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ORIGINAL ARTICLE

Efficacy of denture disinfection methods in controlling *Candida albicans* colonization *in vitro*

RALF BUERGERS[1], MARTIN ROSENTRITT[1], WULF SCHNEIDER-BRACHERT[2], MICHAEL BEHR[1], GERHARD HANDEL[1] & SEBASTIAN HAHNEL[1]

[1]*Department of Prosthetic Dentistry, Regensburg University Medical Centre, Germany and* [2]*Institute of Medical Microbiology and Hygiene, Regensburg University Medical Centre, Germany*

Abstract

Objective. The aim of this study was to rank 10 denture disinfection methods according to their efficacy in reducing *Candida albicans* (*C. albicans*) colonization on soft denture relining material. ***Material and Methods.*** Circular specimens (diameter 8 mm) were made of soft denture relining material (Mucopren E, Kettenbach) and thermally aged. Specimens were incubated with *C. albicans* (strain 1386, DSMZ) followed by 1 of 10 disinfection procedures (6 soaks, 2 microwave irradiation regimes, 1 effervescent commercial cleansing product, and denture left dry overnight). Incubation with phosphate buffered saline (PBS) served as a control. Adhering fungi were quantified using a bioluminometric assay in combination with an automated plate reader for cell quantification. Scanning electron micrographs (SEMs) were made for validation. ***Results.*** Low median luminescence intensities indicated the presence of a few viable fungi after the soaking of specimens in sodium hypochlorite (10 relative luminescence units (rlu)), microwave irradiation immersed in water (8 rlu), and application of effervescent cleansing tabs (22 rlu). No statistically significant difference ($p > 0.05$) to control PBS (200 rlu) was found after immersion in hydrogen peroxide (172 rlu), glutaraldehyde (103 rlu), household vinegar (196 rlu), Listerine coolmint (194 rlu), Plax (222 rlu), dry microwave irradiation (221 rlu) and specimens left dry overnight (165 rlu). SEM displayed *C. albicans* monolayers with different morphologic forms on each surface investigated. ***Conclusions.*** Only soaking in sodium hypochlorite (1%; 10 min), microwave irradiation immersed in water (800 W; 6 min), and application of effervescent cleansing tabs (Blend-a-dent tabs; 10 min) proved to be effective against *C. albicans* colonization on soft denture relining material.

Key Words: *Denture relining, disinfection, fungi, luminescence*

Introduction

Candida albicans (C. albicans) is the most prevalent fungus in the human oral cavity and has been known as the primary cause of denture-related stomatitis [1]. In general, yeast cells have a high potential to adhere to dental material in almost the same manner as to oral tissues, and they are known to be extremely resistant to many disinfectant measures [2]. Mechanical cleansing and chemical disinfection have been recommended for reducing microbial biofilm formation and thus for maintaining the serviceability of prostheses [3,4]. In this context, elderly or handicapped patients, whose manual dexterity may be limited, often prefer chemical denture cleansers over mechanical brushing [4]. A great variety of cleansing agents and disinfection regimes with antifungal properties are available, including soaking in chemical solutions or household fluids, microwave irradiation and exposure to oxygen through air-drying. Sodium hypochlorite (bleach), glutaraldehyde and hydrogen peroxide are traditional dental disinfectants that are widely used for reducing biofilm accumulation on removable prostheses [3,5]. Former studies have shown sodium hypochlorite to be effective in reducing *C. albicans* adhesion *in vivo* and *in vitro* [3,4,6,7]. Many patients prefer commercial mouth rinses and household products such as vinegar for denture disinfection. These solutions are readily available, easy to handle, and well-priced, but there is conflicting evidence of their effectiveness [8,9]. Effervescent cleansing products dissolved in water have become a frequent and effective alternative to conventional disinfectants

Correspondence: Ralf Buergers, University Hospital Regensburg, D-93042 Regensburg, Germany. Tel: +49 941 944 6059. Fax: +49 941 944 6171. E-mail: ralf.buergers@klinik.uni-regensburg.de

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[10]. In general, disinfectant solutions have been known to influence the mechanical parameters of the denture material by reducing hardness, dimensional stability, flexural strength, and color stability [5,11,12]. To overcome these objectionable adverse effects, the use of microwave energy has been suggested for inactivation of pathogens on dentures [13]. Diverse irradiation regimes have been assessed and have proved effective [13–16].

