“Guar gum” as an antimicrobial peptide delivery system to be applied to the control of diabetic foot infections

Hermes Macedo

Abstract

Diabetic foot ulcers are a major complication of Diabetes mellitus. Several microorganisms can colonize and promote opportunistic infections, being Staphylococcus aureus frequently isolated. Antimicrobial peptides have been extensively investigated for new therapies against Gram-positive bacteria, such as nisin, produced by Lactobacillus lactis. Guar gum has been tested as delivery system.

Inhibitory potential of nisin against 23 S. aureus isolates collected from DFU patients was evaluated. The minimum inhibitory (MIC), bactericidal (MBC), minimum biofilm inhibitory (MBIC) and eradication (MBEC) concentrations were determined for nisin, diluted in HCl and incorporated in guar gum gel. The inhibitory activity of nisin incorporated in guar gum gel throughout 6 months, was tested and positively observed.

All isolates tested are considered susceptible to nisin. For nisin diluted in HCl, mean values for MIC, MBC and MBIC were 90±22.8 µg/mL, 495.2±149.9 µg/mL and 150.8±85.5 µg/mL, respectively. MBEC values >1000 µg/mL were observed in 65% of isolates. Regarding the nisin incorporated in guar gum gel, mean values for MIC, MBC and MBIC were 180.8±53.9 µg/mL, 766.7±272.6 µg/mL and 366.7±140.4 µg/mL, respectively. Most isolates (87%) show MBEC values higher than 1000 µg/mL. Statistical differences were observed between MIC, MBC and MBIC, while no significant differences were found between MBEC values for the formulations presented.

Results show the importance of nisin as a substitute or complementary therapy to the current antibiotics used for treating DFU infections. This innovative therapeutic strategy shows a promising delivery system for AMP, allowing the development of novel topical therapies as treatments for bacterial skin infections.

Keywords: diabetic foot ulcers, Staphylococcus aureus, biofilm, nisin, guar gum, minimum inhibitory concentration

1. Introduction

Diabetes mellitus (DM) as a highly prevalent chronic expansion disease, that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it provides [1]. Diabetes occurs in several forms, including type 1, type 2 and gestational diabetes. In the both cases, the concentration of sugar in the blood increases, resulting in a group of metabolic disorders characterized by a chronic hyperglycemic condition [2]. The effects of DM include long-term damage of various organs, leading to dysfunction, failure and death [3]. Diabetes affects 415 million people worldwide. The number of people affected by this disease is set to rise beyond 642 million cases in 2040, which corresponds to 8.8% of the worldwide adult population [4].

Foot infections are the most frequent diabetes complications in the diabetic patients. Diabetic foot ulcers (DFU) and gangrene are common among diabetic patients and represent a major cause of morbidity and mortality [5].
DFU are responsible for amputations in 14 to 20% of cases the patients [6].

DFU infections occur when the layer of skin is broken and the deep tissues are exposed, allowing the colonization by microorganisms. Diabetic foot infections are classified according the International Working Group on the Diabetic Foot. Then, the DFU can be classified as uninfected, mild, moderate or severe infection [6], [7].

The DFU infections can be monomicrobial, being normally mild infection, or be polymicrobial, being classified as a moderate or severe infection [8]. In wild infections, Staphylococcus aureus and the beta-hemolytic streptococci (groups A, B, C and G) usually, are the firsts microorganisms that colonized the wound. Although, in levels higher of infection, the pathogenies present in ulcers besides the staphyloccoci and streptococci, including Pseudomonas aeruginosa, Enterococcus spp. and also anaerobic microorganisms [6], [9].

Methicillin-resistant S. aureus (MRSA) is the most frequent pathogenic microorganism found in all levels of the infection. Usually, the presence of infection by MRSA is a significant indicative of the future amputation of the limb [6], [9].

Antimicrobial resistance ability among pathogenic are increased in worldwide, and in DFU are very well establishment. Diabetic foot infection requires appropriates antibiotics therapy, appropriate wound care, but developing new treatment strategies are required.

