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Povidone iodine treatment is deleterious to human ocular surface conjunctival cells in culture

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ABSTRACT

Objective To determine the effect of povidone iodine (PI), an antiseptic commonly used prior to ocular surgery, on viability of mixed populations of conjunctival stratified squamous and goblet cells, purified conjunctival goblet cells and purified conjunctival stromal fibroblasts in primary culture.

Methods and analysis Mixed population of epithelial cells (stratified squamous and goblet cells), goblet cells and fibroblasts were grown in culture from pieces of human conjunctiva using either supplemented DMEM/F12 or RPMI. Cell type was evaluated by immunofluorescence microscopy. Cells were treated for 5 min with phosphate-buffered saline (PBS); 0.25%, 2.5%, 5% or 10% PI in PBS; or a positive control of 30% H_2O_2. Cell viability was determined using Alamar Blue fluorescence and a live/dead kit using calcein/AM and ethidium homodimer-1 (EH-1).

Results Mixed populations of epithelial cells, goblet cells and fibroblasts were characterised by immunofluorescence microscopy. As determined with Alamar Blue fluorescence, all concentrations of PI significantly decreased the number of cells from all three preparation types compared with PBS. As determined by calcein/EH-1 viability test, mixed populations of cells and fibroblasts were less sensitive to PI treatment than goblet cells. All concentrations of PI, except for 0.25% used with goblet cells, substantially increased the number of dead cells for all cell populations. The H_2O_2 control also significantly decreased the number and viability of all three types of cells in both tests.

Conclusion We conclude that PI, which is commonly used prior to ocular surgeries, is detrimental to human conjunctival stratified squamous cells, goblet cells and fibroblasts in culture.

Strengths and limitations of this study

What is already known about this subject?

- This is the first study to investigate the effects of the commonly used antiseptic povidone iodine (PI) on the viability of cells of the conjunctiva.

What are the new findings?

- Viability of cells grown in culture were used in this study, so the effects of PI on cells in an intact conjunctiva is unknown.
- PI significantly reduces the viability and kills conjunctival cells in culture including goblet cells, fibroblasts and mixed cultures of goblet cells and stratified squamous cells.

How might these results change the focus of research or clinical practice?

- Ophthalmologists should consider pursuing preoperative antisepsis techniques that decrease conjunctival toxicity.

INTRODUCTION

The conjunctiva is a highly vascularised tissue comprised of two layers, the epithelium with goblet cells and the stroma. Three major cells types predominate in the conjunctiva: stratified squamous cells and goblet cells in the epithelial layer, and fibroblasts in the stroma.[1] Undifferentiated epithelial cells are also present in the epithelium. The stratified squamous cells are non-keratinised cells containing the transmembrane mucins MUC4, 16 and 20, that are the constituents of the apically located glycocalyx. The glycocalyx serves to lubricate and protect the ocular surface, and trap bacteria.[2] The glycocalyx is attached to the apical side of the conjunctival epithelium that varies in thickness between 3 and 12 cell layers.[3] In the stratified areas of the epithelium, goblet cells are interspersed among stratified squamous cells and span the entire width of the epithelial layer in rats, but only the apical portion in humans.[4] Goblet cells synthesise and secrete the high molecular weight, gel-forming mucin MUC5AC, which like the transmembrane mucins serve to lubricate and protect the ocular surface, particularly from bacterial adherence.[5] In addition, goblet cells play an important role in the innate immune responses of the conjunctiva.[5–7] The conjunctival stroma is a loose stroma beneath the epithelial layer with fibroblasts present throughout. The fibroblasts secrete cell extracellular matrix proteins. An overgrowth of fibroblasts is

believed to be responsible for conjunctival giant papillae in vernal conjunctivitis.[8]

