Development of tissue conditioner capable of binding with anti-microbial protein lactoferrin

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Abstract

Purpose: This study focused on the anti-microbial protein human lactoferrin (hLF) commonly found in saliva, and tried to develop biocompatible dental materials that have higher anti-microbial effects.

Methods: A lyophilized cation exchange resin was added to tissue conditioner at 4 wt% and 8 wt%. The amount of hLF binding to the tissue conditioner and their anti-microbial effect against Candida albicans was investigated. Then their mechanical properties and cytotoxicity were examined.

Results: Tissue conditioner containing cation exchange resin was bound with hLF and had an anti-microbial effect against C. albicans. In addition, their physical properties were sufficient and they were harmless to human fibroblasts.

Conclusion: The clinical application of cation exchange resin for tissue conditioner can be effective for the prevention and treatment of denture stomatitis and systemic opportunistic infections since it is thought that these materials will increase the local concentration of anti-microbial protein in saliva at the lesion site.

Keywords: Lactoferrin; Tissue conditioner; Antifungal activity; Biocompatibility; Saliva

1. Introduction

Tissue conditioners harden and degenerate with time, and are susceptible to colonization by microorganisms [1]. Unless a tissue conditioner is replaced regularly, it acts as a reservoir for microorganisms that can cause additional complications. In elderly patients who have decreased immunological activity, complications such as pharyngeal and respiratory tract problems [2,3] may occur from microorganisms that have colonized on the tissue conditioners [4]. Accordingly, maintenance of tissue conditioners and the prevention of colony formation by microorganisms are important for oral hygiene.

Candida albicans is commensal yeast that colonizes the oral mucosa of most healthy individuals [5], but it is also the major fungal pathogen of humans, frequently causing denture stomatitis and systemic opportunistic infections [5–7].

Recently, many attempts to incorporate antifungal agents into tissue conditioners have been reported with limited success [8–13]. However, some problems now arise; whether these anti-microbial agents have harmful effects, such as causing allergies, upon human bodies. Therefore, we focused on the anti-microbial protein in saliva, and we have been researching how to develop safe anti-microbial dental materials.

Human lactoferrin (hLF) is an iron-binding protein (77 kDa) present in saliva and gingival cervical fluid that possess in vitro anti-microbial qualities by sequestering the free iron required for microbial growth and by direct interaction with the bacterial surface [14].

The purpose of this study was to determine whether hLF binds to the tissue conditioners containing cation exchange resin and to evaluate their antifungal activity against C. albicans. In addition, their mechanical properties and cytotoxic effect were examined.
2. Materials and methods

2.1. Materials

2.1.1. Anti-microbial protein solution

Commercially available human lactoferrin was used in this study (hLF; Sigma–Aldrich, Steinheim, Germany). The lyophilized hLF was dissolved in sterilized 5 mM potassium phosphate buffer (PPB; K₂HPO₄–KH₂PO₄ [pH 6.0]).

2.1.2. Cation exchange resin

TOYOPEARL CM650 (TOSOH, Tokyo, Japan) was used as a cation exchange resin. This resin is a modified methacrylate polymer which gives the resin a hydrophilic surface due to the presence of hydroxyl groups (–O–CH₂COO–). It also bestows upon the resin excellent pressure/flow characteristics and pH stability. It is suitable for developing the purification conditions of biological target molecules such as proteins or nucleic acids.

2.1.3. Dental materials

The tissue conditioners are soft, resilient materials used to treat inflamed, irritated and distorted tissues as well as to record functional impressions. They are also used as temporary linings of dentures [15]. The tissue conditioner examined in this study was TISSUE CONDITIONER II (Shofu Inc., Kyoto, Japan), which is commercially available. The tissue conditioners were mixed according to the manufacturer’s instructions.

2.1.4. Yeast strains, media and growth conditions

The C. albicans ATCC10231 used in this study was provided by Dr. Shimokawa (Nihon Pharmaceutical University). It is a Candida species involved with denture stomatitis by oral candidiasis and aspiration pneumonia in humans. C. albicans cells from glycerol stocks were cultured on Sabouraud Dextrose Agar (SDA) plates. For the experiments, yeast cells were grown at the stationary phase and subcultured in Sabouraud Dextrose Broth (SDB) to the mid-logarithmic growth phase in a centrifugation tube at 30 °C.