In the pathogenesis of *Candida*-associated stomatitis, denture base and soft relining materials may function as a reservoir of infection and reinfection [17]. In modern prosthetic dentistry, soft lining materials are time- and cost-effective means by which to improve the fit of dentures on a provisional or semi-permanent basis. They are used as components to obturators and to modify removable dentures after implant surgery [3,18,19]. Because of their specific unpolishable surface texture, soft lining materials are even more prone to microbial adhesion than denture base resins and efficient mechanical cleansing is not feasible [3,19].

The aim of the present study was to compare the *in vitro* efficacy of 10 widely used denture disinfection methods (6 soaks, 2 microwave irradiation regimes, 1 effervescent commercial cleansing product, and denture left dry overnight) to reduce *C. albicans* colonization on soft denture relining material. The research hypotheses tested were: (a) that there was no significant difference between the efficacies of the 10 tested disinfection regimes in preventing initial fungal adhesion, and (b) that there were significant differences compared to the control treatment (incubation with PBS).

Material and methods

Specimen preparation

Circular specimens ($n = 132$; diameter 8 mm, thickness 2 mm) of soft denture lining material Mucopren E (Kettenbach, Germany) were prepared using a custom metal mold with calibrated circular holes. The lining material was inserted in the mold and immediately covered with a glass slide (Alfred Becht GmbH, Offenburg, Germany) to obtain smooth specimen surfaces. All specimens were aged in a thermal cycler (Regensburger Kausimulator, EGO, Germany) with 3000 cycles (5°C/55°C), cleansed with ethanol (70%), and fixed onto 96 well-plates (Sarstedt, Newton, NC, USA).

Yeast cell solution

C. albicans human isolate (strain 1386; DSMZ, Braunschweig, Germany) was cultured overnight in yeast broth (3 g yeast extract (Sigma-Aldrich, St. Louis, Mo, USA); 3 g malt extract (Sigma-Aldrich); 5 g Peptone from Casein (Merck, Darmstadt, Germany); 10 g D(+)-Glucose (Merck); 1000 ml distilled water). Cells were harvested by centrifugation at 2300 rpm for 5 min at 18°C. Pellet was washed twice using phosphate buffered saline (PBS; Sigma-Aldrich), and the optical density of the suspension was adjusted to 0.3 at 540 nm (Genesys 10-S; Thermo Spectronic, Rochester, NY, USA).

Disinfection regimes

The *Candida* suspension was incubated with the specimens for 2.5 h at 37°C. To remove non-adhering fungi, all specimens were carefully washed three times in PBS and subsequently randomly subjected to 1 of 10 tested disinfection procedures (Table I). Disinfection solutions were added for the application times given and removed before specimens were carefully washed in PBS. For microwave irradiation procedures, 2 ml distilled water (25°C) was added to half of the specimens; the other half was air-dried. Well-plates were covered and immediately irradiated in an unmodified domestic microwave oven (KOG-6D07, Daewoo Electronics GmbH, Butzbach, Germany; 800 W/2450 MHz) for 6 min. Ten minutes immersion in PBS served as control for all procedures.

Quantification of adhering fungi

All specimens were removed and placed in sterile polypropylene tubes at 4°C; 500 µl perchloric acid (Merck) was added, cups were vortexed for 30 s and 500 µl potassium bicarbonate (Sigma-Aldrich) was added for neutralization. The cups were then centrifuged at 11,900 rpm for 15 min at 4°C; 100 µl of each cup was pipetted onto 96 well-plates (Sarstedt) and 100 µl of bioluminescence adenosine triphosphate (ATP) detection kit VIA Light AMR-Plus buffer (Cabrex Bio Science, Rockland, ME, USA) was added to each well. Luminescence was recorded after 5 min by an automated multi-detection reader (Fluostar optima; BMG labtech, Offenburg, Germany) at wavelengths of 590 nm emission. High relative luminescence intensities correlated with high fungal colonization.

Scanning electron microscopy (SEM)

Three specimens of each material and treatment protocol were used for scanning electron microscopy (SEM) morphological validation. The specimens were directly mounted on aluminum stubs and imaged using SEM (magnification $\times 1700$ and $\times 6000$) (Quanta FEG 400; FEI Company, Eindhoven, The Netherlands).