A healthy ocular surface contains few live bacteria, but a small population of commensal bacteria, namely the coagulase-negative *Staphylococcus epidermidis* can be present.[9] On the healthy ocular surface, these bacteria do not cause active infection due to the effects of multitude of antibacterial proteins secreted into the tears by the lacrimal gland, mucins synthesised and secreted by the cornea and conjunctiva and the blinking action of the lids.[1 10 11] Despite these defence mechanisms, ocular infections do occur and are often attributable to trauma, disease or contact lens wear. Pathogenic bacteria have been identified on the ocular surface of patients with dry eye[7] and infections from *S. aureus*, *Pseudomonas aeruginosa* or *S. pneumonia* can cause vision threatening bacterial keratitis and keratoconjunctivitis.[12] The most common source of endophthalmitis-causing bacteria is the conjunctival and lid flora.[13 14] Following surgical trauma, bacterial flora isolated from patients who developed endophthalmitis were identical to those isolated from the patient's own conjunctiva and eyelid.[15]

To minimise the risk of infections during surgery or ocular injections such as anti-vascular endothelial cell growth factor (VEGF) therapies, ophthalmologists apply the antiseptic povidone iodine (PI) to the conjunctival sac prior to surgery. PI concentrations from 1% to 10% for between 30 s and 10 min reduce the number of bacterial colonies cultured from conjunctiva[15–21] and the rate of endophthalmitis.[15 22] The American Academy of Ophthalmology recommends a concentration of 5% PI to be applied prior to cataract surgery but does not recommend a specific duration or volume. Likewise, the European Society of Cataract and Refractive Surgeons recommends application of between 5% and 10% PI for no longer than 3 min but does not provide guidance on volume.[23] There are, however, no published studies to date on the effect of PI application on the health of cells from the conjunctiva. The purpose of the present study was to determine in culture the effects of PI use on the viability of the three principal cell types present in the human conjunctiva.

MATERIALS AND METHODS

Materials

RPMI, DMEM/F12 media, phosphate-buffered saline (PBS), HEPES, sodium pyruvate, glutamine and penicillin/streptomycin were purchased from Lonza (Portsmouth, New Hampshire, USA). Fetal bovine serum was from Atlanta Biologicals (Flowering Branch, Georgia, USA). Human serum, human insulin, Alamar Blue, calcein AM/ethidium homodimer-1 (EH-1) live/dead assay kit, antibodies against cytokeratin 4 (CK4), cytokeratin 7 (CK7), anti-Ki-67 antibody and vimentin were provided by ThermoFisher (Waltham, Massachusetts, USA). Additional CK4 and CK7 antibodies were purchased from SantaCruz Biotechnology (Dallas, Texas, USA). PI solution (10%) was obtained from CVS (Woonsocket, Rhode Island, USA). Hydrogen peroxide, hydrocortisone, epidermal growth factor (EGF), fluorescein isothiocyanate (FITC)-conjugated lectin from Ulex europaeus agglutinin I (UEA) and lectin Bandeiraea Simplicifolia agglutinin conjugated to FITC were provided by Sigma-Aldrich (St Louis, Missouri, USA). MUC5AC antibody was purchased from Abcam (Cambridge, Massachusetts, USA). Secondary antibodies conjugated to Cy 2 or Cy 3 were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania).

Human conjunctival tissue

Deidentified human conjunctiva was obtained from the eye banks Saving Sight (Kansas City, Missouri) or Eversight (Ann Arbor, Michigan, USA). Tissue was placed in Optisol GS media within 18 hours after death. Tissue was received in Optisol and explants plated within 24 hours. This study was reviewed by the Massachusetts Eye and Ear Human Studies Committee and determined to be exempt and does not meet the definition of research with human subjects as defined by 45 CFR 46.102(d) and (f).

Patient involvement

Patients were not directly involved in the design of this study.

Types of conjunctival cell culture

Mixed population of conjunctival cells

Conjunctival epithelial cells were grown from explants according to García-Posadas *et al*.[24] In short, conjunctival explants were placed in six-well plates with 1 mL DMEM/F12 media supplemented with 5000 units/mL penicillin/streptomycin, 1 µg/mL insulin, 0.5 µg/mL hydrocortisone, 2 ng/mL EGF and 10% human serum. Cells were grown at 37°C and 5% CO_2 for 14 days with media changes every other day. On day 14, cells were passaged onto coverslips and allowed to grow overnight. First-passage cells were used in all experiments. Cell phenotype was determined by immunofluorescence microscopy using unique markers for each phenotype.[25]

Purified goblet Cells

Goblet cells from human conjunctiva were grown in culture and characterised as described previously.[26–34] The cell culture medium consisted of RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/mL penicillin–streptomycin, 87 µM non-essential amino acid (NEAA) solution, 870 µM sodium pyruvate and 8.7 mM HEPES. The tissue plug was removed after fibroblastic outgrowth was observed. Any fibroblasts were scraped away prior to passaging on day 14. First-passage goblet cells, allowed to grow overnight, were used in all experiments. The identity of cultured cells was periodically checked by immunofluorescence microscopy.

