Antibiofilm Effect of Cerium Nitrate against Bacteria and Yeast

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Abstract

Staphylococcus aureus, *Staphylococcus epidermidis*, *Acinetobacter baumannii* and *Candida albicans* are frequent agents of catheter-related bloodstream infections, which increase the time of hospitalization and related medical costs. In order to find new strategies to reduce the colonization of these organisms on indwelling medical devices, this research aimed to determine the antibiofilm effect of cerium nitrate.

XTT and crystal violet assays were used to quantify biofilms challenged with cerium nitrate. Two strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Acinetobacter baumannii* and *Candida albicans* were used, grown on polystyrene surfaces.

Concerning the biofilm metabolic activity of *Staphylococcus epidermidis*, all the tested concentrations of cerium nitrate inhibited significantly both strains, however for *Staphylococcus aureus* only the highest concentration tested inhibited significantly both strains. Referring to the total biomass, cerium nitrate concentrations of 3.2×10^2 or higher inhibited significantly both strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*, however only the higher concentration tested inhibited the total biomass of *Acinetobacter baumannii*.

Concerning the biofilm metabolic activity of *C. albicans*, cerium nitrate at concentrations of 6.4×10^2 mg/L inhibited significantly both strains. Referring to the total biomass, cerium nitrate at a considerably lower concentration (0.8×10^2 mg/L) inhibited both strains of *C. albicans*.

The fact that cerium nitrate inhibited more effectively the total biomass production than the metabolic activity in these strains (except for *Staphylococcus epidermidis*), may be explained by interference with extracellular matrix production or with intercellular communication. Whichever might be the case, cerium nitrate has a potent antibiofilm effect upon these strains and deserves further attention in strategies that could lower microbial colonization of indwelling medical devices.

Keywords: Catheter-related bloodstream infection; Central venous catheters; *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Acinetobacter baumannii*; *C. albicans*. Biofilms; Cerium nitrate.

Introduction

Staphylococcus aureus, *S. epidermidis*, *Acinetobacter baumannii* and *Candida species* are among the most frequently isolated microorganisms from central venous catheters (CVCs) placed in critically ill patients [1]. *Staphylococci* are capable of forming biofilms on implanted devices, causing infections [2]. The emergence of *S. epidermidis* as a pathogen is also linked to the extensive use of catheters. Their capacity to origin infection is mainly related to the ability to form mucoid biofilms on the inert synthetic surfaces of indwelling medical devices [3]. Among bloodstream infections caused by *Candida species*, *C. albicans* is the most frequent isolate, being associated with a high mortality rate. As a whole, *C. albicans* stands on as the fourth leading cause of catheter-related bloodstream infections (CRBSIs) [1,4], which increase the time of hospitalization and related medical costs [5,6].

Data from literature [7], show that over 65% of hospital infections are originated from biofilm forming organisms. A major concern when biofilms develop is the emergence of microbial strains resistant to the host immune system and to antimicrobial therapy [2]. A correlation between *Candida spp.* biofilm formation and resistance to antimicrobial agents was already documented P.K. Mukherjee, et al. [8]. Despite antifungal therapy, the persistence of fungi was noticed.

Therefore, new agents that could inhibit biofilm formation would be highly desirable. Cerium is a rare earth element of the lanthanide group and cerium nitrate is currently used in the management of burn wounds, with a reduction in patient morbidity and mortality [9]. Cerium nitrate was reported to reduce the activity of microbial pathogens due to their action at different cellular targets [10]. This compound might be quite useful in medical applications because toxicity is rare with lanthanides and in addition is an inexpensive compound.

The aim of this research was to determine the antibiofilm effect of cerium nitrate upon *Staphylococcus aureus*, *Staphylococcus*

epidermidis, *Acinetobacter baumannii* and *Candida albicans*, frequent microbial colonizer's of indwelling medical devices like central venous catheters (CVCs).

Materials and Methods

Microbial strains and chemicals

A type strain and a clinical isolate of each of four microbial species were used. The type strains from the American type Culture Collections were *S. aureus* ATCC 29213, *S. epidermidis* ATCC 155, *A. baumannii* ATCC 19606 and *C. albicans* ATCC 90028; the clinical strains of *S. aureus* (SA1), *S. epidermidis* (SE1), *A. baumannii* (AB1) and *C. albicans* (CA1) had been previously isolated from cultures of CVCs removed from critical care patients admitted at Hospital S. Joao (Porto, Portugal). All clinical isolates had been identified by Vitek System (bioMérieux, Vercieux, France).