Statistics

Medians and 25/75% percentiles were calculated for evaluating differences in fungal colonization.

Table I. Assessed disinfection methods

Disinfection method	Manufacturer	Application time
Hydrogen peroxide 3%	Herbeta, Berlin, Germany	10 min soak
Sodium hypochloride 1%	Pharmacy, Regensburg University Medical Center, Germany	10 min soak
Glutaraldehyde 2%	Sigma-Aldrich, Munich, Germany	10 min soak
Household vinegar	Netto, Luebben, Germany	10 min soak
Listerine coolmint (alcoholic-based)	Pfizer, Karlsruhe, Germany	10 min soak
Plax (triclosan 0.3%)	Colgate Palmolive, Hamburg, Germany	10 min soak
Blend-a-dent 2 Phasen tabs (potassium monopersulfate, sodium perborate monohydrate)	Procter & Gamble, Schwalbach, Germany	10 min soak
Microwave irradiation, dry	Daewoo Electronics GmbH, Butzbach, Germany	6 min irradiation
Microwave irradiation, immersed in water	Daewoo Electronics GmbH	6 min irradiation
Left dry overnight		5 h
Phosphate buffered saline (Control)		10 min soak

Statistical analysis was based on the Mann-Whitney U-test ($\alpha < 0.05$) and carried out with statistical software (SPSS 11.5 for Windows; SPSS, Chicago, Ill., USA).

Results

Adhering fungi

Figure 1 displays the medians and 25/75 percentiles from the luminometric quantification of adhering fungi after the assessed disinfection procedures had been carried out and the results from the pairwise tests for significance had been obtained (Mann-Whitney U-test). Median relative luminescence intensities ranged between 8 and 222 rlu. After the control treatment with PBS, a median value of 200 rlu was measured. Comparable values, i.e. without any statistically significant difference ($p \geq 0.05$), were found for Plax (222 rlu), dry microwave irradiation (221 rlu), household vinegar (196 rlu), Listerine (194 rlu), hydrogen peroxide (172 rlu), air drying (165 rlu), and glutaraldehyde (103 rlu). Significantly ($p < 0.05$) lower relative luminescence intensities than for PBS control were found for Blend-a-dent tabs (22 rlu), sodium hypochlorite (10 rlu) and microwave irradiation immersed in water (8 rlu). No statistically significant difference was shown between these three procedures ($p \geq 0.05$).

SEM observations

Examples of the SEMs are displayed in Figures 2–5. *C. albicans* mono-species monolayers were found on all specimens examined. The amount of *C. albicans* cells varied at different locations on the same specimen, as well as among the differently treated specimens. Different morphologic forms, such as blastospores, pseudohyphae, true hyphae and mycelium networks, could be found independently of the applied disinfection regime (Figures 2–4). Two phenotypes dominated the fungal biofilm. Typically, the specimens were covered with dense colonies of

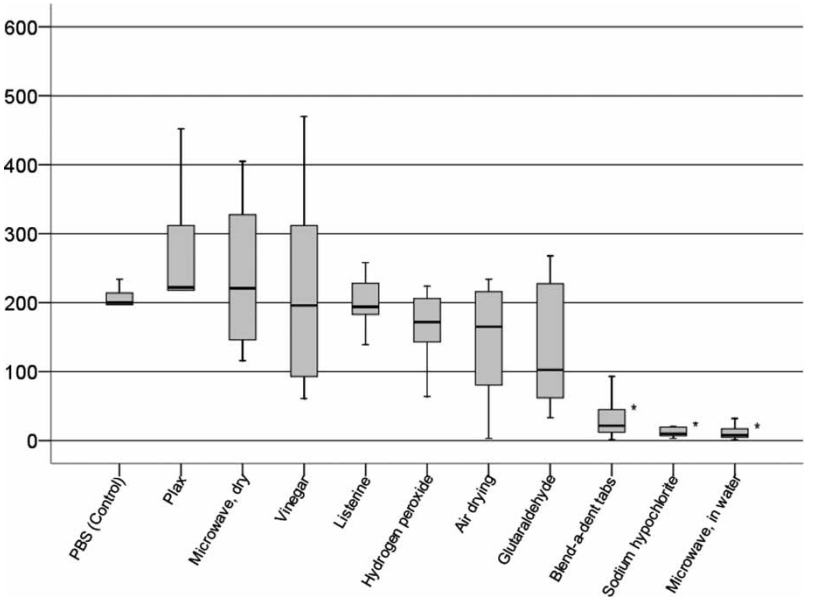

Figure 1. Relative luminescence intensities [rlu] after 10 disinfection procedures (median; 25/75 percentiles); *significantly ($p < 0.05$) lower than PBS control.

