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Non-steroidal anti inflammatory agents decrease bacterial colonisation of contact lenses and prevent adhesion to human corneal epithelial cells

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Abstract

Purpose. To investigate non-steroidal anti-inflammatory agents (NSAIDs), salicylic acid, sodium diclofenac and ketorolac for inhibition of bacterial colonization of contact lenses (CL) and human corneal epithelial cells (HCE).

Methods. CLs pre-colonised with *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Staphylococcus epidermidis* and *Streptococcus pneumoniae* were exposed overnight to NSAIDs and the number of viable bacteria on the CLs were calculated. Cytotoxicity of NSAIDs to HCE cells was evaluated with the MTT assay. Viable counts were used to measure the adhesion of *P. aeruginosa* and *S. epidermidis* to HCE cells in the presence of the least cytotoxic NSAID.

Results. All NSAIDs significantly decreased bacterial colonization of CLs in a dose-dependent manner. Salicylic acid (100 mM) completely inhibited colonisation of all species tested and was the least cytotoxic. Salicylic acid also prevented adhesion of *P. aeruginosa* and *S. epidermidis* to HCE (60% and 58% inhibition at 60 mM at 2 hours).

Conclusions. Salicylic acid demonstrated potential as a compound for incorporation into anti-bacterial strategies to prevent bacterial contamination of contact lenses. This study highlighted the potential for NSAIDs as anti-bacterial agents and indicates that this class of compound should be investigated for other suitable candidates.

Keywords: non-steroidal anti-inflammatory agent; NSAID; contact lens; colonization; corneal epithelial cells

Introduction

Microbial colonization of contact lenses is a significant risk factor for development of infective and infiltrative conditions of the cornea during lens wear. *Pseudomonas aeruginosa* is the predominant organism associated with contact lens related infectious keratitis and a number of reports have shown the organism to be isolated from both the corneal scrape and the contact lens.[1–3] Also, corneal infiltrative events such as contact lens-induced acute red eye (CLARE) are associated with colonization of lenses by significant numbers of gram-negative bacteria such as *P. aeruginosa*, *Haemophilus influenzae* and *Serratia marcescens*.[4–6]

Sodium salicylate, a non-steroidal anti-inflammatory drug (NSAID), decreases the production of capsular polysaccharide of *Klebsiella pneumoniae* in culture.[7] Capsular polysaccharides are said to play a role in the production of biofilms and therefore agents that reduce capsular polysachharide production and are considered to be of benefit in controlling infections. In addition, Farber and Wolff[8] have demonstrated *in vitro* that coating catheters with salicylic acid prevents the adhesion of bacteria and yeasts to these catheters. *In vitro* studies of bacterial adhesion to contact lenses have also

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demonstrated that NSAIDs such as sodium salicylate, diclofenac and bendazac lysine can decrease the adhesion of *Staphylococcus epidermidis*, *P. aeruginosa*[9–11] and *Acanthamoeba*[12] to contact lenses.

We are interested in investigating a range of NSAIDs for their ability to inhibit bacterial colonization of soft contact lenses and prevent adverse events during contact lens wear. In this study we compared the effect of two NSAIDs that are currently used as topical drops to treat ocular inflammation (sodium diclofenac and ketorolac) and salicylic acid, which is not used for ocular therapy. A range of gram-positive and gram-negative bacterial species that are commonly isolated from asymptomatic lens wear or from microbial keratitis and contact lens-related infiltrative events were tested. In addition, we determined whether NSAIDs could inhibit adhesion of bacteria to human corneal epithelial (HCE) cells.

Materials and methods

Micro-organisms and culture conditions

The bacterial strains used in the study are listed in Table 1. Strains of *P. aeruginosa* and *S. epidermidis* were grown overnight at 37°C on nutrient agar (Oxoid, Sydney, Australia) and *Streptococcus pneumoniae* was grown overnight at 37°C on blood agar (Oxoid). *H. influenzae* strains were grown overnight at 37°C with 5% CO_2 on *Haemophilus* test agar supplemented with SR158 (Oxoid).