Bacterial strains were kept frozen in Luria-Bertani broth (LB) (Difco Laboratories, Detroit, MI, USA) supplemented with 20% glycerol, and the yeast strains in yeast potato dextrose medium (YPD) (Difco Laboratories) supplemented with 40% glycerol at -70°C until testing. For each experiment, the microorganisms were subcultured twice on LB agar, 37°C, 24h (bacteria) or Sabouraud agar 35°C, 24h (Difco Laboratories) (yeasts) to assess the purity of the culture and its viability.

The chemical used in the experiments was cerium nitrate cerium (III) nitrate hexahydrate, Sigma-Aldrich.

Biofilm formation

Inoculum preparation: To prepare bacterial inoculum's, one colony of each bacterial isolate was used to inoculate 5 ml of LB medium. The cultures were incubated during 18 h at 37°C, at 180 rpm. Following this period the number of cells in each culture was adjusted to 0.5 MacFarland density in LB medium and subsequent diluted 1:10 in order to obtain 10^7 cells.

Yeast cells were grown for 18 h in Sabouraud broth at 35°C, at 180 rpm. Afterwards, cells were washed in PBS, resuspended in RPMI and adjusted to 0.5 McFarland density in order to achieve 10^6 cells/mL.

Biofilm growth with cerium nitrate: The effect upon biofilm formation was tested with four concentrations of cerium nitrate (6.4×10^2 mg/L, 3.2×10^2 mg/L, 1.6×10^2 mg/L and 0.8×10^2 mg/L), which were selected based on the results described by L. Cobrado & al. [2]. Each concentration was added to 12-well polystyrene microtiter plates containing the inoculum in order to obtain a final volume of 1 ml. In order to obtain biofilm formation, all the strains were incubated for 24 h at 37°C.

Biofilm quantification

Metabolic activity of biofilm cells - XTT assay: A XTT reduction assay was used to determine the *in situ* biofilm metabolic activity. After biofilm formation, supernatants were removed from the plates and 1 ml of PBS buffer was added and gently mixed. Following PBS rejection, 1 ml of XTT [2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] solution was added to each prewashed biofilm and control wells. Plates

were further incubated for 5 h at 37°C, in the dark, and finally the optical density (OD) was measured at 492 nm. The XTT solution was prepared in PBS (4 mg XTT in 10 ml prewarmed at 37°C); this solution was supplemented with menadione prepared in acetone. All the assays were performed in triplicate.

Biomass formation of biofilm cells - crystal violet assay:

Biofilm fixation was performed with 1 ml 99% methanol (15 min), after which supernatants were removed and the plates air-dried. Afterwards, 1 ml of a crystal violet (CV) solution (0.02%; v/v) was added to each well; following 20 min, the excess of CV was removed by washing the plates twice with distilled water. Finally, bound CV was released by adding 1.5 ml of acetic acid (33%; v/v). The optical density (OD) was measured at 590 nm. All the assays were performed in triplicate.

Data analysis

Biofilm metabolic activity and total biomass with cerium nitrate were expressed as the percentage in relation to the control. Values were divided by 1000 and arcsine square root transformed to achieve normal distribution and homoscedasticity [11].

For each concentration and each strain, biofilm formation was compared by one-way ANOVA, followed by a Dunnett's test to identify significant effects. *P* value < 0.05 was considered significant.

Results

Biofilm formation on polystyrene plates

Concerning the biofilm metabolic activity of *C. albicans*, cerium nitrate at 6.4×10^2 mg/L inhibited significantly the clinical strain by 36% (± 12) and the ATCC strain by 28% (± 4) (Figure 1A).

Referring to the total biomass of *C. albicans*, cerium nitrate at 0.8×10^2 mg/L inhibited significantly the clinical strain by 81% (± 11) and the ATCC strain by 50% (± 4) (Figure 1B). Doubling the concentration of cerium nitrate to 1.6×10^2 mg/L, there was a reduction in the total biomass of the clinical strain by 87% (± 11) and of the ATCC strain by 80% (± 5) (Figure 1B).