2.2. Specimen preparation

Lyophilized cation exchange resins were added to tissue conditioner at 4 wt% and 8 wt% as experimental specimens. A previous study has demonstrated the approximate 100–400 μg/ml hLF to kill C. albicans cells [16]. Additionally, we confirmed that 4 wt% and 8 wt% cation exchange resins were able to bind with 200 μg and 400 μg hLF, respectively (data not shown). Therefore, we could expect that the contents of the cation exchange resin in the specimens would exert antifungal activity. Then the specimens were prepared in cylindrical 3 mm × 6 mm Teflon. The specimens were hardened for a few hours at 25 °C after mixing. The specimens were removed from the mold after 24 h and soaked into distilled water after 24 h for testing. The dimensions of the specimens were checked with a micrometer. Conventional powder/liquid type tissue conditioners without cation exchange resins were used in the fabrication of the control specimens.

2.3. Binding of hLF to the tissue conditioner containing cation exchange resin

After the experimental and control specimens were hardened, they were immersed in distilled water for 24 h and then incubated in 1.0 ml of hLF solution (800 μg/ml) for 24 h at 25 °C. After the incubation period, the absorbance of hLF solution was measured by BCA™ Protein Assay Kit (PIERCE, Rockford, IL, USA). The level of hLF binding with the specimens was calculated from the difference between the absorbance of hLF solution soaked specimens and that of hLF solution without specimens (N = 5, respectively).

2.4. Antifungal activity of the tissue conditioner binding with hLF against C. albicans

Each isolate cultured in SDB was suspended in 5 mM PPB and the density of fungal suspensions was adjusted at 10² cells/ml by measuring turbidity at 600 nm. The specimens containing 4 wt% or 8 wt% cation exchange resins immersed in 5 mM PPB, instead of hLF solution, were used as control. The specimens were immersed in 1.0 ml of fungal suspension and incubated for 24 h at 25 °C. After the incubation, the fungal suspensions were harvested on SDA plates and the number of colonies was counted (CFU/ml) (N = 5, respectively). Cell viability (%) was calculated as

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\frac{\text{CFU of the control or experimental specimens}}{\text{CFU of fungal suspension}} \times 100.
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2.5. Cytotoxicity test

2.5.1. Preparation of eluates of specimens

For the preparation of eluates, each specimen was quickly rinsed with 70% ethanol and phosphate-buffered saline (PBS), followed by soaking in 2 ml cell culture medium in a 24-well plate in a humidified incubator at 37 °C with 5% CO₂ and 95% air for 3 days. The surface area to volume ratio was 0.5 cm²/ml which was set according to the ISO standards (0.5–6.0 cm²/ml) [17].

2.5.2. Evaluation of cytotoxicity using WST-8 colorimetric assay

The methyltetrazolium (MTT) test was performed as described [18]. Briefly, Balb/c 3T3 mouse fibroblast cells were plated in a 96-well plate at 1 × 10⁵ cells per well in 100 μl of Dulbecco’s Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin. After incubation at 37 °C overnight, the medium was replaced with 100 μl of the fresh medium containing different concentrations of eluate (80%). The cells were then incubated for 3 days and/or 7 days. The WST-8 colorimetric assay was conducted by adding 10 μl of WST-8 (5 mg/ml) into a well to make a final concentration of 0.5 mg/ml and then incubating the plate at 37 °C for another 2 h. The optical density was measured at 450 nm using an ImmunoMini
microplate reader (Inter Med, Japan). Cell viability (%) was obtained by dividing the OD450 value of the treated specimen by that of the untreated one.

2.6. Mechanical strength test

Lyophilized cation exchange resins were added to tissue conditioner at 4 wt% and 8 wt% as experimental specimens (N = 5, respectively). Conventional powder/liquid type tissue conditioners without cation exchange resins were used in the fabrication of control specimens. Each mix was packed into a stainless steel split mould of 20 mm in length and 10 mm in diameter with a spatula. Glass plates were placed at either ends of the mould and were clamped with sufficient pressure to extrude excess material. After 24 h, the specimens were removed from the mould and compressed to a height of 2 mm at a cross-head speed of 20 mm/min on a RHEOMETER (SUN SCIENTIFIC CO., Ltd., Tokyo, Japan). The stress–strain curves were obtained and the elastic modulus values (EM values) were calculated.