Contact lenses and NSAIDs

Etafilcon A disposable soft contact lenses (FDA group IV, Vistakon, Johnson & Johnson, USA) were used in the study and were washed in phosphate buffered saline (PBS; 8 g/l $NaCl_2$, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4) before use. Salicylic acid (2-hydroxybenzoic acid; Sigma, St Louis, MS, USA) and ketorolac tris salt (Sigma) were prepared as 3 M and 2 M stock solutions, respectively in PBS. Sodium diclofenac ({2-(2,6-diclorophenyl) amino} benzeneacetic acid, monosodium salt; ICN biomedicals Inc., Aurora, OH, USA) was prepared as a 12.5 mM stock solution in tryptone soya broth (TSB; Oxoid).

Effect of NSAIDs on bacterial colonisation of contact lenses

P. aeruginosa, *S. epidermidis* and *S. pneumoniae* strains were sub-cultured at 37°C in 10 ml of TSB and *H. influenzae* strains were sub-cultured at 37°C with 5% CO_2 in 10 ml of brain heart infusion (BHI) with 10 mg/l nicotinamide adenine dinucleotide (NAD; Sigma) and 10 mg/l hemin (Sigma). The cultures were grown to stationary phase without agitation and bacterial cells harvested by centrifugation (3000 × g, 10 min, 20°C), washed twice and suspended in PBS at a concentration of 1×10^7 colony forming units (CFU)/ml (OD of 0.1 at 660 nm).

To induce colonization of contact lenses, the prepared bacteria were added 1 : 10 to fresh TSB, incubated at room temperature with contact lenses in 24 well culture plates on a shaking platform for one or 18 h (very few *H. influenzae* and *S. pneumoniae* adhered to contact lenses after incubation for one hour therefore, the incubation period was extended to 18 h) at ambient temperature. Following incubation, two lenses were randomly selected, placed in 5 ml PBS and homogenized using a hand-held Ultra-Tarrax T-8 dispersing tool (IKA, Rawang, Malaysia). The number of colony forming units per mm^2 of the lens surface were determined by preparing serial dilutions of the homogenates in PBS and culturing 20 µl of a range of dilutions on nutrient agar plates by incubation overnight at 37°C. The remaining lenses were washed twice and incubated overnight in either fresh TSB alone (control lenses) or in fresh TSB with increasing concentrations of salicylic acid, sodium diclofenac or ketorolac (test lenses). Following overnight incubation, lenses were washed gently with PBS to remove loosely adhered bacteria, homogenized and the number of CFU per mm^2 per lens surface were calculated from cultures of serial dilutions of each homogenate on nutrient agar plates as described above. The difference in the number of CFU/mm^2 on test versus control lenses was used to calculate the percentage inhibition

Table 1. Bacterial strains.

Bacterial species	Strain	Source event
Pseudomonas aeruginosa	6294	Microbial keratitis
P. aeruginosa	Paer 1	Contact lens induced acute red eye
Staphylococcus epidermidis	Sepi 5	Asymptomatic contact lens wear
Streptococcus pneumoniae	Spne 8	Contact lens induced acute red eye
Haemophilus influenzae	Hinf 1	Contact lens induced acute red eye
H. influenzae	Hinf 5	Contact lens induced acute red eye

P. aeruginosa 6294 was kindly provided by Dr. Suzi Fleiszig, University of California, Berkeley, California, USA. All other strains were obtained from the culture collection of the Co-operative Research Centre for Eye Research and Technology, University of New South Wales, Sydney, Australia.

of colonisation by the NSAIDs. Each experiment was performed three times.

Human corneal epithelial (HCE) cells and culture conditions

Transformed HCE cells were maintained as described by Araki-Sasaki *et al*.[13] Briefly, cell cultures were grown in modified hormone epithelium medium consisting of an equal volume of Eagle's minimum essential medium (MEM; Life Technologies, Grand Island, NY, USA) and Ham's F12 medium (Trace Bioscience, Sydney Australia) supplemented with 5% fetal bovine serum (Gibco-BRL), 50 µg/ml gentamycin, 100 µg/ml streptomycin, 100 U/ml penicillin, 2.5 µg/ml amphotericin B, 100 µg/ml cholera toxin, 5 µg/ml insulin, 10 ng/ml epidermal growth factor and 0.5% dimethyl sulfoxide (DMSO; all obtained from Sigma). Cells were grown at 37°C in an atmosphere of 5% CO_2 until confluent.