Antimicrobial peptides (AMP) are oligopeptides produced by all living organisms, including animals, plants, bacteria and other. They play an important role as the first line of defense in the innate defense system, impairing the development of most infections [10].

AMP have many intrinsic proprieties which support their future therapeutic application. Studies have report that these molecules have an excellent antimicrobial activity against planktonic cells and against mature biofilms. Therefore, the Food and Drug Administration (FDA) have approved many AMP for clinical use or for other applications, such as food preservation. One example of these AMP is nisin [11], [12].

Nisin is a cationic bacteriocin produced by Lactococcus lactis subsp. lactis, composed 34 amino acids, with a molecular mass of 3354 KDa, that not contain aromatic amino acids [13].

The AMP nisin has a large activity spectrum against by several Gram-positive bacteria and also against spore germination. But, is not effective against Gram-negative bacteria and fungi [14], [15].

Nisin does not require a membrane receptor on the Gram-positive bacteria surface and act by promoting the disintegration of the lipid bilayer of the bacterial cell membranes by simple electrostatic interactions [13], [16].

Antimicrobial properties of nisin support its potential as an alternative for antimicrobial therapeutics, necessary in view of the increase in bacterial resistance to traditional antibiotics, especially against the most prevalent organisms responsible for skin infection, such as S. aureus and MRSA [17].

Nisin (E234) is a bacteriocin approved for application in food preservation in the European Union by Directive 95/2/EC, being classified as Generality Recognised as Safe (GRAS) and the Food Safety Authority has established an Acceptable Daily Intake of 0.13 mg nisin/kg [18].

In spite of nisin antimicrobial properties, their application to the treatment of infected DFU requires the development of an effective delivery system. Guar gum has been tested as a potential AMP delivery system [19].

Guar gum is a natural polysaccharide, derived from the endosperm of the seeds of the plant Cyamopsis tetragonoloba, a member of the Leguminosae family. Being a hydrophilic polysaccharide and uncharged molecule, composed mainly for galactomannan [19].

The distinctive physical-chemical proprieties of guar gum, mainly the presence of a long chain molecular structure and the abundance of hydroxyl groups in the galactomannan molecule, turn this natural gum a strong candidate to be used as excipient in diverse industries, such as food, cosmetics and pharmaceutical industry, being revealed by several studies as a good drug delivery system for human medicine, in treatment of diverse disease, such as cholera, diarrhea, obesity and colorectal cancer [20], [21]. In DM, studies revealed that guar gum reduces the rise in blood glucose and insulin concentrations after meals [22].

The low cost of production and extraction, allied to its non-toxic, biodegradable and
biocompatibility nature, contribute to the increased interest of researchers in this molecule [19]. Besides, FDA regulates the use of gums and has classified the guar gum as GRAS, and has also defined the highest concentrations allowed in many food applications [22], [23]. Therefore, in this study was the evaluation of the guar gum gel as a delivery system for the antimicrobial peptide nisin to be applied to the treatment of infected DFU, using 23 S. aureus isolates obtained and characterized in previous studies.

2. Material and Methods

2.1. Bacterial strains

In 2010, an epidemiological study conducted in Lisbon analyzed the microbiota of infected DFU in patients with DM. From 49 DFU patients it was possible to collect a total of 54 Staphylococcus spp. clinical isolates [24]. All clinical isolates were analyzed based on Pulse Field Gel Electrophoresis, which allowed to select a collection of twenty-three (n=23) representative S. aureus isolates for further research. Isolates were also characterized regarding their virulence profile, antimicrobial resistance traits and biofilm production ability [24]. These isolates were kept at -80°C, in BPW (buffered peptone water) plus 20% of glycerol in cryopreservation tubes [25], and used in this study.

The reference strain S. aureus ATCC®29213™, was also included in this study as a control strain, being a known biofilm producer.

