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Effect of Preservative-Free Artificial Tears on the Antimicrobial Activity of Human β -Defensin-2 and Cathelicidin LL-37 In Vitro

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Abstract

Purpose. Human β -defensin-2 (hBD-2) and cathelicidin LL-37 are salt-sensitive cationic antimicrobial peptides expressed by ocular surface epithelia. The goal of this study was to investigate the effect of preservative-free artificial tears on hBD-2 and LL-37 antimicrobial activity against *Pseudomonas aeruginosa*.

Methods. *P. aeruginosa* was incubated with hBD-2 or LL-37 in the absence or presence (70% vol/vol) of different preservative-free artificial tears—Visine Tears (300 mOsm/kg), Tears Naturale Free (261 mOsm/kg), TheraTears (185 mOsm/kg), and Refresh Plus (325 mOsm/kg)—for 2 hours at 37° C. In some experiments, *P. aeruginosa* was incubated with hBD-2 or LL-37 and Visine Tears or Tears Naturale Free with or without carboxymethylcellulose (0.5% vol/vol final concentration). Plates were inoculated with samples of each reaction mixture and then incubated for 24 hours at 37° C.

Results. Visine Tears and Tears Naturale Free had little or no effect on the antimicrobial activity of 100 μ g/mL hBD-2 or LL-37. In the presence of Refresh Plus and TheraTears, the activity of 100 μ g/mL hBD-2 or LL-37 was reduced by 90% to 100%. Carboxymethylcellulose, at a concentration comparable to that present in Refresh Plus, reduced the effectiveness of hBD-2 or LL-37 by 40% to 90% in the presence of Tears Naturale Free and Visine Tears.

Conclusion. Human β -defensin-2 and cathelicidin LL-37 inhibit the growth of *P. aeruginosa* in vitro, but this activity is markedly reduced in the presence of Refresh Plus and TheraTears. These results suggest that carboxymethylcellulose-containing artificial tears may reduce the activity of the endogenously produced antimicrobial peptides.

Keywords

Carboxymethylcellulose; Cationic antimicrobial peptides; Human β -defensin-2; Human cathelicidin LL-37; Preservative-free artificial tears; *Pseudomonas aeruginosa*

Secretion of small cationic antimicrobial peptides by epithelia is an important component of the innate immune response.¹ These peptides, which include β -defensins and cathelicidins, insert themselves into microbial cell membranes and, by pore formation or electrostatic disruption, cause release of intracellular contents leading to death of the organism.² By regulating mammalian cell functions, such as chemotaxis and proliferation, defensins and

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cathelicidins also provide a link between the innate and adaptive immune systems and regulate wound-healing processes.^{3,4}

The human corneal epithelium expresses three β -defensins. Human β -defensin (hBD)-1 and hBD-3 are constitutively expressed, whereas hBD-2 expression is induced by proinflammatory cytokines and bacterial products and after injury.⁵⁻⁷ The authors have recently identified that the corneal epithelium also expresses LL-37, an antimicrobial peptide of the cathelicidin family, which is up-regulated after injury.⁸ This pattern of expression suggests that hBD-1 and hBD-3 provide baseline defense against infection, whereas after injury, additional antimicrobial protection may be afforded by hBD-2 and LL-37. It also raises the possibility that hBD-2 and LL-37 may have important roles in modulating corneal epithelial cell behavior during wound healing. Not only are defensins and LL-37 important endogenously expressed molecules, but they have great potential as pharmaceutical agents that would simultaneously provide antimicrobial protection and stimulate corneal epithelial healing.

Preservative-free artificial tears have become one of the most widely used modalities for the relief of discomfort from dryness of the eyes and for conservative treatment of ocular surface diseases, such as meibomian gland dysfunction.⁹ The goal of this study was to determine whether commonly used preservative-free artificial tear solutions affect the antibacterial activity of hBD-2 and LL-37 against a common ocular pathogen, *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Preparation of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (ATCC 27853 and two clinical isolates from corneal scrapings of patients with bacterial keratitis) was tested in this study. ATCC 27853 is known to invade an intact cornea and produce severe ocular infection in experimentally infected animal models of bacterial keratitis.^{10,11} Most of the current studies were carried out by using ATCC 27853, and selected experiments were repeated with the two clinically isolated *P. aeruginosa* strains.