Cytotoxicity of NSAIDs to HCE cells

Cytotoxicity assay was performed as described by Thuruthyil *et al*.[14] Briefly, HCE cells were grown in modified hormone epithelium medium in 24 well tissue culture plates to a confluent monolayer. The medium was then aspirated and 200 µl of fresh medium containing various concentrations of each NSAID (salicylic acid, sodium diclofenac or ketorolac) was added to wells. The cell monolayers were then incubated for 2 h, and the culture suspensions were aspirated and replaced with 200 µl of fresh medium supplemented with 20 µl of 12 mM 3-(4,5-dimethlthiazol-2-yl)–2,5-diphenyl-tetrazolium bromide (MTT; Sigma) and re-incubated for a further 3 to 4 h to allow the vital cells to form insoluble purple formazan precipitates. The contents of the wells were aspirated and 0.5 ml DMSO was added to each well to dissolve the formazan crystals. After 5 min incubation, 100 µl of the purple solution was transferred to a 96 well plate (Nunc Products, Denmark) and the absorbance was measured at 570 nm (Spectrafluor Plus, Tecan, Austria GmbH, Austria). The percentage of dead cells was calculated by subtracting the absorbance of cells incubated with each concentration of NSAID from the absorbance of control cells. Each experiment was performed three times.

Effect of NSAIDs on bacterial adhesion to HCE cells

P. aeruginosa (Paer 1) and *S. epidermidis* (Sepi 5) cells were grown in TSB, then harvested by centrifugation (3000 × g, 10 min, 20°C), washed twice in PBS and re-suspended in MEM:F12 containing 0.035% $NaHCO_3$ and 0.6% bovine serum albumin (BSA, Sigma) at 1×10^7 CFU/ml (0.1 at 660 nm). HCE cell monolayers were washed three times with pre-warmed PBS and incubated with increasing concentrations of salicylic acid containing bacterial cells (1×10^6 CFU/ml) in MEM buffered with 0.035% $NaHCO_3$ and containing 0.6% BSA at 37°C in an atmosphere of 5% CO_2 for 2 h. The culture supernatants were then aspirated and the monolayers were washed three times with pre-warmed PBS to remove any unbound bacteria. The bacteria adhered to HCE cells were recovered by adding 0.5 ml solution of 0.25% Triton X-100 for 15 min and the number of CFUs were counted after serial dilutions were cultured on nutrient agar plates overnight as previously described. The assay was performed at least twice using duplicate wells in each experiment.

Statistical analysis

Data is presented as the mean ± SE of experiments. The number of colonies per mm^2 lens surface (CFU/mm^2) and the differences in the effect of each concentration of NSAID on bacterial adhesion to contact lenses, cytotoxicity and adhesion of bacteria to HCE were tested using a one-way ANOVA. Differences were considered significant at $p < 0.05$.

Results

Bacterial colonisation of contact lenses

The ability of each of the bacterial strains to colonise contact lenses was compared by measuring the mean number of CFU/mm^2 of contact lens after 1 and 18 hours incubation. Both *P. aeruginosa* strains (6294 and Paer 1) adhered to contact lenses in greater numbers than other bacterial strains after incubation for 1 and 18 h (Table 2). The *H. influenzae* strains and the *S. pneumoniae* strain colonised contact lenses at low concentrations and only after incubation for 18 h.

Effect of NSAIDs on bacterial colonisation of contact lenses

Inhibition of colonisation was compared by measuring the ability of each NSAID to inhibit biofilm formation on contact lenses. All three NSAIDs demonstrated a progressive dose-dependent inhibition of colonisation of bacteria to contact lenses (Fig. 1). Salicylic acid was the only NSAID to completely inhibit colonisation of all strains at the concentrations tested. At 50 mM, salicylic acid completely inhibited colonisation of the two strains of *H. influenzae* (Hinf 1 and Hinf 5), and *S. pneumoniae* Spne 8 and at 100 mM inhibited colonisation of *S. epidermidis* Sepi 5 and *P. aeruginosa* Paer 1 and 6294 by >99%. Colonization of all strains was significantly lower compared to controls for all concentrations of salicylic acid tested ($p < 0.05$).