2.2. Nisin stock and Guar gum incorporation

Nisin stock solution was prepared using a nisin powder and hydrochloric acid (HCl) 0.02 M (Merck®). For preforming a nisin stock solution of 1000 µg/mL corresponding to 40 000 IU/mL, 1 g of nisin powder was dissolved in 25 mL of 0.02M HCl. Next, using a 0.22 µm Millipore filter (Frilabo®) the nisin stock solution was sterilized and kept at 4°C, until further use. Serial dilutions of nisin solution were prepared for following use.

A guar gum gel of 1.5% (w/v) was prepared using guar gum powder (Sigma-Aldrich®) and sterile distilled water. 0.75 g of guar gum were dissolved in 50 mL of sterile distilled water and heat sterilized by autoclave. In a proportion of 1:1, the serial dilutions of nisin were integrated within the gel guar gum, obtaining final gel suspensions of 0.75% (w/v).

2.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination

MIC and MBC were determined using the microtiter broth dilution method [26]. Bacterial strains were grown in nonselective Brain Heart Infusion (BHI) agar medium (VWR Chemicals®) at 37°C for 24h. Subsequently, bacterial suspensions were prepared in sterile normal saline (Scharlau®) with approximately 10^6 CFU/mL using a 0.5 McFarland standard reference. For the MIC and MBC assays, these bacterial suspensions were diluted in BHI broth, at a concentration of approximately 10^7 CFU/mL.

Various suspensions of nisin were distributed in 96-well flat-bottomed polystyrene microtiter plates (Nunc, Thermo Fischer Scientific®), at a volume of 25 µl in the case of nisin in HCl solution, and 50 µl when combined with guar gum gel. Concentrations range from 5 µg/mL to 1000 µg/mL, corresponding to 5 IU to 1000 IU per well, respectively. All wells were inoculated with 150 µl of the 10^7 CFU/mL bacterial suspensions, except for the wells corresponding to the negative control, which contained only fresh broth medium. Microplates were statically incubated at 37°C for 24 h, and MIC was considered as the lowest concentration of nisin that visually inhibited the microbial growth [26].

MBC was determined by inoculating 3 µl of the suspensions from the wells where no visible growth was observed on BHI agar plates and incubated at 37°C for 24 h. MBC was considered as the lowest nisin concentration that impaired bacterial growth on to the agar plates.

2.4. Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MEBC) determination

MBIC was determined using a modified version of the Calgary Biofilm Pin Lid Device [27].

For broth MBIC and MBEC determinations, bacterial suspensions were prepared in sterile normal saline (Scharlau®) as described before,
and diluted in Tryptic Soy Broth (TSB) (VWR Chemicals®) plus 0.25% (w/v) glucose (Merck®) to an approximated concentration of 10^6 CFU/mL.

Next, except in the negative control well, 96-well flat-bottomed polystyrene microtiter plates (Nunc, Thermo Fisher Scientific®) 200 µL of each bacterial suspensions were distributed per well, which contained only fresh TSB plus 0.25% (w/v) glucose. Plates were covered with 96-peg polystyrene lids (Nunc-TSP, Thermo Fisher Scientific®) and incubated at 37°C for 24 h, allowing biofilm formation on pegs. After washing the peg-lids three times in sterile normal saline (Scharlau®) to remove planktonic bacteria, the peg lids were transferred to new microplates. These new microplates contained where nisin suspensions in HCl solution or combined with guar gum gel, the supplemented gel was placed on BHI agar plates containing a lawn formed by a culture of the isolate with a approximated 10^8 CFU/mL. BHI agar plates were incubated at 37ºC for 1 h. Afterwards, absorbance (A) of each well was determined using a microplate reader (BMG LABTECH®) at 570 nm and 600 nm. MBEC value was defined as the lowest nisin concentration resulting in ≤ 50% of Alamar Blue reduction.

