical disruption using a scraper. The biofilm suspensions were serially diluted from 10 to 10^8. 10 μL of diluted the biofilm suspensions was inoculated on sabouraud dextrose agar and incubated at 37°C. The colony forming unit of *C. albicans* was counted.

Comparison of antifungal activity of various electrolyzed water against *C. albicans* biofilm

C. albicans biofilm was treated with 1 mL of tap water and electrolyzed water using copper, silver and palladium electrode for 12 minutes and then immediately aspirated the solutions by vacuum pump. *C. albicans* biofilms were added with 1 mL of sabouraud dextrose broth and resuspended by mechanical disruption using a scraper. The biofilm suspensions were serially diluted from 10 to 10^8. 10 μL of diluted the biofilm suspensions was inoculated on sabouraud dextrose agar using multi-pipette and incubated at 37°C. The colony forming unit of *C. albicans* was counted.

Statistical analysis

Differences between Control and samples were analyzed by Kruskal-wallis and Mann-Whitney test using SPSS 10 (SPSS Inc., Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Investigation of culture condition for blastoconidia and hyphae of *C. albicans*

C. albicans has yeast and hyphal morphology according to growth conditions. Furthermore, the both types of *C. albicans* have different characteristics such as virulence and antifungal tolerance, and *C. albicans* exists with hyphal type in oral cavity. Therefore, the growth conditions of *C. albicans* were investigated using various media and atmospheres. When *C. albicans* was cultured by sabouraud dextrose broth at 37°C, the fungus formed blastoconidia (Fig. 1A). Also, when *C. albicans* was cultivated by using F-12 nutrient media at 37°C in 5% CO_2 conditions, they formed hyphae (Fig. 1B).

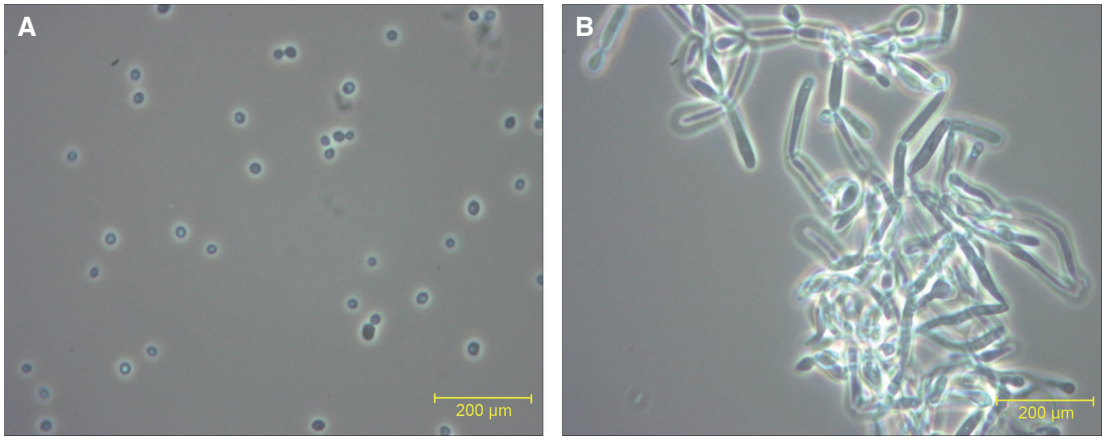

Fig. 1. Blastoconidia and hyphal type of *C. albicans*. *C. albicans* was cultivated by sabouraud dextrose broth (A) or F-15 nutrient medium (B) at 37°C in aerobic condition or 5% CO_2 conditions, respectively. (A) Blastoconidia form, (B) Hypae form.

Antifungal activity of the electrolyzed water against *C. albicans*

The electrolyzed water using palladium electrode (EWP) as generally using electrolyzed water was investigated antifungal activity against yeast or blastoconidia type of *C. albicans*. In comparing to tap water, the electrolyzed water showed antifungal activity for *C. albicans* significantly (Fig. 2). The electrolyzed water and listerine exhibited fungicidal effect on *C. albicans* from 2 minutes and 4 minutes, respectively. Also, tap water has antifungal effect on *C. albicans* from 8 min compared to control.