Colonisation of the *H. influenzae* and *S. pneumoniae* strains was completely inhibited at 50 mM ketorolac. The highest concentration of ketorolac tested (150 mM) completely inhibited colonisation of *S. epidermidis* Sepi 5 and inhibited colonization of *P. aeruginosa* Paer 1 and 6294 by 78 and 79%, respectively. Colonization of all strains was significantly lower compared to controls for all concentrations of ketorolac tested ($p < 0.05$).

Table 2. Bacterial colonisation of contact lenses.

Strain	Mean number of colony forming units/mm^2 of contact lens surface*	
	1 hour incubation	18 hour incubation
Pseudomonas aeruginosa 6294	$2.17 \times 10^3 \pm 2 \times 10^2$	$1.22 \times 10^6 \pm 139$
P. aeruginosa Paer 1	$7.07 \times 10^2 \pm 1.01 \times 10^2$	$9.08 \times 10^5 \pm 153$
Staphylococcus epidermidis Sepi 5	42 ± 13	$1.7 \times 10^2 \pm 22$
Streptococcus pneumoniae Spne 8	–	37 ± 4
Haemophilus influenzae Hinf 1	–	65 ± 9
H. influenzae Hinf 5	–	111 ± 21

*mean ± SE of 3 independent experiments.

Colonisation of the *H. influenzae* and *S. pneumoniae* strains was completely inhibited at 6.25 mM sodium diclofenac. The highest concentration of sodium diclofenac tested (12.5 mM) inhibited colonisation of *S. epidermidis* Sepi 5 by 92% and inhibited colonisation of *P. aeruginosa* Paer 1 and 6294 by 81% and 67%, respectively. Higher concentrations of sodium diclofenac were not soluble in TSB and could not be tested. The mean colonization of the *S. epidermidis*, *H. influenzae* and *S. pneumoniae* strains was significantly lower than the control for all concentrations of sodium diclofenac ($p < 0.05$). The two *Pseudomonas* strains exhibited significant inhibition of colonization with ≥6.25 mM sodium diclofenac ($p < 0.05$).

Cytotoxicity of NSAIDs to HCE cells

The monolayers of epithelial cells with and without NSAIDs were compared following incubation for 2 h (representative photographs shown in Fig. 2). There was zero cytopathic effect on HCE cells in the un-inoculated wells and controls. All the test samples demonstrated a cytopathic effect (Fig. 2c & 2d) with salicylic acid being the least cytotoxic and diclofenac the most (Fig. 3). Cell death was less than 4% and 14% with 20 mM and 60 mM salicylic acid. Salicylic acid continued to exhibit less than 20% cell death even after 24 h at a concentration less than 20 mM (data not shown).

Adhesion of bacteria to HCE cells in the presence of salicylic acid

This experiment was done to determine whether salicylic acid affects bacterial adhesion to HCE cells. Salicylic acid was chosen because it was the least cytotoxic compound. The inhibition of adhesion of *P. aeruginosa* Paer 1 and *S. epidermidis* Sepi 5 to HCE cells by salicylic acid was similar for both strains across the range of concentrations that were tested. After 2 h 20 mM and 60 mM salicylic acid inhibited the adhesion of *P. aeruginosa* to HCE cells by 28% and 66%, respectively and inhibited the adhesion of *S. epidermidis* by 22% and 58%, respectively (Fig. 4). Adhesion data are expressed as the percentage reduction relative to the adhesion of the control sample without salicylic acid.

Discussion

This study compared the effects NSAIDs on colonisation of contact lenses by a range of gram-positive and gram-negative bacteria isolated from contact lens wearers, usually during an adverse response. Overall the rates of inhibition of bacterial colonization varied with the type and concentration of each NSAID and bacterial species. This is the first report of the ability of ketorolac to inhibit bacterial colonisation of contact lenses and demonstrated that this NSAID was able to inhibit colonisation of most bacterial strains. However, salicylic acid was able to inhibit colonisation completely of all strains to contact lenses.

