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ScienceDirect
Biomolecular Engineering 24 (2007) 483–488
Biomolecular Engineering
www.elsevier.com/locate/geneanabioeng

Antibacterial functionalization of an experimental self-etching primer by inorganic agents: Microbiological and biocompatibility evaluations

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Abstract

Antibacterial activities have been demonstrated on oral bacteria with inorganic antibacterial agents (ABAs) after their incorporations into an experimental self-etching primer (ESP) before curing. This study was to assess their biocompatibility and antibacterial activity after curing. Six ABAs were incorporated respectively into ESP for treating specimens. After curing, their bactericidal activities on *Streptococcus mutans* and influences to the early bacterial colonization were assessed by direct contact and viable count. Systemic toxicity in rats after short-term oral exposure and direct contact cytotoxicity with NIH3T3 fibroblasts were tested. Incorporation of ZnOw AT-83, Longbei antibiotic, Antim-AMS2 or IONPURE-H significantly enhanced the antibacterial effect of ESP after curing, even after 1 month aging. Specimens treated by ESP with ZnOw AT-83, Longbei antibiotic or Antim-AMS2 showed slightly less bacterial adhesion than control. Animal experiments revealed neither toxic signs nor significant differences in body weight gain between control and other groups. Cell vitality or proliferation rates were ranged from 76% to 100% with respect to controls. Basic magnesium hypochlorite, ZnOw AT-83 and ZnOw AT-88 were less toxic. Toxicity only observed in areas beneath the specimens and/or in the direct vicinity of the specimen edge. From microbiological and biocompatibility aspects, the tested ABAs can be effectively incorporated in ESP to provide antibacterial activity against *S. mutans*. ZnOw AT-83 was the most promising one.

Keywords: Caries; Antibacterial; Inorganic agents; Self-etching primer; *Streptococcus mutans*; Biocompatibility

1. Introduction

Persistent attempts have been made to develop antibacterial dental restorative materials (Jedrychowski et al., 1983; Ohashi et al., 2004; Imazato et al., 2006), as dental caries is an infectious disease of bacterial origin. Such kind of materials aim at killing the residual bacteria in the cavity and inactivating the invading bacteria via microleakage, thus will offer a new pattern of caries management, which can be called ''preventive-oriented treatment''. It will surely prolong the life expectancy of restorations, and is possible to realize a complete cure of caries. Nevertheless up to now, there is only one bonding system newly developed by Kuraray Co. Ltd. (Japan), which is reported with substantial antibacterial activity (Imazato et al., 2006). Much more efforts are still in great need to provide antibacterial series of dental restorative materials.

An experimental self-etching primer ESP was developed by our group and has been proved for having good etching effect and satisfactory bond strength on teeth (Zhang et al., 2004, 2005). The antibacterial effects of six inorganic ABAs on several cariogenic bacteria strains (Fang et al., 2006) and the antibacterial activities after their incorporation into ESP before polymerization have been testified. Since self-etching primers evolve from unpolymerized to polymerized state during their functioning, the antibacterial activity of ESP incorporating different ABA after polymerization, which is a critical point of its preventive effect, should be investigated. Additionally, preliminary biocompatibility tests are also essential to assess and characterize the potentially harmful effects to human tissues. This study was performed in both purposes.

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doi:10.1016/j.bioeng.2007.07.013

2. Materials and methods

2.1. Inorganic antibacterial agents and experimental self-etching primer

Six ABAs were used: ABA1 was Basic magnesium hypochlorite $[Mg_2ClO(OH)_3{\cdot}H_2O]$ (Hunan Research Institute of Chemical Industry, China); ABA2 was Cu, Zn-loaded zeolite—Longbei inorganic antibiotic powder (Jiangsu Changtai Nanometer Material Co. Ltd., China); ABA3 was Zn, Ag, Cu-loaded silicate—Antim-AMS2 (Beijing ChamGo Nano-Tech Co. Ltd., China); ABA4 was Ag, Cu-loaded glass—IONPURE-H (Ishizuka Glass Co. Ltd., Japan); ABA5 and ABA6 were both zinc oxide whisker (ZnOw) from Advanced Technologies & Crystal-wide Co. Ltd., China—the former, ZnOw AT-83, loaded with Ag and Cu, and the latter was ZnOw AT-88 only with Cu. The newly developed experimental self-etching primer ESP was tested in this study, of which main components are maleic acid, 2-hydroxyethyl methacrylate and acetone.

2.2. Preparation of cured specimens and artificial aging procedure

Melinex® OD polyester Film (DuPont TeijinFilms™, Ø14.9 mm), with good biocompatibility, was used as a substitute for resin. Adhesion procedure was performed in reversal order of clinical application to create specimen whose surface simulated the restorative interface contacting with tooth. 5 μL of Adaper™ Singlebond 2 (3M ESPE) was applied onto its surface and spread to a thin layer, followed by light curing for 20 s (Kerr Optilux Demetron VCL 401). 5 μL of ESP with or without ABAs (at 0.5%, w/v) was applied as described above. After light curing for 40 s, the specimen was immersed in distilled water and agitated for 1 h to remove off the uncured components. Aging procedures were undertaken by immersing specimens separately in artificial saliva and incubating in CO_2 incubator at 37 °C with 100% relative humidity for 1 month.