One single isolated *P. aeruginosa* colony was used to inoculate 5 mL of nutrient broth overnight at 37°C. Fifty microliters of this bacterial suspension was used to inoculate 50 mL of fresh nutrient broth, which was then incubated for 2.5 hours with vigorous shaking at 37°C to achieve mid log phase growth. Twenty-five milliliters of the warm *P. aeruginosa* culture was centrifuged at 3,100g for 10 minutes, and the bacterial cell pellet was resuspended in cold phosphate buffer (8.2 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). Optical density of the suspension was adjusted to 0.2 at 620 nm (approximately 10⁷ colony-forming units [CFU]/mL) by adding an appropriate volume of phosphate buffer.

Effect of Preservative-Free Artificial Tears or Sodium Chloride on the Antimicrobial Activity of hBD-2 and LL-37

Human β -defensin-2 (Peninsula, San Carlos, CA) and LL-37 (American Peptide Company, Sunnyvale, CA) were dissolved in 0.01% acetic acid at a concentration of 1 mg/mL and stored at -20°C. The antimicrobial assay procedure was adapted from that described by Tomita et al.¹² Reaction mixtures (final volume of 50 μ L) containing 10 μ L 10⁷ CFU/mL *P. aeruginosa*, 5 μ L hBD-2 or LL-37 diluted in phosphate buffer (each at a final concentration of 100 μ g/mL) were incubated in the absence and presence of 70% vol/vol preservative-free artificial tears. The peptide concentration was chosen based on studies in which the authors found 100 μ g/mL to be 100% effective against *P. aeruginosa* (data not shown) and published reports of 90% lethal dose and minimum inhibitory concentration values.^{13,14} Four different preservative-free artificial tears were tested: Visine Tears (Pfizer, Inc., New York, NY), Tears Naturelle Free (Alcon, Fort Worth, TX), TheraTears (Advanced Vision Research, Woburn, MA), and Refresh

Plus (Allergan, Irvine, CA). Osmolality of the artificial tears was measured using a vapor pressure osmometer (Vapro 5520; Wescor, Logan, UT). The osmolality and the ingredients of the four preservative-free artificial tears tested are summarized in Table 1. Because of other constituents of the reaction mixture, 70% vol/vol was the maximum artificial tear concentration obtainable in these experiments. Reaction mixtures were incubated at 37°C for 2 hours with vigorous shaking. In each experiment, reaction mixtures containing 5 µL 0.01% acetic acid, the vehicle for diluting hBD-2 or LL-37, acted as a control. At the end of the incubation, each reaction mixture was serially diluted with nutrient broth, and then samples at the same dilution factor were used to inoculate nutrient broth agar plates. Samples were spread evenly over the surface of the plates by using sterile glass spreaders. After incubation at 37°C for 24 hours, the agar plates were placed on a light board, and a digital image was captured using an Alpha Imager documentation system (Alpha Innotech, San Leandro, CA).

In some experiments, sodium chloride was used in place of artificial tears so that the effect of a solution with osmolality matched to that of Refresh Plus (325 mOsm/kg, the highest osmolality among the four artificial tears tested) could be determined. In other experiments, antimicrobial assays were performed with hBD-2 or LL-37 (100 µg/mL) in the absence or presence of Visine Tears or Tears Naturale Free, with or without added carboxymethylcellulose (CMC) (0.5% vol/vol final concentration). CMC is the cellulose polymer present in TheraTears (0.25% vol/vol CMC) and Refresh Plus (0.5% vol/vol of CMC).

RESULTS

Effect of Artificial Tears on hBD-2 and LL-37 Antimicrobial Activity Against *Pseudomonas aeruginosa*

When tested in the presence of Refresh Plus and TheraTears, the antibacterial activity of 100 µg/mL hBD-2 and LL-37 against *P. aeruginosa* (ATCC 27853) was eliminated completely ($n = 5$). In contrast, Visine Tears and Tears Naturale Free had no effect on the antimicrobial activity of hBD-2 or LL-37 ($n = 5$). Figure 1 is representative data from one of the five experiments. The same results were obtained when experiments were repeated twice with each of the two other *P. aeruginosa* strains.