At 100 mM, salicylic acid almost completely inhibited colonisation of contact lenses by all bacterial strains (>99%) and at 60 mM inhibited colonisation of all strains by more than 80%. In addition, this NSAID demonstrated low cytoxicity to HCE cells and prevented adhesion of *P. aeruginosa* strains and *S. epidermidis* Sepi 5 to HCE cells in a dose-dependent manner. Salicylic acid has previously been shown to inhibit *P. aeruginosa* and *S. epidermidis* colonisation of contact lenses,[9] but this is the first report showing that salicylic acid has broad-spectrum anti-bacterial activity and low toxicity. These results indicate that it is a strong candidate for potential ocular applications designed to prevent bacterial contamination of contact lenses or for the treatment of corneal infection.

The concentrations of sodium diclofenac and ketorolac that were tested in this study were much higher than the concentrations of these compounds when used as therapeutic ocular drops (0.5% ketorolac is equivalent to approximately 13 mM and 0.1% diclofenac is equivalent to approximately 3.1 mM). At high concentrations, ketorolac (150 mM) and sodium diclofenac (12.5 mM) inhibited colonisation of most bacterial species by approximately 80% or more however both NSAIDs demonstrated unacceptable cytotoxicity to

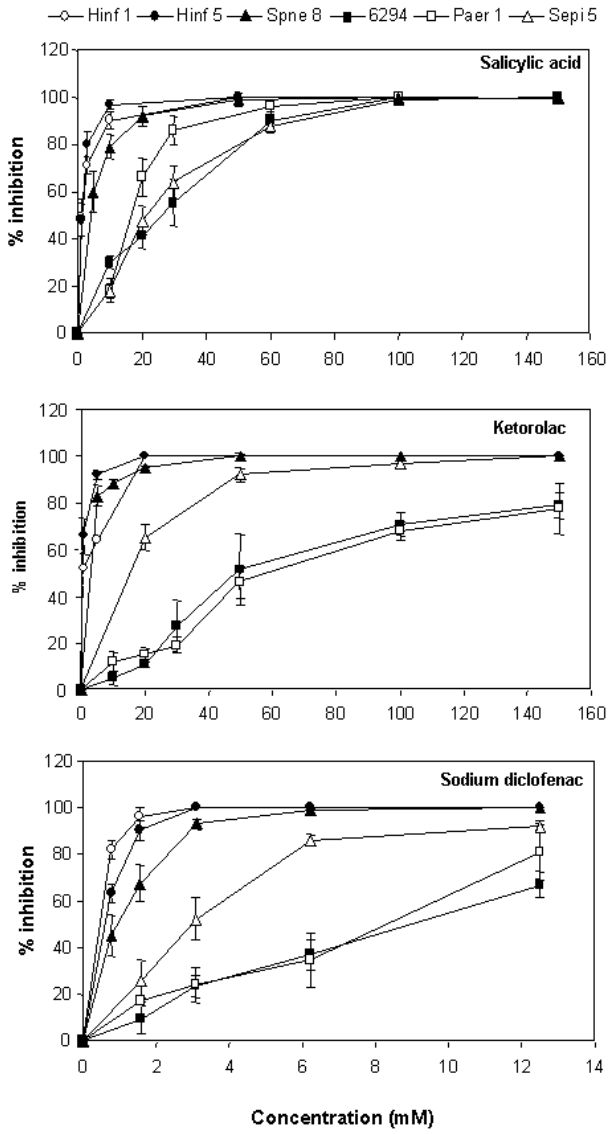

Figure 1. Percentage inhibition of colonization of *Haemophilus influenzae* strains Hinf1 and Hinf8, *Streptococcus pneumoniae* Spne8, *Pseudomonas aeruginosa* strains 6294 and Paer 1, and *Staphylococcus epidermidis* Sepi 5 on contact lenses after overnight exposure to salicyclic acid, ketoroloac and sodium diclofenac relative to control lenses in the absence of NSAIDs. Results represent the mean ± SE of three independent experiments.