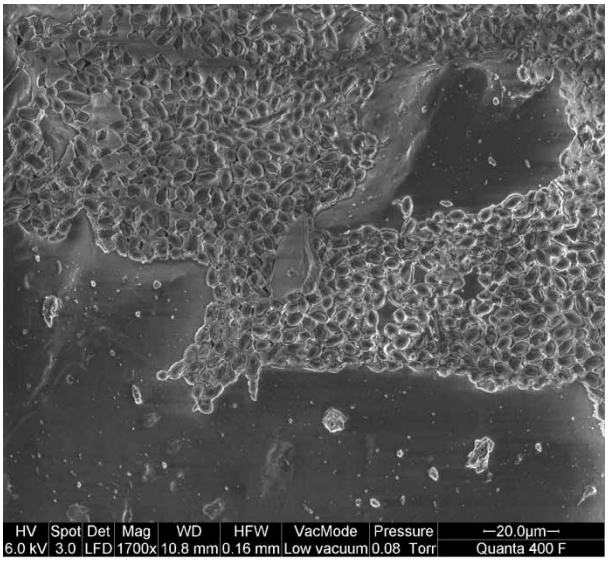

Figure 2. SEM (original magnification ×1700) after application of hydrogen peroxide. Dense monolayer of *C. albicans* blastospores with a few hyphal forms on soft relining material.

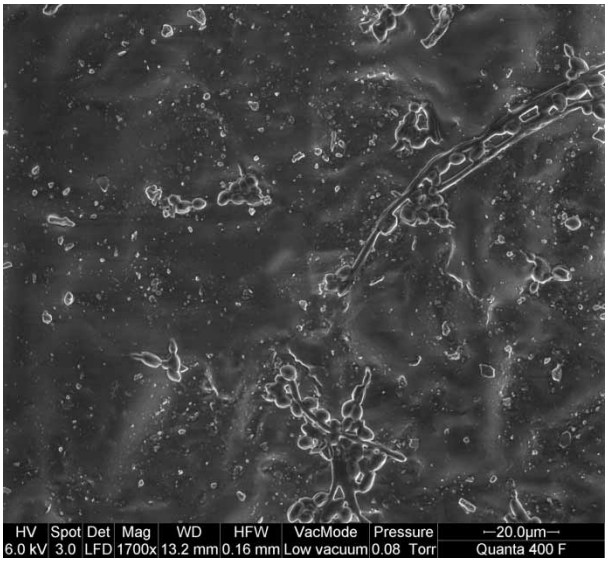

Figure 4. SEM (original magnification ×1700) after application of sodium hypochlorite. A few remaining blastospore and hyphal cells attached tightly to soft relining material.

oval blastospores in different budding stages (ranging between 2 and 5 µm) or a mixture of sparsely packed yeast cells with scattered long hyphal cells (extended as long as 100 µm) (Figures 2 and 3). The proportions between hyphae, pseudohyphae and blastospores varied. Vesicular remnants (approximately 0.2 µm) derived from broken cells were found as an extracellular matrix on the smooth surface of yeast and hyphal cells (Figure 5).

Discussion

Ten methods of denture disinfection were ranked according to their potential to reduce *C. albicans* colonization on soft denture lining material. Although various other species are involved, *C. albicans* has been considered as the major microbiological factor in the pathogenesis of denture stomatitis and has therefore been used as a test organism in this investigation [1]. As brushing seems to be ineffective in removing adhering microorganisms from denture surfaces [20], a simple and safe disinfection procedure with a high antifungal activity is recommended in support of mechanical cleansing [3,4].