Sodium Chloride Solution (322 mOsm/kg) Does Not Affect hBD-2 and LL-37 Antimicrobial Activity Against *Pseudomonas aeruginosa*

Previously, it has been shown that the antimicrobial activity of some cationic peptides, including hBD-2 and LL-37, is attenuated in the presence of high salt content.^{15–18} These effects depend on the concentration of peptide being used, with the higher concentrations being little affected.¹⁹ Therefore, at the peptide concentration used in the current study, one would not expect to find that antimicrobial activity is compromised by salt present in the artificial tear solutions. To confirm this hypothesis, the authors tested the antimicrobial activity of the peptides in the presence of a solution of sodium chloride at 322 mOsm/kg. This osmolality was approximately equivalent to that of Refresh Plus (325 mOsm/kg), which had the highest osmolality of the four artificial tears tested. That the antimicrobial activity of the peptides was not reduced under these conditions indicates that salt in the artificial tear solutions was not responsible for loss of peptide activity (Fig. 2).

Carboxymethylcellulose Reduces hBD-2 and LL-37 Antimicrobial Activity Against *Pseudomonas aeruginosa*

Refresh Plus and TheraTears contain CMC, an anionic polymer, whereas Tears Naturale Free and Visine Tears contain hydroxypropylmethylcellulose (HPMC), a nonionic polymer (Table 1). To determine whether the CMC in Refresh Plus and TheraTears was responsible for the loss of hBD-2 and LL-37 antimicrobial activity, antimicrobial assays were performed in which

CMC was added to Tears Naturale Free and Visine Tears to give a final concentration of 0.5% vol/vol CMC. As shown in Figure 3, in the presence of added CMC, Tears Naturale Free partially (40%–50%) and Visine Tears almost completely (80%–90%) impaired hBD-2 and LL-37 antibacterial activity against *P. aeruginosa* (ATCC 27853).

DISCUSSION

Preservative-free artificial tears are often recommended instead of preserved artificial tears for patients as a palliative therapy for ocular surface disorders because of potential adverse reactions to commonly used ophthalmic preservatives.²⁰ Because cationic antimicrobial peptides, such as hBD-2 and LL-37, are important components of the innate immune defense produced by ocular surface epithelia in response to infection and inflammation,^{5,6,8} the effect of preservative artificial tears on hBD-2 and LL-37 antibacterial activity was studied.

The current study showed that the ability of hBD-2 and LL-37 to inhibit the growth of *P. aeruginosa* in vitro was markedly reduced in the presence of Refresh Plus and TheraTears, but not Tears Naturale Free or Visine Tears. Comparable findings were obtained using a laboratory and two clinical strains of *P. aeruginosa*. It has been shown that the antimicrobial activity of some defensins, including hBD-2 and LL-37, is attenuated in the presence of high salt concentrations.^{15–18} However, this response depends on the concentration of the peptide being used, so by using a relative high concentration of hBD-2 and LL-37 (100 µg/mL), the authors eliminated any effect of salt present in the artificial tears. The exact concentration of hBD-2 and LL-37 at the ocular surface in vivo has yet to be determined, although Oren et al.²¹ recently showed that physiologic concentrations of these peptides are approximately 10 µg/mL in other epithelial tissues. Although the current study used a supraphysiologic concentration of the peptides, the authors would expect to see that artificial tears have the same effect on physiologic levels.

The current data suggest that the differential effects of the preservative-free artificial tears are the result of the difference in the cellulose polymers present in the solutions. CMC, an active ingredient in Refresh Plus and TheraTears, is an anionic compound, and through ionic interaction, it would be capable of binding to hBD-2 and LL-37, which are both positively charged peptides. HPMC, an active ingredient in Tears Naturale Free or Visine Tears, is a nonionic compound, which would not be expected to interact with the peptides. Based on the differences in the chemical properties between CMC and HPMC, the loss of hBD-2 and LL-37 activity in the presence of Refresh Plus is likely the result of the peptides being bound to CMC. It is noteworthy that similar findings were reported in a recent study that showed that binding of CMC to polyhexamethylene biguanide, a cationic disinfectant, was accompanied by reduced antibacterial activity of polyhexamethylene biguanide.²²

When comparing the outcome of added CMC, the authors observed that reduction of hBD-2 and LL-37 antimicrobial activity was greater with Visine Tears plus CMC than with Tears Naturale Free plus CMC. This may be attributed to differences in the composition between the two preservative-free artificial tears. Variation in the ionic makeup of Tears Naturale Free and Visine Tears may differentially influence the putative binding of CMC to the peptides, leading to a change in the availability of hBD-2 and LL-37 and, hence, variability in their antimicrobial effectiveness.