HCE cells. Sodium diclofenac was extremely cytotoxic, exhibiting significant HCE cell death, even at low concentrations. Ketorolac demonstrated limited cytoxicity at low concentrations (20 mM), but at these concentrations had very little effect on the colonisation of contact lenses by *P. aeruginosa*. Repeat and long-term use, even at low concentrations can lead to an accumulation of drug in ocular tissues and potentially cytotoxic effects. There are many reports in the literature of adverse events associated with long-term use of sodium diclofenac and ketorolac. These adverse events range from allergic conjunctivitis to punctate corneal keratopathy, corneal infiltrates and corneal melts leading to perforation.[15–18] It is unlikely that either of these NSAIDs would be acceptable candidates for anti-bacterial applications during contact lens wear at the concentrations tested in this study. Interestingly, however, a recent report indicates that the vitamin E based solubilizer in a diclofenac ocular preparation (not used in the present study) triggers the corneal toxicity associated with topical application of diclofenac.[19] Therefore, further work is needed to obtain more information and to identify the cytotoxic effects of diclofenac.

The mechanism by which the NSAIDs tested decreased bacterial colonization was not addressed in this study. In addition to reducing formation of capsular polysaccharide in *K. pneumoniae*,[7] salicylic acid also reduces the production of extracellular polysaccharide[20] and cell wall slime-associated proteins and teichoic acids,[21] that play an important role in the formation of *S. epidermidis* biofilm. Perilli *et al*.[10] showed that diclofenac had a bacteriostatic effect on *S. epidermidis* and inhibited production of extracellular material. They also demonstrated that diclofenac has a significant effect on formed biofilm and causes total disorganization of the biofilm structure. In this study we demonstrated that ketorolac disrupted biofilm formation by interfering with microbial attachment during colonisation of contact lenses and therefore may have a similar mode of action to salicylic acid and sodium diclofenac. In addition, salicylic acid is reported to reduce colonisation of *Escherichia coli* to human lung epithelial cells (Hep-2) by inhibiting attachment that is mediated by fimbrial adhesins.[22] Clearly further work is needed to investigate the mode of action of these compounds in inhibiting biofilm formation and preventing initial adhesion to both contact lenses and corneal epithelial cells.

All compounds used in this study inhibited colonization of contact lenses by *S. epidermidis*, and salicylic acid prevented adhesion of this micro-organism to HCE cells. *S. epidermidis* is the most common isolate from the external ocular surface and is frequently found as a contaminant during wear of soft contact lenses.[23,24] Although *S. epidermidis* may become an opportunistic pathogen, this micro-organism is not associated with adverse events or symptomatic lens wear.[6,25] A better understanding of the consequences of disrupting the normal ocular microbiota in the long-term is necessary before these compounds can be used in for example, contact lens solutions.

This study demonstrates the potential of salicylic acid for preventing bacterial contamination of contact lenses by a range of ocular pathogens and highlights the potential for NSAIDs as anti-bacterial agents, indicating that this class of compound be investigated further for other suitable candidates.