Percent of Alamar Blue reduction was calculated the according to equation 1, where \( \varepsilon_{ox} = \text{molar extinction coefficient of Alamar Blue oxidized form} \) \((\varepsilon_{ox1} = 80.586 \text{ and } \varepsilon_{ox2} = 117.216)\), \( \varepsilon_{red} = \text{molar extinction coefficient of Alamar Blue reduced form} \) \((\varepsilon_{red1} = 155.677 \text{ and } \varepsilon_{red2} = 14.652)\), \( A = \) absorbance of test wells, \( A' = \) absorbance of negative control well, \( \lambda_1 = 570 \text{ nm} \) and \( \lambda_2 = 600 \text{ nm} \) [27].

\[
\frac{(\varepsilon_{ox})\lambda_2A_1 - (\varepsilon_{ox})\lambda_1A_2}{(\varepsilon_{red})\lambda_2A_1 - (\varepsilon_{red})\lambda_1A_2} \times 100 \tag{1}
\]

Experiments were performed for all isolates, including the reference strain, and conducted in triplicate. Independent replicates were performed at least three times at different days.

2.5. Guar gum viability assay

To estimate the effect of storage period and temperature on the inhibitory effect of nisin diluted in guar gum, the supplemented gel was stored at five different temperatures, namely -18, 4, 20, 37 and 44°C, during six months. After 1, 3 and 6 months incubation, its inhibitory ability was tested against two S. aureus isolates. At each time, a 3 µL drop of the nisin incorporated guar gum gel was placed on BHI agar plates containing a lawn formed by a culture of the isolate with a approximated 10^7 CFU/mL. BHI agar plates were incubated at 37°C for 24 h, and inhibition halos diameters were measured. Assays were performed in triplicate.

2.6. Statistical Analysis

Statistical analysis was performed using the STATISTICA Data Miner Software, (StaSoft R version 13). Wilcoxon Matched Pairs Tests was applied to determine the significance of the variables under study and a two-tailed p-value < 0.05 was considered to be statistically significant. Quantitative variables, related with triplicate experiments, were expressed as means ± standard derivation.
3. Results and Discussion

DFU are common in Diabetes mellitus patients. Infections in DFU patients occur after the colonization of a traumatic injured member by diverse pathogenic bacteria, mainly S. aureus [6]. Among S. aureus strains, MRSA have been reported as a major cause of antimicrobial resistant related infections worldwide [9].

Antimicrobial resistant bacteria represent huge clinical, economic and social problems, together with the incapacity of antibiotics to act on resistance and biofilm produced by bacteria, new therapy must be developing for decrease these problems [6].

AMP offers a new resource for the development of novel antibacterial agents, due to their extraordinary antimicrobial potential against a large range of bacteria [12]. Nisin is an AMP with inhibitory action against Gram-positive bacteria, including S. aureus, and for this reason it has been used for many years as a food additive for control of pathogens [13].

Natural polysaccharide guar gum has been studied extensively due to its intrinsic proprieties that render him an excellent source for diverse applications, especially in pharmaceutical and medical industries, since the natural gums are regarded as safe for humans [19].

3.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination

For the MIC value determination a microtiter broth dilution method was used [29]. All DFU isolates, and also the reference strain S. aureus ATCC®29213™, were tested against the nisin diluted in HCl and incorporated in the guar gum gel. This test was also used for determining the MBC value.

All 23 S. aureus isolates and the reference strain tested were considered susceptible to nisin. The MIC values for the nisin diluted in HCl ranged from 40 to 100 µg/mL, with an average value of 90 ± 22.8 µg/mL. In the case of MBC, values were around 5-fold higher than the MIC ones. The average MBC value, was 495.2 ± 149.9 µg/mL, and only three isolates presented a MBC > 800 µg/mL (Figure 1).

In the case of the nisin incorporated in guar gum gel, all the strains investigated in this study, including the reference strain, were also considered susceptible to nisin. The MIC concentrations ranged from 40 to 300 µg/mL and the average value was 180.8 ± 53.9 µg/mL. The difference between MIC values for the nisin diluted in HCL incorporated in guar gum gel were significantly different (p-value < 0.05). The same significantly difference was observed for the MBC values. In the case of nisin incorporated in guar gum gel, only three isolates presented a MBC > 1000 µg/mL, and the average MBC value was 766.7 ± 272.6 µg/mL (Figure 2).

