In vivo antibiofilm effect of cerium, chitosan and hamamelitannin against usual agents of catheter-related bloodstream infections

L. Cobrado1–3, A. Silva-Dias1,2, M. M. Azevedo1,2, C. Pina-Vaz1,2,4 and A. G. Rodrigues1–3*

1Department of Microbiology, Faculty of Medicine, University of Porto, Porto, Portugal; 2Cardiovascular Research & Development Unit, Faculty of Medicine, University of Porto, Porto, Portugal; 3Burn Unit and Department of Plastic and Reconstructive Surgery, Hospital S. João, Porto, Portugal; 4Department of Microbiology, Hospital S. João, Porto, Portugal

*Corresponding author. Tel: +351-913-235-755; Fax: +351-225-513-662; E-mail: agr@med.up.pt

Received 8 May 2012; returned 30 May 2012; revised 30 July 2012; accepted 24 August 2012

Objectives: Catheter-related bloodstream infections (CRBSIs) are common healthcare-associated infections associated with increased morbidity and medical costs. Antiseptic- and antibiotic-coated central venous catheters (CVCs) have been proposed to reduce the incidence of CRBSIs, with variable success. The aim of this study was to determine the in vivo antibiofilm activity of biocompatible and inexpensive compounds, such as cerium nitrate, chitosan and hamamelitannin, against usual agents of CRBSIs.

Methods: The antibiofilm effect of cerium nitrate, chitosan and hamamelitannin was tested against Staphylococcus epidermidis, Staphylococcus aureus, Acinetobacter baumannii and Candida albicans in a mouse foreign body infection model, using polyurethane catheter segments. Biofilm formation was assessed with a crystal violet assay to quantify the total biomass, with a tetrazolium reduction assay to quantify the metabolic activity and with scanning electron microscopy.

Results: At subinhibitory concentrations, cerium nitrate significantly reduced biofilm formation by C. albicans, chitosan significantly decreased biofilm formation by S. epidermidis and C. albicans, and hamamelitannin significantly inhibited all bacterial biofilms.

Discussion: The in vivo antibiofilm effect of cerium nitrate against C. albicans and of chitosan against C. albicans and S. epidermidis, at subinhibitory concentrations, makes them promising alternatives to coat CVCs. Moreover, the microbicidal effect on a wider range of CVC colonizers was previously reported in vitro for both compounds, at higher concentrations. For all bacterial strains, the highest in vivo antibiofilm efficacy was achieved with hamamelitannin. For A. baumannii, this is the first report of in vivo inhibition.

Keywords: nosocomial infections, biofilm inhibition, CRBSIs

Introduction

Catheter-related bloodstream infections (CRBSIs) are common healthcare-associated infections associated with increased morbidity, hospital stay and medical costs.1 Antiseptic- and antibiotic-coated central venous catheters (CVCs) have been proposed to reduce the incidence of CRBSIs; catheters impregnated with chlorhexidine–silver sulfadiazine appear to be effective in reducing colonization and infection, but hypersensitivity reactions have been documented.2 Silver-impregnated catheters are not associated with a lower rate of colonization3 and, despite catheters coated with minocycline and rifampicin significantly decreasing the incidence of CRBSIs, some concern may still exist regarding the development of antimicrobial resistance.4 Therefore, additional strategies are being developed in order to reduce CVC colonization.

Among promising compounds, cerium nitrate, extensively used in the management of burn patients, has been shown to exhibit antimicrobial activity.5 Chitosans are hydrophilic biopolymers obtained by N-deacetylation of chitin, with a proven antimicrobial effect.6 Moreover, microbicidal activity was recently demonstrated for low molecular weight chitosan (LMWC).7 Hamamelitannin is a polyphenol extracted from the bark of Hamamelis virginiana, and belongs to the family of tannins. In addition to inhibiting device-associated infections in vivo caused by Gram-positive bacteria,7 in vitro activity against Acinetobacter baumannii has been documented.8 Overall, cerium nitrate, LMWC and hamamelitannin are biocompatible and relatively inexpensive compounds, displaying a wide range of antimicrobial activity. In order to determine their in vivo antibiofilm activity against the usual agents of CRBSIs, such as Staphylococcus epidermidis, Staphylococcus aureus,
A. baumannii and Candida albicans, polyurethane catheter segments were used in a mouse foreign body infection model.

Materials and methods

Chemicals

Cerium nitrate [cerium(III) nitrate hexahydrate; Sigma–Aldrich, Schnell-dorf, Germany], chitosan [LMWC 107 kDa, 75%–85% deacetylated; Sigma–Aldrich, St Quentin Fallavier, France; stock solution of 4% (w/v) prepared in 1% acetic acid, pH 4.0] and hamamelitannin [hamamelofuranone 2',5-digallate, 2-C-(hydroxymethyl)-α-ribofuranose 2',5-digallate; Sigma–Aldrich] were used in the experiments.