The Via Light kit was used for quantifying viable fungal cells in this study. This bioluminometric test assay is based on the determination of cellular adenosine triphosphate (ATP), which is present in all metabolically active cells. The use of ATP

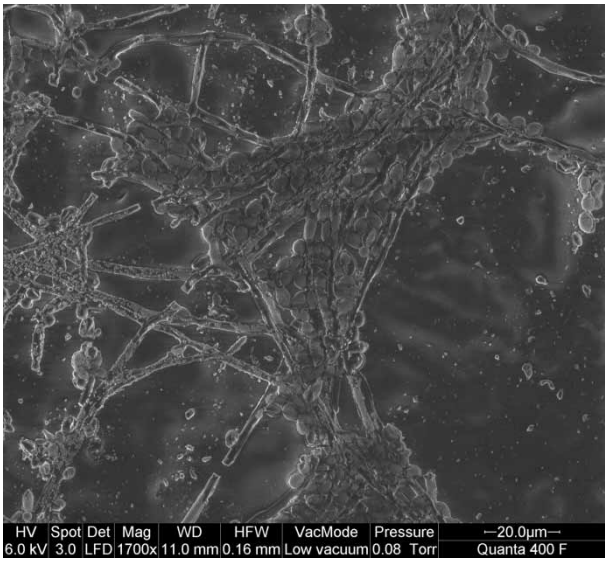

Figure 3. SEM (original magnification ×1700) after dry microwaving. A few yeast cells surrounded by a mature network of true hyphal and pseudohyphal extensions (mycelium).

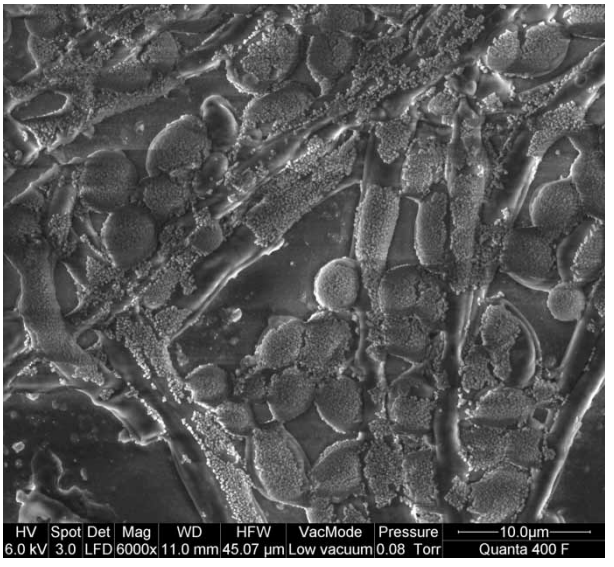

Figure 5. SEM (original magnification ×6000) after dry microwave application. Cluster of interconnected smooth-walled blastospores on hyphal extensions, covered with vesicular deposits.

luminescence as a measure of cell viability and quantity is accepted as reproducible and highly sensitive in the literature [21,22]. In previous studies, many different disinfection regimes have been tested against *C. albicans*, including a range of denture soaks, microwave irradiation, and air-drying procedures. Any comparison of the results of these studies would be inappropriate because of the use of a variety of *Candida* strains, different growth conditions, concentrations, application times, and methods of analysis. Consequently, one of the main concerns of this study was to include a wide range of different disinfection regimes in one investigation and subsequently to rank them according to their potential to reduce *C. albicans*. Generally, the luminometric quantification of viable *C. albicans* conducted in the present study revealed a statistically significant difference in the amounts of adhering fungi after the various assessed disinfection protocols. In some cases, high variances in measured luminescence intensity were found on different specimens of the same material. This is in agreement with other studies that also displayed large standard deviations [5–7].