MIC values, for diluted nisin in HCl indicate a higher antimicrobial activity against planktomic cells, with MIC ≤ 100 µg/mL for majority of isolates tested and MBC 5.5 times higher (Figure 1). French referred that antimicrobial agents are generally classified as bactericidal if the MBC values are no more than four times higher than the MIC values [30]. In our study,
the averages of MBC values are higher than that which allowed to conclude that nisin is a bacteriostatic agent against the tested isolates.

*S. aureus* DFU isolates from this study showed susceptibility to nisin, while diluted in HCL or incorporated in a guar gum gel, showing the potential of this compound as a delivery system for nisin (Figure 1 and Figure 2). Several researchers have demonstrated that nisin is a promising compound to be applied for the control of bacterial infections, such as respiratory tract infections, gingivitis or *S. aureus* infections in atopic dermatitis [31], [32].

Okuda and collaborators [33] investigated the effects of diverse bacteriocins on MRSA clinical isolates and demonstrated that nisin showed a higher bactericidal activity against both free-floating and biofilm cells.

The antimicrobial activity of nisin was also assessed in studies using the oral microbiota. In one of these studies, performed by Shin and collaborators, it was observed that nisin inhibited planktonic growth of oral bacteria in lower concentrations (2.5 to 50 µg/mL), that the ones used in our study regarding *S. aureus* DFU isolates [31].

When incorporated in guar gum gel, nisin also were effective against all of the *S. aureus* DFU isolates, which suggests that the guar gum gel permits the diffusion of the nisin polypeptide. As observed for nisin diluted in HCl, the MBC values in this case were higher than the MIC values, and nisin worked as a bacteriostatic agent (Figure 2).

A previous study [34], which used natural polymers for topical delivery of cationic antimicrobial peptides to *S. aureus*, showed that incorporation of antimicrobial peptides within gel formulations presents a potential viable for treatment of wound skin. The results from this study also suggest that guar gum gel is a possible delivery system for nisin against *S. aureus* present in polymicrobial DFU [8], [35].

### 3.2. Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) determination

The method used in this study for MBIC and MBEC determinations consisted in a modified version of the Calgary Biofilm Pin Lid Device [27], in which the two formulations of nisin were tested.

Concerning the nisin diluted in HCL, MBIC values ranged from 20 to 300 µg/mL and the average value was 150.8 ± 85.5 µg/mL. In case of MBEC, values observed were higher than the respective MIC. For present nisin diluted in HCl, the MBEC values were > 1000 µg/mL for 65% of the isolates tested (n=15) (Figure 3). MBEC values were obtained by visual observation and quantified using an Alamar Blue protocol.

In the case of nisin incorporated in guar gum gel, the MBIC values ranged between 100 to 600 µg/mL, and the average value was 366.7 ± 85.5 µg/mL. MBEC values determinated by visually observation were higher than the respective MIC, and a large majority of the isolates presented MBEC values >1000 µg/mL, namely 87% of DFU isolates (n=20) (Figure 4).
for the nisin within the guar gum gel, reflecting in a significant difference (p-value < 0.05) between results. However, the inhibitory action of this AMP associated with the guar gum gel was only 2-fold higher than the one from nisin diluted in HCL, demonstrating that this delivery system acts not only in planktonic cells but also in established bacterial biofilms.

Regarding the MBEC it was also observed that values were higher for the nisin incorporated in the guar gum gel, but the difference was not no significant (p-value ≥ 0.05) when compared with the values obtained for the nisin in HCl.

MBEC values were higher than MBIC and MBC, as expected since it is more difficult to eliminate established biofilms than planktonic bacteria. In fact, only 13% of performed biofilms were eradicated by the concentrations used in this study. These results can be explained by the fact that assays were performed under ideal conditions, temperature and nutrients and no inhibitors that affect the formation of a biofilm matrix were present [36].