No relevant biofilm metabolic activity inhibition was found for *S. aureus* with cerium nitrate at concentrations $\leq$ than (3.2×10^2 mg/L) (Figure 2A), however an inhibition in total biomass was found for concentrations $\geq 3.2 \times 10^2$ mg/L ($p < 0.01$) (Figure 2B).

Cerium nitrate at all the tested concentrations reduced significantly *S. epidermidis* biofilm metabolic activity ($p < 0.01$) [Figure 3A], and concentrations $\geq 3.2 \times 10^2$ mg/L significantly inhibited total biomass ($p < 0.05$) [Figure 3B].

In respect of *A. baumannii* none of the tested concentrations inhibited biofilm metabolic activity (data not shown), nevertheless the higher tested concentration inhibited total biomass $21 \pm 0\%$ (Figure 4A).

Figure 1: Effect of cerium nitrate on metabolic activity (A) and total biomass (B) (as a percentage of the control) by *Candida albicans* clinical strain (CA) and ATCC strain. (*) P value <0.05.

Figure 2: Effect of cerium nitrate on metabolic activity (A) and total biomass (B) (as a percentage of the control) by *S. aureus* clinical strain (SA1) and ATCC strain. (*) P value <0.05.

Figure 3: Effect of cerium nitrate on metabolic activity (A) and total biomass (B) (as a percentage of the control) by *S. epidermidis* clinical strain (SE1) and ATCC strain. (*) P value <0.05.

Figure 4: Effect of cerium nitrate on total biomass (A) (as a percentage of the control) by *A. baumannii* clinical strain (AB1) and ATCC strain. (*) P value <0.05.

Discussion

The frequent use of indwelling devices in medical care makes patients more prone to develop biofilm-related infections, with a negative impact on the clinical outcome and hospital costs. The ability of nosocomial pathogens such as *C. albicans* to form biofilms is clinically relevant, since their formation impairs the efficacy of antimicrobial therapy [12]. Therefore, it is crucial to find new modes of prevention and effective alternatives to antimicrobial treatment.

Cerium nitrate is thought to enter the cytoplasm of microorganisms and inhibit cellular respiration, glucose metabolism and, eventually, induce disruption of the cell membrane (Dwight and Johnson 1967). According to the

results of J.P. Garner & et al.[9], cerium nitrate exhibits a bacteriostatic effect, but further testing demonstrated a microbicidal effect against a wide range of microbial pathogens [13]. Moreover, a potent antibiofilm activity against *C. albicans* grown on polyurethane catheter segments was documented with concentrations lower than MIC: cerium nitrate, at 1.7×10^3 mg/L, inhibited biofilm formation by ~60%. The results were similar either at 24 h or 48 h of incubation.

The present study confirms the antibiofilm effect of cerium nitrate against *C. albicans* even at lower concentrations. This work also reveal that cerium nitrate at all the tested concentrations inhibited significantly *S. epidermidis* biofilm metabolic activity, however only concentrations $\geq 3.2 \times 10^2$ mg/L significantly inhibited total biomass [14,15]. The effect of cerium nitrate on biofilm inhibition is specie and concentration dependent. Despite both XTT and CV assays were reproducible, as estimated by the low values of standard deviation, major differences in the quantification of the biofilm were observed. The XTT assay is based on the reduction of tetrazolium salts by mitochondrial dehydrogenases of yeasts and it is used for the quantification of metabolically active cells. The less expensive and time-consuming CV assay is commonly used for the quantification of biofilm biomass, staining the cells (living and dead) and the matrix produced [3]. The fact that cerium nitrate inhibited more effectively the total biomass production than the metabolic activity of the yeast cells, *S. aureus* and *A. baumannii* strains, may be explained by its interference with intercellular communication or with extracellular matrix production. Whichever might be the case, further studies are being held to clarify the mechanism of action and the endovascular biocompatibility of cerium nitrate, in order to support the clinical use of such promising compound to coat indwelling medical devices.

Conclusion

Nowadays medical device colonization constitutes a global threat that may contribute to antimicrobial resistance. The present study clearly demonstrated the antibiofilm effect of cerium nitrate against bacteria and yeast strains. Further studies are being conducted in order to clarify the mechanism of biofilm inhibition and the biocompatibility of cerium nitrate, which may play a promising role in biomedical applications in the future.

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