According to our results, only soaking in sodium hypochlorite, microwave irradiation immersed in water, and application of effervescent cleansing tabs (Blend-a-dent tabs) exhibited distinct antifungal properties. These findings are in agreement with former *in vitro* studies that have also found sodium hypochlorite and microwave irradiation immersed in water to be highly efficient methods for eliminating *C. albicans* [3,4,6,13–16,23]. In contrast to microwaving and immersion in sodium hypochlorite, little information is available on the efficacy of effervescent commercial denture cleansing products, although they represent one of the most common methods for denture cleansing [10,20]. The active ingredients of Blend-a-dent tabs, which were selected as a representative effervescent cleansing product in this study, are potassium monopersulfate and sodium perborate monohydrate. Sodium hypochlorite is one of the most widely used household disinfectants and is reported to be highly bactericide and antifungal, even in short-term immersion [3,4,6,7,23]. Its efficacy has been proved by the results of the present investigation. In contrast, sodium hypochlorite is known to have discoloring and corrosive effects on denture base materials [15,24]. The use of microwave energy has been suggested to overcome the adverse effects of chemical soaks such as sodium hypochlorite [13]. An unmodified microwave oven was chosen for all tests in the current investigation because of its similarity to the household appliances commonly used by denture wearers at home. Campanha *et al.* [13] reported *C. albicans* cell membrane damage after microwaving immersed in water, but there is conflicting evidence about the mode of inactivation by microwave exposure. Machado *et al.* [25] claimed that reduction of microorganisms results from non-thermal effects of microwave energy, whereas other authors believe thermal decoction to be the cause [13,14]. In the present study, and in agreement with the results of Dixon *et al.* [14], dry irradiation proved to be completely ineffective against *C. albicans*. These findings suggest thermal effects to be responsible for the killing of microorganisms by microwaving. Hydrogen peroxide and glutaraldehyde were tested as alternative chemical soaks to sodium hypochlorite, but neither chemical showed any significant antifungal effect on adhering *C. albicans*. Furthermore, these chemical solutions are known to dissolve the surface of denture base materials and cannot thus be recommended for denture disinfection [12,26]. Some patients prefer household products and commercial mouth rinses as denture soaks because these are readily available, well-priced, and considered less harmful compared to chemical disinfectants. Vinegar (acetic acid solution) and alcoholic-based mouth rinses have been reported as ineffective in reducing microorganisms [8,9]. Our findings concur with those reports. Listerine (alcoholic-based), Plax (containing 0.3% Triclosan = 5-chloro-2-(2, 4-dichlorophenoxy) phenol), and household vinegar showed no antifungal potential compared to the control. Because of their aversion to chemicals and time-consuming disinfection procedures, some denture wearers limit denture hygiene to simply removing the prosthesis from the mouth and letting it dry for a couple of hours or overnight [4]. Baysan *et al.* [4] found this procedure, i.e. exposure to oxygen through air-drying, to be significantly less effective than microwave irradiation and soaking in sodium hypochlorite. Accordingly, the removal of adhering *C. albicans* by leaving the specimens dry for 5 h was found to be ineffective in our study, too.

SEM observations confirm the hypothesis that *C. albicans* adheres directly to the surface of dental materials [27–29]. The establishment of an acquired pellicle by saliva components or prior adhesion of early colonizing bacteria seems not to be an indispensable prerequisite for *C. albicans* adhesion to an artificial substratum. Furthermore, SEMs are in accordance with the results of the luminometric quantification. After application of the tested disinfection regimes, dense fungal colonization was found on all specimens except those being treated with sodium hypochlorite, effervescent cleansing tabs (Blend-a-dent tabs), and microwave irradiation.

Dendritical hyphae were found on various locations on all investigated specimens (Figures 3–5). This is noteworthy, because Gow & Gooday [30]

have stated earlier that hyphae start budding laterally after 15 h. In the present study, this phenomenon could be observed already after 2.5 h of incubation. The *C. albicans* cells in the present study grew in different morphological forms (blastospores, pseudohyphae, true hyphae and mycelium), which confirms the polymorphic character of this yeast [31]. The form of growth depended on environmental conditions, e.g. temperature, pH and nutrient solution [30]. Nevertheless, describing changes in fungal phenotype as a result of specific disinfection effects remains fairly speculative. Additionally, it seems inadequate in our opinion to conclude from this *in vitro* investigation that specific fungal forms might be more potent sources of infection than others. Future studies have to clarify whether the occurrence of different morphological forms influences the pathogenous capacity of a (fungal) biofilm.

In conclusion, all 10 tested disinfection procedures showed statistically significant differences in the reduction of viable fungi. Only soaking in sodium hypochlorite (1%; 10 min), microwave irradiation immersed in water (800 W; 6 min), and the application of effervescent cleansing tabs (Blend-a-dent tabs; 10 min) proved to be effective against *C. albicans* colonization on soft denture relining material.

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