An obvious conclusion from the current study is that CMC-containing artificial tears may reduce the activity of endogenously produced cationic antimicrobial peptides. Furthermore, because hBD-2 and LL-37 have been shown to modulate cell migration and proliferation and, as a consequence, have been implicated as regulatory factors for wound healing,^{23–25} it is possible that CMC-containing tear solutions may also impair the ability of these peptides to

stimulate corneal wound healing. However, extrapolation of the in vitro data to the in vivo situation should be done with extreme caution. Indeed, the ocular surface is equipped with several other antimicrobial substances²⁶ and numerous factors believed capable of regulating wound healing.²⁷ Thus, even if the activity of cationic antimicrobial peptides is compromised by the use of CMC-containing solutions, redundancy at the ocular surface in most cases will ensure that adequate antimicrobial protection and responses to injury still exist.

Defensins and LL-37, in addition to being antimicrobial, are known to be chemotactic for various immune cells.^{28–32} They have also been implicated as mediators of inflammation simulating the release of cytokines and histamine from innate and adaptive immune cells.^{33–36} Furthermore, several studies have shown high concentrations of antimicrobial peptides, such as defensins, to be cytotoxic to various mammalian cell types.³⁷ Therefore, if present in excess, these peptides may be detrimental to the ocular surface for a variety of reasons, such as uncontrolled inflammation or cellular toxicity. Notably, CMC-based solutions have been shown to improve objective measurements in patients with dry eye³⁸ and preserve ocular surface health after laser in situ keratomileusis.^{39,40} Therefore, in keeping with the concept that CMC has cytoprotective properties,²² an alternative interpretation of the results of the current study is that neutralization of hBD-2 and LL-37 by CMC at the ocular surface may actually be advantageous in preventing unwanted effects that may result from having an excess of these peptides.

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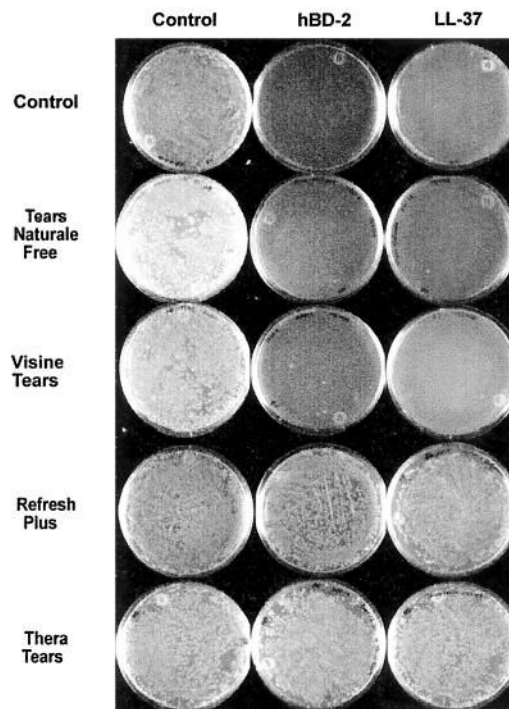
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**FIG. 1.**

Effect of preservative-free artificial tears on hBD-2 and LL-37 antimicrobial activity. *Pseudomonas aeruginosa* (10^7 CFU/mL ATCC 27853) was incubated with 0.01% acetic acid (control), with hBD-2 and LL-37 (100 μ g/mL), or with hBD-2 and LL-37 (100 μ g/mL) in the presence of four preservative-free artificial tears, at 37°C for 2 hours. When tested in the presence of Refresh Plus and TheraTears, the antibacterial activity of 100 μ g/mL hBD-2 and LL-37 was markedly reduced ($n = 5$). Tears Naturale Free or Visine Tears did not reduce the activity of hBD-2 or LL-37 ($n = 5$).