2.3. Bactericidal activities of cured specimens

An overnight culture of *S. mutans* ATCC 25175 was adjusted to about 1.0×10^7–1.0×10^8 CFU/mL in Brain-Heart Infusion (BHI, Difco) broth. 100 μL of this suspension was added on each specimen. Then sterilized polyethylene films were covered onto the specimens that would undergo a 24 h contact (CLIS, 2003), to make the bacterial suspension distribute homogeneously and avoid desiccation. After incubation for 1 or 24 h, bacteria were collected by vigorously shaking the specimen in 9.9 mL Ringer solution (RS) containing 0.5% Tween 80, which is helpful for rinsing off the bacteria from the specimens. The bacterial suspensions were inoculated and the number of recovered viable bacteria was determined by counting the colonies after 24–48 h anaerobic incubation at 37 °C. The test was repeated seven times.

2.4. Inhibition of early bacterial adhesion of cured specimens

One specimen of each group was put in a Petri dish (Ø90 mm) with conditioned surface upwards. Bacterial suspension (12 mL) prepared as mentioned above was added into the dish. After 2 h incubation, the specimens were taken out and passed through sterilized RS to dislodge non-adherent bacteria. They were then vortexed separately in 10 mL RS containing Tween 80 for 30 s to remove the adherent bacteria for culture and quantitation. The reason to choose 2 h exposure was that complete biofilm formation in oral cavity normally occurs in 2–4 h (Montanaro et al., 2004). This test was repeated seven times.

2.5. Systemic toxicity tests in rats following short-term oral exposure

According to the Chinese Medical Industry Standard YY/T0244-1996 (CMIS, 1996), 40 male and 40 female Sprague–Dawley rats (130–150 g) were randomly divided into eight groups. ESP with or without 5.0% (w/v) ABA was formulated at a concentration of 20% in 2% (w/v) starch solution, and administered via oral gavage each day after fasting for 4 h, at a dose of 5 mL/kg. Pure starch solution was used for negative control. Administration was lasted for a week body weights of rats were recorded prior to dosing and their clinical signs were observed twice daily for 14 days. Then all rats were sacrificed and subjected to autopsy. Main internal organs were examined macroscopically, followed by histopathological microscopy analysis.

2.6. Cytocompatibility tests

Thermanox® cover slips (Nunc, Ø13 mm), similar as Melinex, were used for preparation of cured specimens as mentioned above. Based on ISO 10993-5 (ISO, 1999), direct contact test was performed with an established cell line NIH3T3 mouse connective tissue fibroblast ATCC CRL 1658 in six-well culture plates. Thermanox without any surface treatment were served as carrier control, and the bottom of wells without any treatment were used as negative control. Ninety thousand cells in 5 mL Dulbecco's modified MEM medium (Gibco), supplemented with 10% decomplemented new-born calf serum (Gibco) were seeded into each well and incubated at 37 °C in 5% CO_2 atmosphere for 48 h to reach subconfluency. Medium was removed and one specimen was carefully put into each well with 2 mL renewed medium. Three specimens were used for each group. After incubation for another 24 h, specimens and culture medium were taken out and replaced by 2.5 mL medium containing 10% Alamar Blue (Interchim), which is a nontoxic dye frequently used for precise indication of cell function due to its reduction by intracellular enzyme activity (O'Brien et al., 2000). Fluorescence measurements were recorded by a Fluorometer (TwinkleTM LB 970, Berthold Technologies) after 3 h incubation. Cell proliferation was measured successively by a cell counter Z1 (Coulter Electronics) following trypsinization. Results are expressed as the mean percentage of five separate triplicate assays for each specimen. Similar cell seeding and specimen exposure were done using Petri dishes (Ø60 mm) and cells were stained with 2% crystal violet for morphological observations.

2.7. Statistical analysis

Data of bacteriological tests were analyzed by non-parametric Kruskal–Wallis test and Mann–Whitney's *U*-test. Body weights of rats were analyzed by ANOVA and LSD test. Results of cytocompatibility tests were subjected to ANOVA and Scheffe test.

3. Results

3.1. Bactericidal activities of cured specimens

The incorporation of ABA1, ABA2, ABA3, ABA4 or ABA5 significantly improved the bactericidal activity of cured specimens after aging ($P < 0.05$), regardless of incubation time (Fig. 1). Among them, ABA5 was the most effective one. Results were improved with prolonged incubation time for each material. When the specimens were incubated with bacteria for 1 h, there were no significant differences in the percentage of bacteria recovered between pre- and post-aging for each group. Nevertheless when prolonged incubation time (24 h) was tested, significant increases of data were revealed from the specimens after 1 month aging in artificial saliva for each group, comparing with those of pre-aging ($P < 0.05$).

3.2. Inhibition of early bacterial adhesion of cured specimens

Melinex specimens conditioned by ESP incorporating with ABA5, ABA2 or ABA3 had slightly less bacterial adhesion than those without any treatment (Fig. 2). There were no significant differences among nine groups. Significant differences were found between ESP and Melinex control, adhesive and Melinex control, ESP and ABA5-containing ESP ($P < 0.05$). No other