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Benzydamine Bactericidal Effect Results from a Permanent Cell Membrane Lesion

Ferdinando F Andrade[1,2*], Irene Pina-Vaz[3,4], Acácio G Rodrigues[1,4], Cidália Pina-Vaz[1,3,4]

[1]Laboratory of Microbiology, Department of Pathology, Faculty of Medicine, University of Porto, Portugal

[2]Farmanimal Veterinary Centre, Caldas da Rainha, Portugal

[3]CINTESIS - Center for Health Technology and Services Research, Faculty of Dental Medicine, University of Porto, Portugal

[4]CINTESIS - Center for Health Technology and Services Research, Faculty of Medicine, University of Porto, Portugal

[*]**Corresponding author:** Ferdinando F Andrade, Laboratory of Microbiology, Department of Pathology, Faculty of Medicine, University of Porto, Portugal

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Abstract

Benzydamine is a pyrozolone compound often used topically in Medicine and Veterinary Medicine, with demonstrated anti-inflammatory and anti-microbial properties. In the present study, the antibacterial mechanism of benzydamine was unveiled. The antibacterial activity of benzydamine was assessed *in vitro* by classic broth microdilution test and its mechanism of action reveled through flow cytometric assays. A total of 120 strains (57 Gram negative bacilli and 63 Gram positive cocci) including control ATCC strains and clinical isolates from the bacterial collection of the Microbiology Laboratory of Porto, Faculty of Medicine were selected. The assays demonstrated a potent bactericidal activity against a wide variety of pathogens including antibiotic resistant phenotypes like ESKAPE (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp). In most cases the minimal inhibitory concentration ranged between 100-400 µg/ml, being usually higher for Gram-negative bacteria specially *Pseudomonas aeruginosa*. The bactericidal effect resulted from a primary lesion of the cell membrane.

Keywords: Benzydamine; Bactericidal activity; ESKAPE; Flow cytometry

Introduction

Antimicrobial resistance is a growing global threat and soon we risk to be confronted with the unavailability of these conventional drugs to treat infectious diseases. While few novel drugs are arising-most of the putative targets were already explored-bacteria are quite rapid to develop resistance whenever a new antibiotic is made available. Disinfectants are an important tool to prevent infection especially with the increasing burdens of antimicrobial resistance and emerging novel pathogens, as is the case with the actual pandemic. Benzydamine is a pyrozolone compound, acting through the inhibition of cyclooxygenase. It exhibits anti-inflammatory [1] and local anesthetic effects. Antimicrobial properties such as antifungal [2] and antibacterial effects have also been described [3,4]. Bacteria can be found in their natural habitats in the planktonic state, that is, isolated cells suspended in a fluid media; an alternative way to thrive involves its adhesion to solid surfaces, forming biofilms. The oral and vaginal cavities are among a multitude of microenvironments in which bacteria do survive and multiply actively in both states.

Bacterial plaque actually involves a set of mixed bacterial populations, of a multitude of species, included in a matrix that is consistently adherent to the teeth. From a microbiological point of view, it should be understood as a very dynamic system, influenced by environmental factors, in particular by the physical-chemical and biological conditions of the oral milieu [5]. The genital tract, in particular the vaginal microbiome, although less rich in its microbial diversity, also suffers from similar endogenous influences although its native pH is subject to less fluctuation [2]. Despite good gastrointestinal absorption of benzydamine following oral administration (87%), its systemic absorption is poor (<10%) when used as a mouthwash or after use as a vaginal solution or cream [6]. Benzydamine is also poorly absorbed through the skin and non-specialized mucous membranes, making its topical use safe, despite reports of hallucinations following its administration for recreational purposes, although always involving other distinct

routes [7].

Concerning its effects and it adsorption and permanence at the mucosal surfaces, benzydamine is recommended for the treatment of thrush, periodontitis, pharyngitis, gingivitis and tonsillitis [6,8,9] as well as to control vulvovaginal infections [10,11], most authors highlighting mainly its anti-inflammatory and analgesic effects. Nevertheless, the mechanisms involved in its antimicrobial effect, remain unknown. While there are several studies addressing its pharmacokinetics, even in distinct animal species [6,12-17], knowledge regarding its pharmacodynamics is still very scarse. The objective of this study involves the assessment of the bactericidal efficacy of benzydamine and the elucidation of its mechanism of action, particularly its target among bacterial cell structures. Flow cytometry is an excellent tool to evaluate antimicrobial susceptibility [18,19] but also to clarify the mechanism of action of compounds not classically considered antimicrobial agents.

Methods

Strains

One-hundread-twenty strains with different antimicrobial susceptibility phenotype were tested: 57 Gram-negative bacilli (39 *Enterobacterales*, 10 *Pseudomonas aeruginosa* and 8 *Acinetobacter baumannii*) and 63 Gram-positive cocci (23 *Staphylococcus* spp and 40 *Enterococcus* spp). Bacterial strains - deposited at the clinical strain collection of the Microbiology laboratory of Porto Faculty of Medicine - correspond to isolates from different biological products such as blood cultures, bronchial secretions, urine and wounds. American Type Culture Collection strains such as *E. coli* 25922, *Pseudomonas aeruginosa* 27853, *S. aureus* 29213 and *E. faecalis* 29212 were also included as control strains. ESKAPE (*Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp) microorganisms, recognized by the World Health Organization (WHO) as the most difficult to treat, were also included on this study (Table 1).