As already mentioned, MBEC values registered by visual observation using microtiter-plate test is one of the most frequently used techniques for quantifying biofilm susceptibility [37]; however, the addition of Alamar Blue allowed the MBEC quantification (Figure 3 and Figure 4). MBEC quantification with Alamar Blue [28] was used for nisin diluted in HCL and for nisin integrated in the guar gum gel. When comparing the MBEC results obtained by both methods, no significant differences were detected between both MBEC determinations methods, for both nisin formulations (p-value ≥ 0.05). This indicates that the visual direct observation of biofilm inhibition offers correct MBEC determinations, avoiding the need for the applications of a very expensive methodology.

Results also allowed to continuum that eradication of the S. aureus established biofilm is difficult, since only 35% of isolates presented MBEC values below 1000 µg/mL (Figure 4). These results are in agreement with previous studies. Okuda and collaborators [33] used this AMP against MRSA biofilms, obtaining similar results. Emel Mataraci and Sibel Dosler [38] also obtained similar results in the in vitro evaluation of diverse antimicrobial cationic peptides, including the polypeptide nisin, against MRSA strains.

DFU bacteriology is normally quite diverse, being usually polymicrobial infections. One study performed by Shin and collaborators [31], that applied nisin against saliva derived multi-species biofilms, showed that nisin has no cytotoxicity to human oral cells and that this AMP retarded the development of multi-species biofilms at concentrations above 1 µg/mL. These results suggest that nisin is useful for controlling polymicrobial infections.

According to Lewis [39], in biofilm there are differences between the cells in the surface and the ones inside the biofilm matrix. However, nisin acts against cells promoting pore formation. In fact, results showed that nisin is an excellent AMP because it can act against established biofilms as opposed to other antimicrobials polypeptides, such as lactacin Q, lactoferrin or pexiganan [10], [40].

This activity was also observed for nisin incorporated in guar gum delivery system, which suggests that guar gum gel can be used as a delivery system for nisin against established biofilms present on DFU.

3.3. Guar gum viability assay

A six months assay was performed in order to evaluate the effect of the period and temperature of storage in the inhibitory activity of nisin incorporated in guar gum.

Results showed that nisin maintained its antimicrobial activity in all time periods and all temperatures of storage, although there were variations in the inhibition potential of this AMP. Nisin maintained its antimicrobial activity probably due to the physical and chemical characteristics of the guar gum gel formulation, such as its viscosity, stability over a wide range of pH, due its non-ionic nature, and its polymeric nature and functional groups in its structure, and also due to the intrinsic characteristics of the nisin [22], [41], [42].

It was also observed that the 0.75% (w/v), guar gum gel retained used its viscosity when applied to the human surface skin, which indicates its potential for topical therapeutically administration.

According to O’Driscoll and collaborators [35], the gel formulations prepared from natural polymers offer new topical delivery systems for
wound treatment, allowing direct and continued release of integrated antimicrobial agents, thus ensuring a steady-state concentration of the agent in the wound environment. Also Zhang and collaborators [43] refered that freeze-dried wafer formulations prepared from natural polysaccharides are new formulas for antibacterial agents delivery, not showing toxicity and immunogenicity problems. In fact, gel formulations seen to be able to promote mucoadhesion, targeting of specific tissues and reduction of the inflammatory response, adding to the many benefits that contribute to wound healing.

In conclusion, this study shows that nisin has the capacity for inhibiting the planktonic cells and establishment biofilms at concentrations lower than the established for acceptable daily intake [18], [20]. Nisin is considered GRAS for oral consumption [18]. Therefore, it can be assumed that the nisin integrated in guar gum gel can be safely and effectively applied topically to clinical patients with DFU.

4. References


[15] V. Suganthi, E. Selvarajan, C. Subathrdevi and V. Mohanasrinivasan,