Microbial strains and inoculum preparation

Four type strains from the ATCC were used: S. aureus ATCC 29213, S. epidermidis ATCC 155, A. baumannii ATCC 19606 and C. albicans ATCC 90028.

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

For the inoculation preparation, the microbial strains were grown overnight in LB broth (bacteria) or Sabouraud broth (yeast) at 37°C and 180 rpm; cells were harvested by centrifugation (10000 g, 10 min), washed with PBS, counted in a Newbauer chamber and the concentration was standardized to 1×10⁷ cells/mL for bacteria and 1×10⁶ cells/mL for yeasts, in PBS.

Polyurethane catheter implantation procedure

A mouse subcutaneous foreign body infection model as described by Rupp et al.⁸ was used, with slight modifications. Eight-week-old pathogen-free female BALB/c mice (Charles River) weighing 20 g were used. Animals were housed in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) criteria. Animal experiments were approved by the Animal Ethics Committee of the Faculty of Medicine, University of Porto.

Prior to catheter implantation, mice were anaesthetized by intraperitoneal injection (1 mg/kg) of a mixture of xylazine (20 mg/mL) and ketamine (100 mg/mL) in a ratio of 1:2 (v/v) and the lower back of each animal was shaved with a hair clipper and disinfected with 0.5% chlorhexidine in 70% alcohol. A 2 mm incision was made longitudinally and the subcutis was dissected. Using an aseptic technique, six 1 cm animal was shaved with a hair clipper and disinfected with 0.5% chlorhexidine in 70% alcohol. A 2 mm incision was made longitudinally and the subcutis was dissected. Using an aseptic technique, six 1 cm

Scanning electron microscopy

Catheters were washed in PBS, sectioned lengthwise and fixed overnight (4% paraformaldehyde and 1% glutaraldehyde v/v in PBS). The samples were rinsed in PBS and air dried in desiccators.¹ Samples were coated with gold/palladium (40%/60%) and observed in CEMUP (Materials Centre of the University of Porto) using a scanning electron microscope (JOEL JSM 6301F/Oxford INCA Energy 350) in high-vacuum mode at 15 kV.

Data analysis

Biofilm metabolic activity and total biomass formed in the presence of cerium nitrate, LMWC and hamamelitannin 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. For each compound, each concentration and each microorganism, biofilm formation was compared by one-way ANOVA, followed by a Dunnett’s test to identify significant effects; P<0.05 was considered significant.

Results

Cerium nitrate, at a concentration of 6.5×10² mg/L, significantly reduced the biofilm metabolic activity of S. epidermidis by 48.3% (+12.4%) and its total biomass by 37.0% (+8.9%) (Figure 1a). For C. albicans and at 1.3×10³ mg/L, cerium nitrate inhibited the metabolic activity by 66.4% (+7.7%) and the biomass by 28.0% (+4.3%) (Figure 1b).

LMWC, at 80 mg/L, significantly decreased the biofilm metabolic activity of S. epidermidis by 57.6% (+14.3%) and its total biomass by 41.3% (+5.6%) (Figure 1c). Concerning C. albicans, at 2.5×10⁷ mg/L, inhibitions of the metabolic activity by 43.5% (+16.6) and of the total biomass by 23.2% (+13.7) (Figure 1d) were found.

Hamamelitannin significantly reduced all bacterial biofilms at subinhibitory concentrations: at 50 mg/L, S. aureus biofilm metabolic activity decreased by 59.3% (+19.1%) and its total biomass by 48.4% (+8.0%) (Figure 1e); at the same concentration, S. epidermidis biofilm metabolic activity was reduced by 36.3% (+12.4%) and its total biomass by 34.6% (+6.9%) (Figure 1f); and at 30 mg/L, hamamelitannin inhibited A. baumannii biofilm metabolic activity by 36.7% (+3.4%) and its total biomass by 31.6% (+9.2%) (Figure 1g).

At other subinhibitory concentrations, no significant biofilm inhibition was found for the tested microbial strains (data not shown).

In order to support the biofilm quantification by XTT and CV assays, scanning electron microscopic examination was used to determine the architectural differences between biofilms. Untreated biofilms (Figure 2a, b, i, j, m, n, q and r) comprised a more dense network of microbial cells and exopolymeric matrix than treated biofilms (Figure 2c–h, k, l, o, p and s–v).