Bacteria	n	MIC range µg/ml
Gram-negative	57	200-400
E. coli	17	200-300
Klebsiella pneumoniae	12	200-300
Enterobacter spp	10	200-400
Pseudomonas aeruginosa	10	300-400
Acinetobacter baumannii	8	200-400
Gram-positive	63	100-200
Enterococcus faecalis	20	100-200
Enterococcus faecium	20	100-200
S. aureus	23	100-200
Total	100	100-400

Table 1: Minimal Inhibitory Concentration (MIC) of Benzydamine.

Microdilution susceptibility assay

Microplates were prepared with serial concentrations of benzydamine (ranging from 50-1200 µg/ml) using the microdilution protocol recommended by Clinical & Laboratory Standards Institute (CLSI) and growth evaluated visually by turbidity; the Minimal Inhibitory Concentration (MIC) was recorded after 24hr incubation time at 37°C.

Flow cytometric assays

A bacterial suspension (0.5 McFarland) was prepared with each strain, diluted in Muller-Hinton broth, incubated during 1 h at 35ºC with 100, 200, 300 and 400 µg/ml of benzydamine and propidium iodide (PI-Sigma Aldrich, St. Louis) - a fluorescent probe that can only stain the cells whenever the cell membrane is permeable, meaning a dead cell. Afterwards, flow cytometric analysis using a CytoFLEX (Beckmam) flow cytometer was performed.

Results

For all bacteria tested, MIC values ranged between 100µg/ml and 400µg/ml. The highest values were registered in case of *Pseudomonas* while the lowest corresponded to *Enterococcus* (Table 1). Flow cytometry showed that benzydamine, after 1 hour of incubation resulted in permeabilization of the bacteria cell membrane. The PI staining of the bacterial cells after such a short incubation time corresponds to a primary lesion on the cell membrane. A shift of the population stained with PI compared to control, non-treated cells, was registered with all bacteria tested (Figure 1). This effect is higher in cocci than in bacilli, a finding that is in agreement with the MIC values.

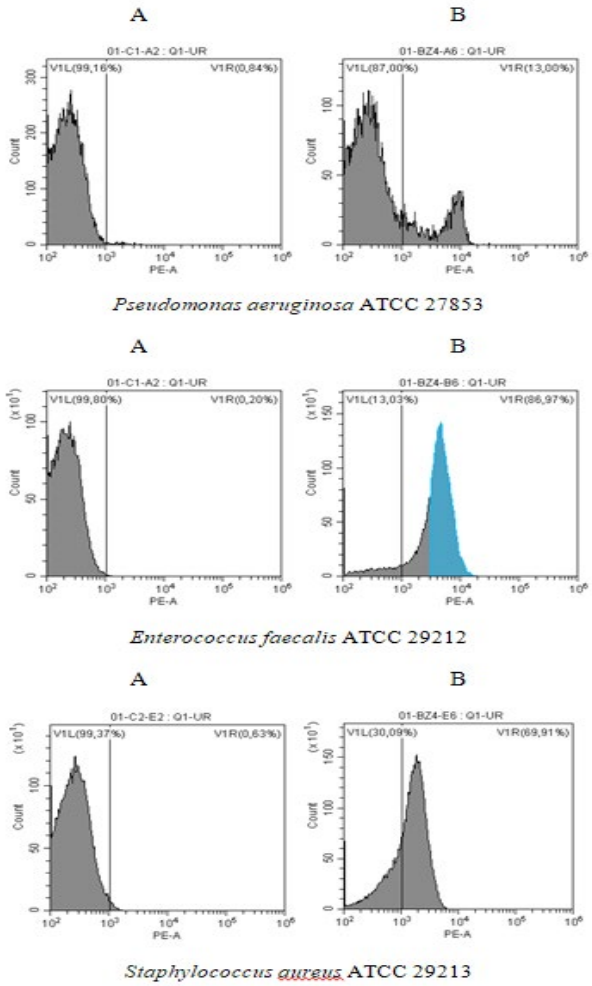

Figure 1: Histograms representing 3 ATCC strains included in the study: *Pseudomonas aeruginosa* 27853, *E. faecalis* 29212 and *S. aureus* 29213. A- represents non-treated cells (control) and B - represents cells treated with 400 µg/ml of benzydamine after incubation for 1 h. An increase of the intensity of fluorescence (shift to the right) is evident after benzydamine exposure.

Conclusion

Our results demonstrate a potent bactericidal activity for benzydamine against a wide range of microorganisms such as Enterobacterales (i.e. *E. coli, Klebsiella*, *Enterobacter*), *Pseudomonas, Acinetobacter, Staphylococcus* and *Enterococcus*. Previously, authors had demonstrated the bactericidal efficacy of benzydamine upon bacteria such as *P. aeruginosa* and *S. aureus*. The lesion of the cell membrane hereby described could explain, apart its effect as a single drug, the synergistic effect previously described with combinations of benzydamine with antibiotics such as ampicillin, tetracycline or chloramphenicol [3,4]. The cell permeability is increased following exposure to benzydamine, thus allowing the entrance of other compounds. Nevertheless, benzydamine is a per se powerful bactericidal against a wide variety of organisms, even at concentrations lower than those used in anti-inflammatory treatment [3,20-22]. Our results reveal a higher activity upon Gram-positive bacteria, which is consistent with a putative protective effect of the lipid outer membrane of Gram-negative bacteria that limits the access of molecules from the outside. Previously antifungal effect was shown and also the lesion of the cell membrane was demonstrated by flow cytometry and afterwards confirmed by electron microscopy [2]. Notably, the cell membrane lesion could result in side effects upon human cells, since it represents a less selective target comparing with conventional antibiotics. Nevertheless, topic applications of benzydamine, either in humans, veterinary medicine or clinical environment are of clinical major interest, particular in setting of growing antimicrobial resistance and the need to spare as much as possible still active antibiotics, thus meeting the challenges of One-Health policy.

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