Purified fibroblasts

Fibroblasts were grown from human conjunctival explants in RPMI supplemented with 10% fetal bovine serum,

2 mM L-glutamine, 100 µg/mL penicillin–streptomycin, 87 µM NEAA solution, 870 µM sodium pyruvate and 8.7 mM HEPES. Goblet cells rather than fibroblasts were scraped away prior to passage on day 14, and first-passage fibroblasts were used in all experiments. Fibroblasts were identified morphologically and by immunofluorescence microscopy.

Immunofluorescence and immunohistochemical microscopy

First-passage cells grown on glass cover slips overnight were fixed in 10% methanol diluted in PBS (145 mM NaCl, 7.3 mM Na_2HPO_4 and 2.7 mM NaH_2PO_4, pH 7.2) for 10–15 min at 4°C. The coverslips were rinsed for 5 min in PBS and stored at 4°C in PBS until use. On removal from storage, coverslips were rinsed with PBS and placed in blocking buffer containing 1% bovine serum albumin, and 0.2% Triton X-100 in PBS for 30–45 min at room temperature. All primary antibodies were used at a concentration of 1:100 overnight at 4°C. Secondary antibodies and lectins were used at a dilution of 1:150 or 1:200 for 1 hour at room temperature. Negative control experiments included incubation with the isotype control antibody, where appropriate. Coverslips were mounted with mounting media containing 4′,6-diamino-2-phenylindole (DAPI). Cells were viewed by fluorescence microscopy (Eclipse E80i; Nikon, Tokyo, Japan) and micrographs were taken with a digital camera (Spot; Diagnostic Instruments, Inc, Sterling Heights, Michigan, USA and PCO Panda sCMOS 4mp Camera; Micro Video Instruments, Avon, Massachusetts, USA).

In order to determine the cell types present in mixed cultures, coverslips were fixed in methanol and antibodies against two cell markers for each cell type were used: UEA, a lectin that selectively binds to goblet cell secretory product, and cytokeratin (CK) 7, a cytoskeletal marker of goblet cells were used to identify goblet cells. Stratified squamous cells were identified using CK4, a cytoskeletal marker of stratified squamous cells and lectin from Bandeiraea Simplicifolia, that preferentially binds to stratified squamous cell secretory product.[20] Representative photos of each of the three preparations were taken and cells of a specific type were counted using ImageJ (National Institutes of Health, USA). The total number of cells was determined from DAPI staining. Only cells expressing both cell specific markers were counted as either goblet or stratified squamous cells.

Goblet cell cultures were periodically checked with UEA and CK7 and cultures containing <80% goblet cells were discarded.

The phenotype of fibroblast cultures was confirmed in a similar manner with cultures prepared and fixed as previously described. An antivimentin antibody was used to identify fibroblasts and was applied at 4°C overnight. Cultures that were not exclusively fibroblasts were discarded.

Cell viability methods

Alamar Blue

To determine cell number, first-passage cells were seeded into 96-well plates and allowed to attach overnight. The media was removed, and cells rinsed twice with PBS before addition of increasing concentrations of PI (0%–10%) or a positive control 30% H_2O_2 diluted in PBS for 5 min. The cells were rinsed again with PBS to remove PI and Alamar Blue assay reagent added for 30 min. Fluorescence was read on fluorescence ELISA reader (model FL600; Bio-Tek, Winooski, Vermont, USA) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Fluorescence obtained with PBS was considered maximum viability and was set to 1. Data were expressed as fold change compared with PBS.

In select preparations of goblet cell cultures, following the Alamar Blue assay, cells were washed with PBS, placed in fresh media and allowed to recover for 0–7 days. After recovery, cells were fixed and stained with an anti-Ki-67 antibody to mark proliferating cells.