Antifungal activity of the electrolyzed water against *C. albicans* biofilm

C. albicans inhabits with biofilm of hyphal morphology in oral cavity. Thus, the antifungal activity of electrolyzed water against *C. albicans* was examined after forming the biofilm on rough surface of the 6-well plate. The EWP and listerine significantly reduced the level of *C. albicans* in the biofilm from 2 minutes and 4 minutes, respectively (Fig. 3, 4). However, tap water did not show significant difference compared with control.

Comparison of antifungal activity of various electrolyzed water against *C. albicans* biofilm

Finally, it was investigated that the antifungal effect of electrolyzed water against *C. albicans* showed whether in using all metal electrodes or in using palladium electrode. The EWP showed strongly antifungal activity. However, the electrolyzed water using copper and silver electrode did not exhibit the antifungal effects (Fig. 5).

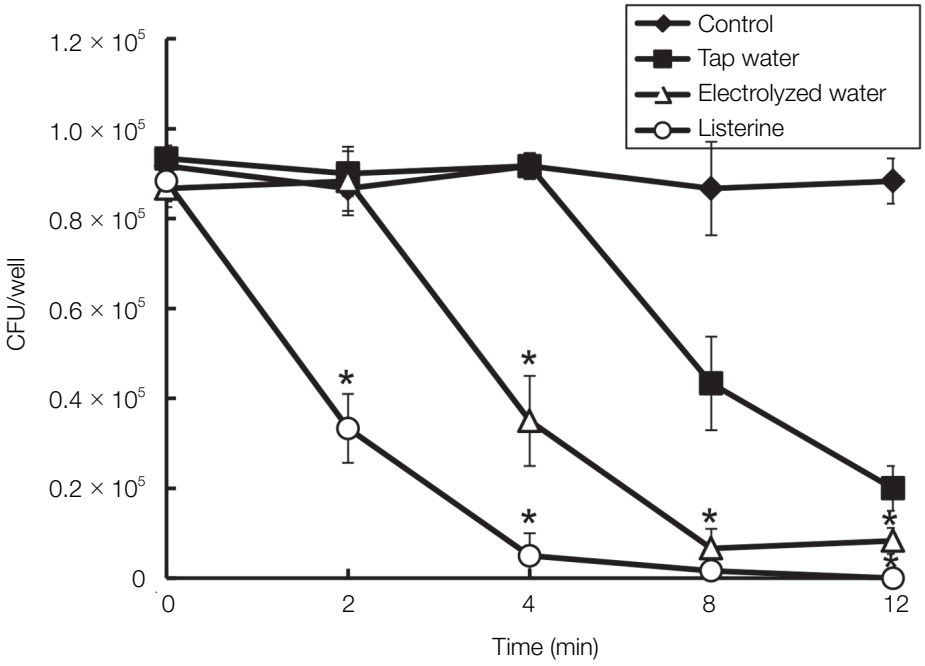

Fig. 2. The antifungal activity of the EWP against blastoconidia type of *C. albicans*. *C. albicans* was cultivated by using sabouraud dextrose broth in aerobic condition overnight and then harvested by centrifugation. The pellet of *C. albicans* was treated with tap water, EWP and listerine in various times. The live *C. albicans* was counted by colonies after plating on sabouraud dextrose agar. The examinations were performed three times. * Indicates a significant difference compared with control ($P < 0.05$).