Acknowledgments

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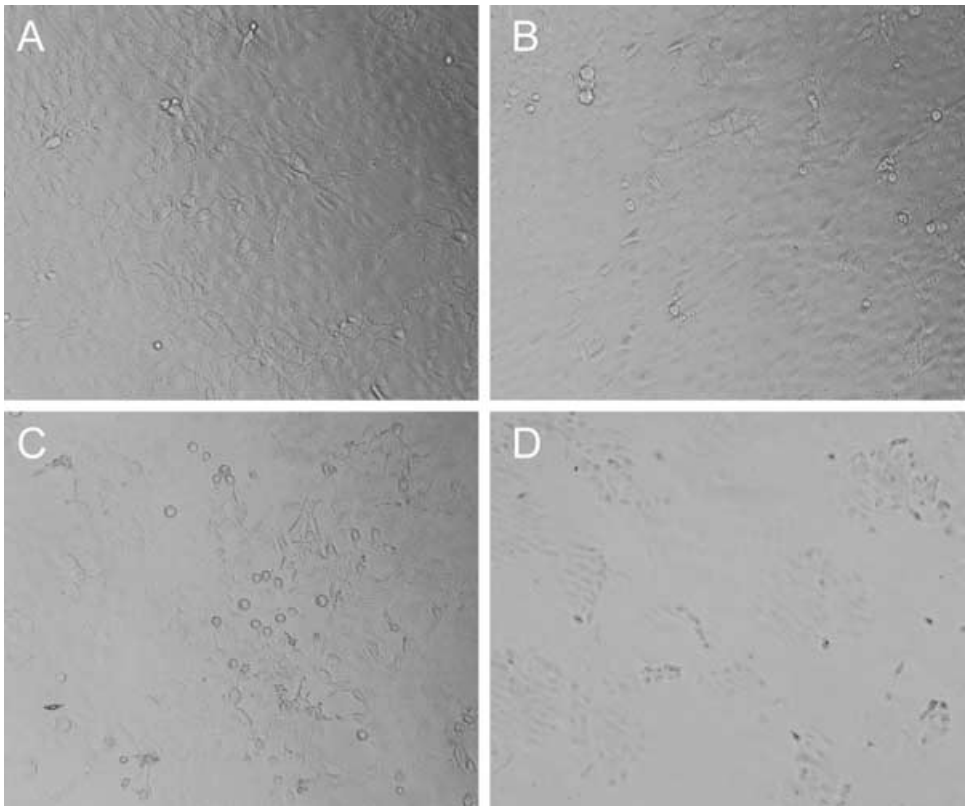

Figure 2. Phase-contrast microscopy of human corneal epithelial cells after 2 h exposure to three NSAIDs. (a) Control sample without NSAIDs, (b) cells treated with 20 mM salicylic acid, (c) cells treated with 12.5 mM sodium diclofenac and (d) cells treated 50 mM ketorolac. Cells in (c) and (d) show cytopathic effects.

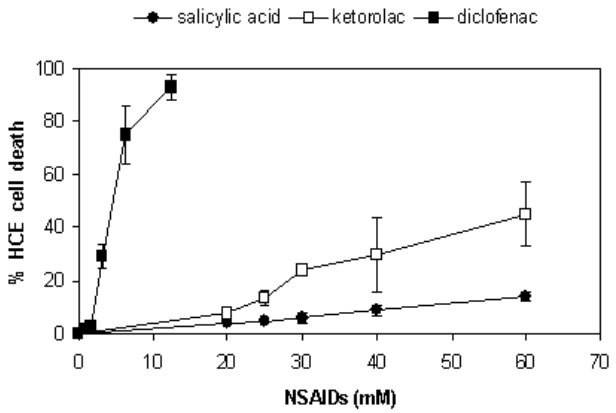

Figure 3. Comparison of *in vitro* cytotoxicity on human corneal epithelial cells (2 h) for salicylic acid, ketorolac and sodium diclofenac. Results are expressed as the mean percentage corneal cell death relative to controls that were incubated without NSAIDs.

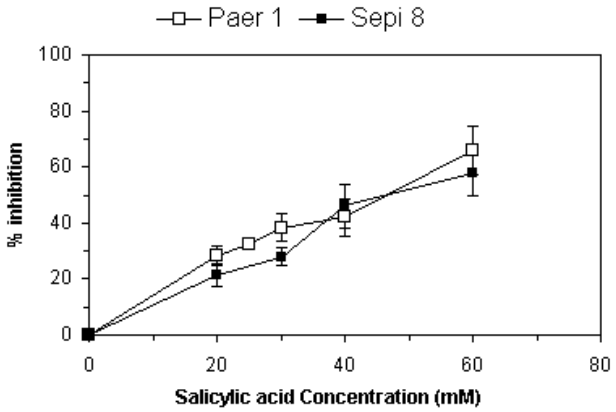

Figure 4. Mean inhibition of adhesion bacteria to human corneal epithelial (HCE) cells in the presence of salicylic acid. Results are expressed as the mean percentage reduction compared to HCE cells incubated with bacteria and without salicylic acid.

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