Live/dead assay

To determine cell viability, first-passage cells were grown on coverslips overnight. Cells were washed twice with PBS before addition of increasing concentrations of PI (0%–10%) diluted in PBS, PBS alone or 30% H_2O_2 for 5 min. The cells were rinsed again with PBS to remove PI and the live/dead viability assay was performed. Calcein/AM, which is converted to fluorescent calcein (green fluorescence) in live cells, was used to count the number of surviving cells after each treatment while EH-1, which penetrates the damaged cell membranes of dead cells and produced red fluorescence in the nucleus, was used to identify dead cells. Cells were incubated in a solution containing 2% calcein/AM and 2% EH-1 in PBS for 30 min at room temperature. Cells were viewed by fluorescence microscopy (Eclipse E80i; Nikon, Tokyo, Japan) and micrographs were taken with a digital camera (Spot; Diagnostic Instruments, Inc, Sterling Heights, Michigan and PCO Panda sCMOS 4mp Camera; Micro Video Instruments, Avon Massachusetts). The number of live cells (green), dead cells (red) and total number of cells (sum of live and dead cells) were counted in a masked fashion using ImageJ. Cell mortality (%) was calculated from the number of dead cells and the number of total cells.

Statistical analysis

Results are presented as mean±SEM. A three-way analysis of variance was performed comparing treatment concentration, predominant cell population and treatment identity (ie, PBS, PI or H_2O_2) and was followed by a Tukey's multiple comparison test.

RESULTS

Culture of conjunctival epithelial cells

Three different populations of conjunctival cells were used in the present study, mixed stratified squamous and

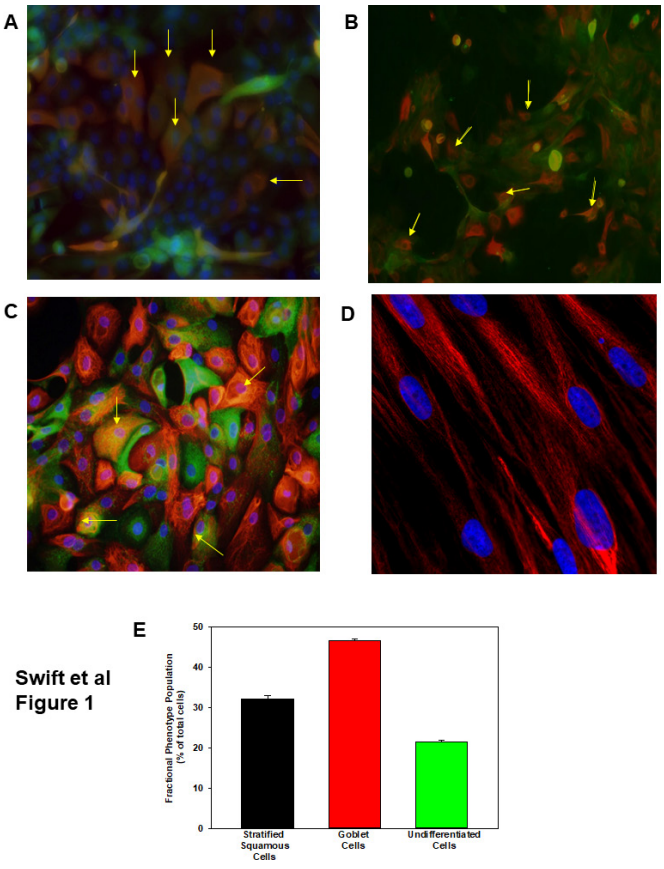

Figure 1 Identification of cell types in cultured conjunctiva. Mixed populations of cells containing populations of stratified squamous cells (A) and goblet cells (B) were grown on coverslips, fixed and labelled against unique markers for each cell type and used to determine the fractional cell population in mixed preparations. Stratified squamous cells were identified from mixed populations using antibodies against cytokeratin 4 (red), Bandeiraea Simplicifolia lectin (green) and DAPI (blue). Goblet cells were identified from mixed populations of cells (B) or purified goblet cell cultures (C) using antibodies against cytokeratin 7 (red), and Ulex Europeaus Agglutin 1 (green). Fibroblasts (D) were identified in purified cultures using antibodies against vimentin (red), and DAPI (blue). Representative stratified squamous and goblet cells are indicated with arrows in A and B. Cells were analysed from three individuals in A and B and four individuals in C and D. Cell types were counted and percentage of cell type is shown in E. Data are expressed as mean±SEM. DAPI, 4′,6-diamino-2-phenylindole.