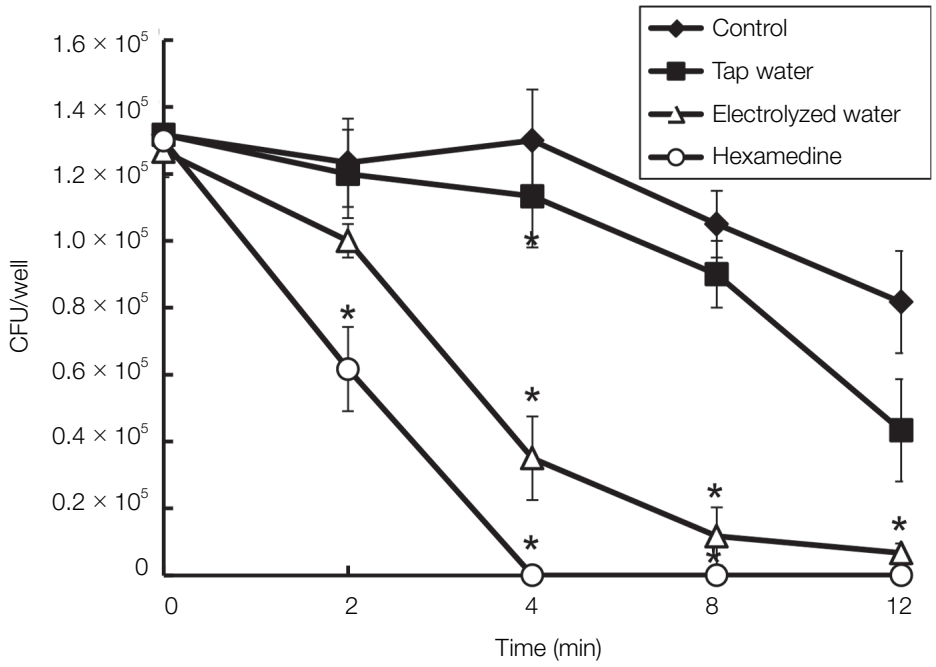

Fig. 3. The antifungal activity of the EWP against *C. albicans* biofilm. *C. albicans* was cultivated by using F-12 media in 5% CO_2 condition overnight and then harvested by centrifugation. The pellet of *C. albicans* was treated with tap water, EWP and listerine in various times. The live *C. albicans* was counted by colonies after plating on sabouraud dextrose agar. The examinations were performed three times. * Indicates a significant difference compared with control ($P < 0.05$).

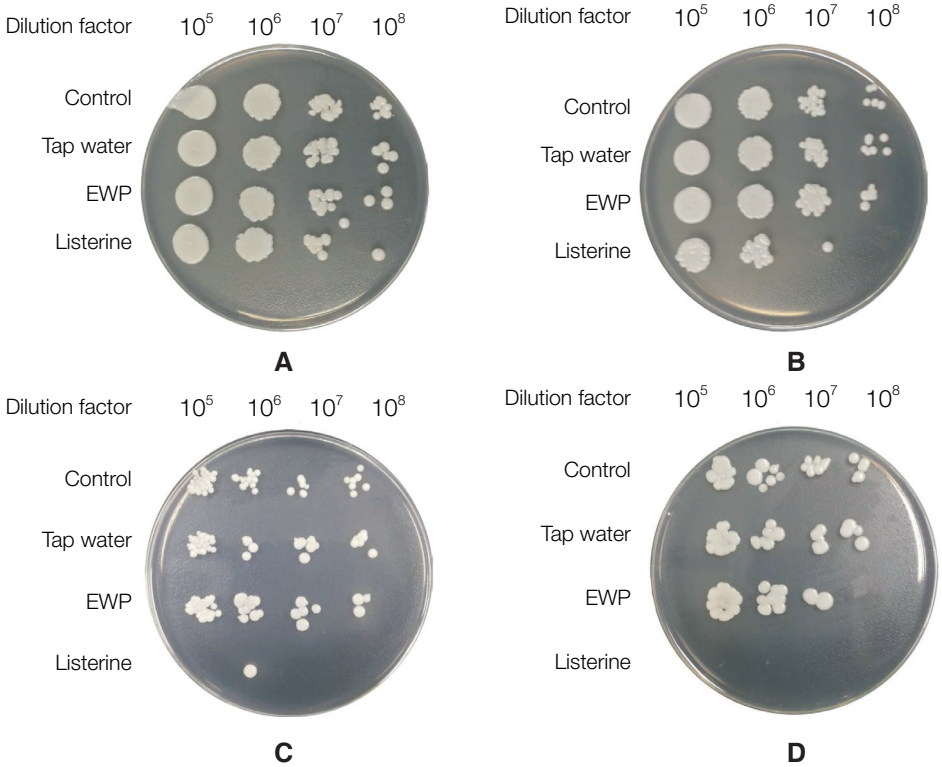

Fig. 4. The image of antifungal activity against *C. albicans* biofilm. *C. albicans* was cultivated using F-12 media in 5% CO_2 condition overnight and then harvested by centrifugation. The pellet of *C. albicans* was treated with tap water, electrolyzed water and listerine in various times and then inoculated on sabouraud dextrose agar. (A) 2 minutes, (B) 4 minutes, (C) 8 minutes, (D) 12 minutes. EWP, electrolyzed water using palladium electrode.