Discussion

In the long quest aiming to prevent medical device-related infections in a more effective way, many different strategies have been proposed. Until now, this objective remains to be achieved. Cerium nitrate, LMWC and hamamelitannin are biocompatible and relatively inexpensive compounds that could be used to coat CVs and other medical devices. Differences in their
mechanisms of action might explain the pattern of antimicrobial activity; whereas cerium nitrate and LMWC may disrupt the cell membrane,\textsuperscript{6,11} hamamelitannin seems to inhibit bacterial cell communication.\textsuperscript{7} The microbicidal effect of cerium nitrate and LMWC upon usual CVC colonizers was previously shown in vitro,\textsuperscript{5} as was the antibiofilm activity of hamamelitannin on polyurethane catheter segments.\textsuperscript{5} However, in vivo experiments with subinhibitory concentrations of the compounds were still required, as the results obtained under in vitro conditions do not account for the interaction between the host cells, immune system and biofilm.

Garner and Heppell,\textsuperscript{11} studying the action of cerium nitrate on the treatment of severe burns, reported that it has limited antimicrobial properties. However, minimal lethal concentrations (MLCs) and MICs have been published more recently for both bacteria and yeasts.\textsuperscript{5} In vivo, at subinhibitory concentrations, cerium nitrate exhibited an antibiofilm effect only against \textit{C. albicans}. Such a selective finding should not preclude its

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Effect of cerium nitrate (a and b), LMWC (c and d) and hamamelitannin (e, f and g) on biofilm formation by \textit{S. epidermidis} (a, c and f), \textit{S. aureus} (e), \textit{A. baumannii} (g) and \textit{C. albicans} (b and d). The XTT assay was used to determine the biofilm metabolic activity and the CV assay was used to measure the total biomass. *$P<0.05$.}
\end{figure}
Figure 2. Scanning electron microscopic (SEM) examination of untreated biofilms of *S. epidermidis* (a and b), *S. aureus* (i and j), *A. baumannii* (m and n) and *C. albicans* (q and r). SEM examination of *S. epidermidis* biofilms treated with cerium nitrate at $6.5 \times 10^2$ mg/L (c and d), LMWC at 80 mg/L (e and f) and hamamelitannin at 50 mg/L (g and h), *S. aureus* biofilms treated with hamamelitannin at 50 mg/L (k and l), *A. baumannii* biofilms treated with hamamelitannin at 30 mg/L (o and p) and *C. albicans* biofilms treated with cerium nitrate at $1.3 \times 10^3$ mg/L (s and t) and LMWC at $2.5 \times 10^3$ mg/L (u and v).
biomedical applicability since in medical burn wound care it is used routinely as an effective antiseptic, at much higher concentrations and with no known toxicity. In addition, Candida organisms are important pathogens causing CRBSIs.

The microbicidal activity of LMWC against usual CVC colonizers has already been documented in vitro. Using an in vivo CVC model, Martinez et al. demonstrated the efficacy of chitosan against Candida species biofilms. Our study not only supports these findings concerning C. albicans, at subinhibitory concentrations of chitosan, but further documents the antibiofilm efficacy against S. epidermidis, a more frequent CVC colonizer. Given the known biocompatibility of chitosan, promising results could be achieved in the prevention of CRBSIs by a wider range of microbial colonizers if higher concentrations were used for treating the surface of CVCs.

At subinhibitory concentrations, the highest antibiofilm efficacy was obtained with hamamelitannin; a reduction in the metabolic activity and total biomass was documented for S. aureus, S. epidermidis and A. baumannii. This is the first report of in vivo biofilm inhibition for A. baumannii. Moreover, the antibiofilm efficacy was achieved at lower concentrations than expected considering the previous in vitro results for all the tested bacteria, a fact that may be attributable to differences in environmental conditions. In the case of staphylococci, Kiran et al. attributed such variation to the specific quorum-sensing inhibitor effect of hamamelitannin. For pre-soaked grafts, the same authors reported no signs of staphylococcal infection in vivo with hamamelitannin concentrations of 30 mg/L, but the present study found a statistically significant reduction in biofilm formation by no more than ~50%, even at 100 mg/L. Nonetheless, hamamelitannin may be an excellent compound to coat medical devices given its bacterial range of biofilm inhibition.

A more durable coating strategy for cerium nitrate, chitosan and hamamelitannin and their further testing with a CVC model in vivo is the next step in the path to develop a more cost-effective and biocompatible catheter that can prevent CRBSIs more effectively than the current alternatives.

Acknowledgements
We would like to thank the excellent technical support of Daniela Silva (CEMUP).

Funding
This study was supported by internal funding.

Transparency declarations
None to declare.

References