2.7. Statistical analysis

All data were analyzed by ANOVA and Scheffe’s multiple range tests (P < 0.05).

3. Results

Fig. 1a shows the resultant values by the protein assay of hLF solution in which the specimens were immersed for 24 h. The amount of hLF binding to the specimens calculated from the protein concentration of the hLF solution. The vertical bars indicate standard deviation for five replicates. Asterisks indicate significant difference among specimens (*P < 0.05, ANOVA and Scheffe’s multiple range tests).

Fig. 2 shows the assessment of antifungal activity against Candida albicans after incubation for 24 h. The viabilities in 4 wt% and 8 wt% of the experimental specimens were significantly lower than those of the control. However, the antifungal activity of 4 wt% and 8 wt% were not significantly different (P > 0.05).

Fig. 1b shows the amount of hLF binding to the specimens calculated from the difference in protein concentration of hLF solution before and after 24 h incubation. The amount of bound hLF in the specimens was 52.3, 228.0 and 450.5 mg at the control, 4 wt% and 8 wt%, respectively. The amount of bound hLF of the control, 4 wt% and 8 wt% was significantly different from each other (P < 0.05).

Fig. 2 shows the assessment of antifungal activity against C. albicans after incubation for 24 h. The viabilities in 4 wt% and 8 wt% of the experimental specimens were significantly lower than those of the control. However, the antifungal activity of 4 wt% and 8 wt% were not significantly different (P > 0.05). The CFU values of the control specimens were higher than those of fungal suspension (data not shown). In other words, the viabilities of the control specimens were above 100%.

The cell viability (%) of the experimental and control specimens using eluates with specimens incubated for 3 and 7 days are presented in Fig. 3. The control (104.1 ± 12.6), 4 wt% (103.5 ± 4.4) and 8 wt% (120.2 ± 16.0) were not significantly
different from each other for the 3-day incubation \((P > 0.05)\) (Fig. 3a). Likewise, the control \((95.6 \pm 10.2)\), 4 wt\% \((97.1 \pm 1.3)\) and 8 wt\% \((100.4 \pm 6.2)\) were not significantly different from each other for the 7-day incubation \((P > 0.05)\) (Fig. 3b).

The EM values of the experimental and control specimens are presented in Fig. 4. The EM values of 4 wt\% and the control were not significantly different \((P > 0.05)\). The EM values of 8 wt\% were significantly higher than those of the control and 4 wt\% \((P < 0.05)\).

4. Discussion

Recently, numerous attempts have been made to prevent oral diseases by adding anti-microbial agents to dental materials [19,20]. However, it is possible that these materials can have harmful effects, such as allergies, upon a living body. Therefore, this study focused on the anti-microbial protein, human lactoferrin, which is commonly present in the oral cavity.

In addition, the present study demonstrated that tissue conditioner containing cation exchange resin bound with hLF and had an antifungal effect against \textit{C. albicans}.

A small amount of hLF bound to the control specimens, thus suggesting that hLF may adsorb to tissue conditioner nonspecifically because hLF gets caught in the pores of tissue conditioner surfaces (Fig. 1a and b). Nevertheless, the amount of bound hLF in the experimental specimens was significantly greater than the control. This means that the specimens of 4 wt\% and 8 wt\% increased its binding by about four and nine times, respectively, in comparison with the control (Fig. 1b).

The concentration of lactoferrin in the saliva of healthy adults is known to be approximately 20 \(\mu\)g/ml [21], and the salivary flow rate is known to be 1000–1500 ml per day [22]; that is, the amount of hLF binding to the experimental specimens, which is accumulated from the whole saliva secreted for a day, was about 10–20 times as much as that of hLF in 1 ml saliva. The polymorphonuclear leukocytes (PMN) in gingival crevicular fluid have extremely high hLF contents [23]. Consequently, the accumulation of lactoferrin in the saliva of healthy adults is known to be approximately 20 \(\mu\)g/ml [21], and the salivary flow rate is known to be 1000–1500 ml per day [22]; that is, the amount of hLF binding to the experimental specimens, which is accumulated from the whole saliva secreted for a day, was about 10–20 times as much as that of hLF in 1 ml saliva. The polymorphonuclear leukocytes (PMN) in gingival crevicular fluid have extremely high hLF contents [23]. Consequently, the accumulation of lactoferrin at high levels is predicted. Moreover, there will be atrophy in the salivary glands with aging, resulting in decreased salivary flow. Therefore, these results suggest that the experimental specimens could contribute to the concentration of lactoferrin in the oral cavity for elderly people in particular.