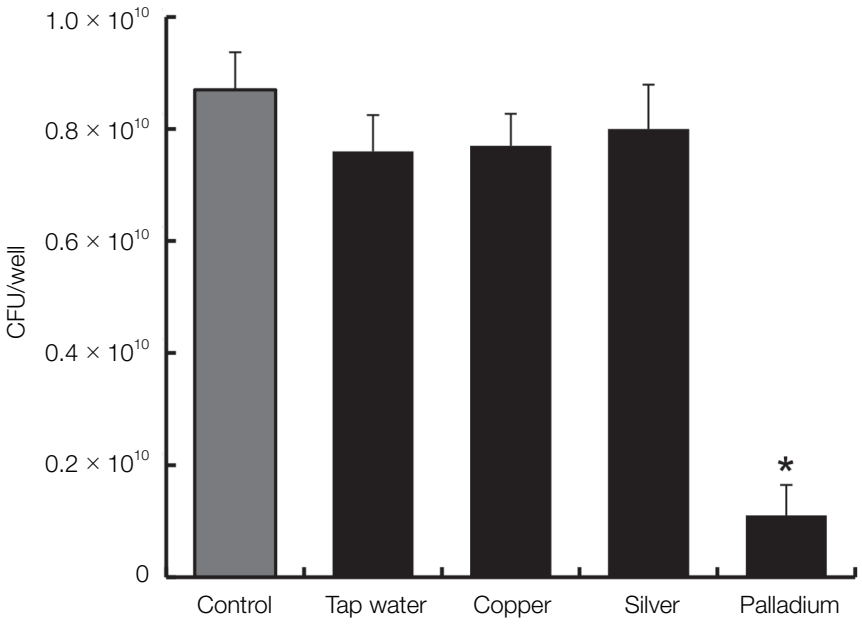

Fig. 5. The antifungal activity of the electrolyzed water using various electrodes against *C. albicans*. *C. albicans* was cultivated by sabouraud dextrose broth in aerobic condition overnight and then harvested by centrifugation. The pellet of *C. albicans* was treated with tap water and electrolyzed water using various electrodes for 12 minutes. The live *C. albicans* was counted by colonies after plating on sabouraud dextrose agar. The examinations were performed three times. * Indicates a significant difference compared with control ($P < 0.05$).

Discussion

Candida albicans is normally a harmless commensal fungus in oral cavity. However, they can cause mucosal disease in some immunologically weak patients, xerostomia patients and older people.[1] *C. albicans* grows with yeast (blastoconidia) or hyphal morphology according to growth conditions.[3] One of the major factors to the virulence of *C. albicans* is morphological flexibility that results in adaptation to environments.[17] Thus, they easily have resistant ability for antifungal agents. Also, *C. albicans* is frequently found on denture materials and associated with denture-related stomatitis.[18]

Electrolyzed water is generated by electric current passed via water using metal electrodes. Also, most electrolyzed water is generated using palladium electrode and has been showed antibacterial activity for oral bacteria.[15] However, antifungal effect on *C. albicans* has not been reported. Thus, this study investigated antifungal activity of EWP on *C. albicans* biofilm of hyphal type.

C. albicans formed blastoconidia in cultivating using sabouraud dextrose broth at 37°C. Also, they formed hyphae in cultivating using F-12 nutrient media at 37°C in 5% CO_2 condition. The blastoconidia and hyphal type of *C. albicans* showed different virulence.[17] Also, *C. albicans* exists with hyphae or hyphal biofilm in oral cavity.[19,20] Therefore, *C. albicans* was investigated susceptibility test for EWP after forming hyphae and hyphal biofilm. The EWP significantly showed antifungal effects on blastoconidia and hyphae of *C. albicans*. The hyphae exhibited more resistance for electrolyzed water and listerine than blastoconidia. Next, *C. albicans* was formed hyphal biofilm on rough surface of plastic wear. *C. albicans* biofilm was observed and confirmed by phase contrast microscope and then the biofilm was treated with the

EWP for various times. The EWP showed antifungal activity for *C. albicans* biofilm from 4 minutes. *C. albicans* produces extracellular matrix as glucan which is a barrier for antifungal agents. Thus, they are more resistant against antifungal agent than yeast type of *C. albicans*.[21] The electrolyzed water is not chemical solution. Thus, the electrolyzed water do not have side effect and is an environment-friendly solution. Although, the electrolyzed water affects antifungal activity by long time reaction, the electrolyzed water may have enough antifungal activity for application and prevention of denture stomatitis.