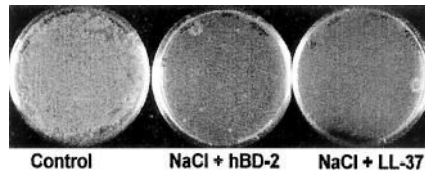


FIG. 2.

Effect of sodium chloride (NaCl) solutions on hBD-2 and LL-37 antimicrobial activity. *Pseudomonas aeruginosa* (10^7 CFU/mL ATCC 27853) was incubated with 0.01% acetic acid, with hBD-2 and LL-37 (100 μ g/mL), or with hBD-2 and LL-37 (100 μ g/mL) in the presence of sodium chloride solution (322 mOsm/kg) at 37°C for 2 hours ($n = 3$).

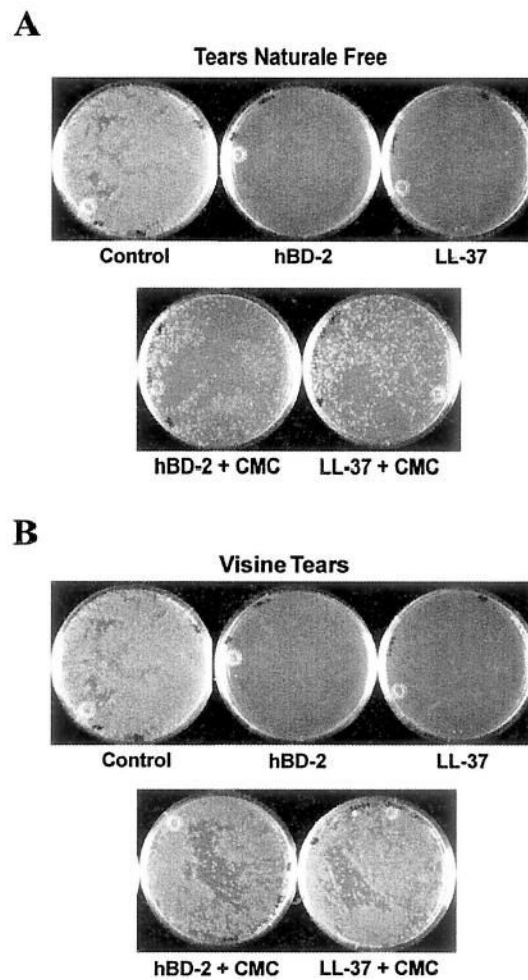


FIG. 3. Effect of carboxymethylcellulose (CMC) on hBD-2 and LL-37 antimicrobial activity against *Pseudomonas aeruginosa*. *P. aeruginosa* (10^7 CFU/mL ATCC 27853) was incubated with 0.01% acetic acid (control), with hBD-2 and LL-37 (100 μ g/mL), or with hBD-2 and LL-37 (100 μ g/mL) in the presence of Tears Naturele Free or Visine Tears containing 0.5% CMC at 37°C for 2 hours. With the addition of CMC, Tears Naturele Free (A) and Visine Tears (B) partially or completely impaired the activity of hBD-2 and LL-37 ($n = 2$).

TABLE 1
Active Ingredients and Osmolality of the Preservative-Free Artificial Tear Solutions

	Refresh Plus	Tears Naturale Free	Visine Tears	TheraTears
Osmolality (mOsm/kg)	325	261	300	185
Dextran 70 (0.1%)		X		
Hydroxypropylmethylcellulose (0.2–0.3%)		X	X	X
Carboxymethylcellulose CMC (0.5%)	X		X	
Polyethylene glycol 400 1%			X	
Glycerin 0.2%			X	

Inactive ingredients for Refresh Plus: CaCl₂, MgCl₂, KCl, NaCl, purified water, sodium lactate. May also contain HCl and NaOH to adjust pH.

Inactive ingredients for Tears Naturale Free: KCl, purified water, sodium borate, NaCl. May also contain HCl and NaOH to adjust pH.

Inactive ingredients for Visine Tears: ascorbic acid, dextrose, glycine, MgCl₂, KCl, purified water, sodium citrate, sodium lactate.

Inactive ingredients for TheraTears: borate buffer, CaCl₂, MgCl₂, KCl, NaCl, purified water, sodium bicarbonate, sodium phosphate.