Antifungal activity of the experimental specimens against \textit{C. albicans} was significantly greater than that of the control (Fig. 2). However, the concentration dependence of cation exchange content (4 wt\% and 8 wt\%) did not significantly affect the antifungal property. The experimental specimens of 4 wt\% could even reduce the viability of the yeasts sufficiently.

The viabilities of control specimens containing 4 wt\% and 8 wt\% cation exchange resin were above 100%. Individual compounds (e.g. plasticizer, ethanol) eluting from control specimens by the addition of cation exchange resin might have caused the fungal growth; the facilitation of \textit{C. albicans} growth might have been due to the alternation of hardened tissue conditioner composition. It has been reported that ethanol gradually eluting from the tissue conditioners was utilized as a carbon source for \textit{Candida} spp. [24].

In the present cytotoxicity study, a Balb/3T3 fibroblast cell line was used to examine the \textit{in vitro} cytotoxicity of the
experimental specimens and compare 4 wt% and 8 wt% with the control with the help of the MTT assay. Both 4 wt% and 8 wt% showed high cell viability after cell exposure to the 3- and 7-day eluate (Fig. 3). It has been reported that the cell viabilities of some tissue conditioners containing silver-zeolite (SZ) decreased with increasing SZ content and one of the reasons for this might be that silver ions released from SZ caused the cytotoxic effects [25]. In another study, the addition of Microban (a broad-spectrum anti-microbial compound containing triclosan) to soft denture liner did not show signs of cytotoxicity but also did not show any significant antifungal activity [26]. However, in our study, the experimental specimens showed no reduction of cell viability and showed sufficient antifungal activity by binding with hLF (Figs. 2 and 3). In this preliminary study, an in vitro cytotoxicity study of the new tissue conditioner was conducted with Balb/3T3 fibroblast cells. In the future, it will be necessary to test the cellular response of the new tissue conditioner on dental pulp cells (more clinically relevant cells) and conduct in vivo animal studies.

The use of soft liners produced a dramatic improvement in masticatory function and satisfaction compared with hard based dentures. Furthermore, the mechanical properties of the experimental specimens were found to be an important factor in bringing about this improvement. There were no significant differences among the experimental specimens containing 4 wt% resin and the control (P > 0.05) (Fig. 4). In contrast, the experimental specimens containing 8 wt% resin showed higher elastic modulus values than the control (Fig. 4). But there was considerable variability in the physical and mechanical properties of soft liners [27], and the required essential properties for soft liners are as yet not determined. Ranges of the EM values of edentulous oral mucosa were very large (0.66–4.36 MPa) and the average value was 2.73 MPa [28]. In the present study, the EM values of 4 wt% and 8 wt% were 0.27 MPa and 0.33 MPa, respectively. These values were lower than those of edentulous oral mucosa. The compatibility of these values with oral mucosa may be varied depending on the condition of the oral tissue.

In regard to the thermal stability of hLF, a previous study showed that native hLF maintained its structural integrity and iron-binding property at high temperature (72 °C) [29]. Another study reported that hLF exerted the antifungal activity against C. albicans cells at 37 °C [16]. Therefore, hLF binding with the specimens will be used to improve the health of the oral cavity without denaturation under various high temperatures simulating intraoral circumstances.

Taken together, the clinical application of cation exchange resin for prosthesis may be available for the prevention of denture stomatitis and systemic opportunistic infections, since the concentration of anti-microbial protein in saliva is increased in the oral cavity by this procedure. The major advantages of this application could be for elderly people with restricted manual dexterity or cognitive disturbances. Further studies should be conducted addressing the antibacterial effects against C. albicans by the application of cation exchange resin to denture base acrylic resin as well as tissue conditioner. Although the use of tissue conditioner to improve the adaptation of the denture in cases of denture stomatitis is part of routine treatment, it has been shown that it also promotes or supports in vivo Candida tropicalis and Candida glabrata colonization [30]. C. glabrata is known to more pathogenic and resistant to hLF than C. albicans. Therefore, it might be necessary to examine the long-term antifungal effect of this material against other Candida spp. [31].

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References