Finally, all electrolyzed water using various metal electrodes were examined effect of antifungal activity on *C. albicans* biofilm. Copper and silver electrode did not affect *C. albicans* biofilm except palladium electrode. The electrolyzed water has various characteristics according to metal electrode. When tap water was electrolyzed using metal electrode, metal electrodes produce weakly alkaline water or acidic water.[10] However the EWP is pH 7.2. Antibacteria related electrolyzed water was generated from electrolyzing NaCl in separated water chamber.[22] Acidic electrolyzed water in cathode chamber has strongly antibacterial activity and has used to wash foods.[23] However, the EWP in this study is neutral pH. The reason of the different antifungal activity was searched in conductivity. The chemical reaction is follows, $H_2O \rightarrow 1/2O_2 + 2H^+ + 2e^-$, $2Cl^- \rightarrow Cl_2 + 2e^-$ and $Cl_2(aq) + H_2O \leftrightarrow HClO + HCl$ in anode side; $H_2O + 2e^- \rightarrow 1/2H_2 + OH^-$ in cathode side. However, high conductivity of palladium electrode compared to copper and silver electrode may produce radical oxygen (O_2^-), free chloride (Cl^-) and reactive hydrogen (H). These ions can bind components in bacterial surface and destruct cell wall. The concentration and reactive time of free chloride are important factors for bactericide.[24] For the reasons, the EWP may have antifungal activity for *C. albicans* biofilm.

Conclusion

To prevent candidiasis, mechanical cleaning as scrubbing with brush and chemical cleansing as mouth gargle and denture cleanser were used. Also, it can not easily prescribe steroid-line drug to older patients. In case of dental wearers, although the chemical cleansing is considered to be the most effective method for removing *C. albicans*, the chemicals are possible to change physical characteristics of denture wearers such as surface roughness, porosity and solubility. Also, some denture cleansers impair resilient liners and cause gastric perforation. However, the EWP has been not reported side effect and is not chemical solution. In this study, the EWP has antifungal activity against candidal biofilm. Therefore, the EWP may be possible to use a gargle solution and a soaking solution for prevention of oral candidiasis and denture-related stomatitis.

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Candid albicans 바이오필름에 대한 전기분해 수소수의 항진균 효과

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목적: *Candida albicans*는 면역력이 약한 환자에게서 점액질환을 야기하며, 의치성 구내염과 밀접하게 관련되어 있다. 전기 분해 수소수는 금속 전극을 통해서 전기를 흘려 전기분해 한 물이며, 구강세균에 대해서 항균효과를 보였다. 본 연구에서는 칸디다 바이오필름에 대한 전기 분해 수소수의 항진균효과를 조사하였다.

연구 재료 및 방법: *C. albicans*는 발아세포 및 균사형태를 형성시키기 위해서 사부로포도당 액체배지 및 F-12 영양 배지를 이용하여 호기상태 및 5% 이산화탄소 환경에서 각각 배양하였다. 여러 금속 전극을 이용하여 수돗물을 전기분해 한 후에 발아세포 형태와 균사형태의 칸디다를 전기분해 수소수로 처리하였다.

결과: 사부로포도당배지와 F-12 영양배지를 이용하여 칸디다를 배양하였을 때, 각각 발아세포 형태와 균사 형태를 보였다. 전기 분해 수소수는 발아세포 형태의 *C. albicans*에 대해서 항진균 효과를 보였다. 또한 칸디다 바이오필름에 대해서도 항진균 효과를 보였다. 여러 종류의 금속 전극을 이용한 전기분해 수소수중에 백금전극을 이용한 전기분해 수소수만 항진균 활성이 있는 것으로 나타났다.

결론: 백금 전기분해 수소수는 구강 칸디다증과 의치관련 구내염을 예방하기 위한 구강 청결제로 사용 가능 할 것이다.

(구강회복응용과학지 2015;31(3):212-20)

주요어: 전기분해 수소수; *C. albicans*; 항진균 효과; 칸디다 바이오필름

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