goblet cells, purified goblet cells and fibroblasts. To identify goblet cells and stratified squamous cells in mixed culture preparations and determine the fractional population, stratified squamous cells (figure 1A) and goblet cells (figure 1B) from multiple preparations (n=3 for stratified squamous cells and n=4 for goblet cells) were identified by immunofluorescence microscopy. Only cells expressing both cell markers CK4 and the lectin Bandeiraea Simplicifolia for stratified squamous cells (figure 1A) or CK7 and UEA for goblet cells (figure 1B) were counted. In agreement with our published results[26–34] goblet cells grown in specialised media (RPMI) were almost entirely goblet cells as measured by CK7 and UEA1 staining (figure 1C). Finally, fibroblast cultures were grown and cells identified by immunofluorescence microscopy by antivimentin antibody (figure 1D). Cultures were overwhelmingly pure fibroblasts. An average of 32.1%±0.9% of total cells in the mixed-cell population were phenotypically mature stratified squamous cells and 46.5%±0.6% of total cells (DAPI positive) in each case were phenotypically mature goblet cells (figure 1E).The remaining cells (21.4%±0.3%) were not identified and were considered to be undifferentiated cells or fibroblasts.

Effect of PI on conjunctival cell viability as determined by Alamar Blue assay

The effects of different concentrations of PI on conjunctival cell viability were tested using the Alamar Blue assay. Cells of each type of preparation were incubated for 5 min with 0% (PBS, cell viability control), 0.25%, 2.5%, 5% and 10% PI. Cells were also incubated for 5 min with 30% H_2O_2 as a cell death control. A decrease in fluorescence intensity indicated substantial cellular damage. When mixed-cell cultures were treated with PI (figure 2A), the fluorescence intensity decreased from 1.00±0.00 with PBS to 0.05±0.03, 0.02±0.02, 0.03±0.02, 0.06±0.04 fold at 0.25%, 2.5%, 5% and 10% PI, respectively. H_2O_2 decreased fluorescence to 0.04±0.04 fold. All concentrations of PI damaged the stratified squamous and goblet cells.

When purified goblet cells were treated with PI, all concentrations of PI also significantly decreased fluorescence intensity (figure 2B). Fluorescence intensity decreased from 1.00±0.00 (PBS) to 0.05±0.04, 0.06±0.05, 0.06±0.05 and 0.01±0.12 at 0.25%, 2.5%, 5% and 10% PI, respectively. In these experiments, H_2O_2 decreased fluorescence intensity to 0.06±0.05 fold. All concentrations of PI damaged the goblet cells.

Additionally, select preparations of purified goblet cells (n=3) were washed with PBS following the Alamar Blue assay, placed in fresh media and allowed to recover for 0, 1, 3 or 7 days before staining with anti-Ki-67 antibody to detect proliferation. This staining did not reveal any proliferating cells in any of the treatment groups. Proliferating cells were only observed 1, 3 and 7 days (26.6% of total cells as assessed by DAPI, 16.6% of total and 27.2% of total goblet cells, respectively) following treatment with PBS alone (data not shown). These results suggest that the purified goblet cells did not functionally recover after the PI treatment.

Use of cultured conjunctival fibroblasts (n=3) demonstrated that all concentrations of PI significantly decreased fluorescence compared with PBS control (figure 2C). Fluorescence intensity decreased from 1.00±0.00 (PBS) to 0.03±0.01, 0.03±0.006, 0.007±0.014 and 0.02±0.0006 at 0.25%, 2.5%, 5% and 10% PI, respectively. In these experiments, H_2O_2 decreased fluorescence intensity to 0.02±0.0007 fold. Much like the mixed cultures and goblet cells, the fibroblasts were also damaged with PI treatment.

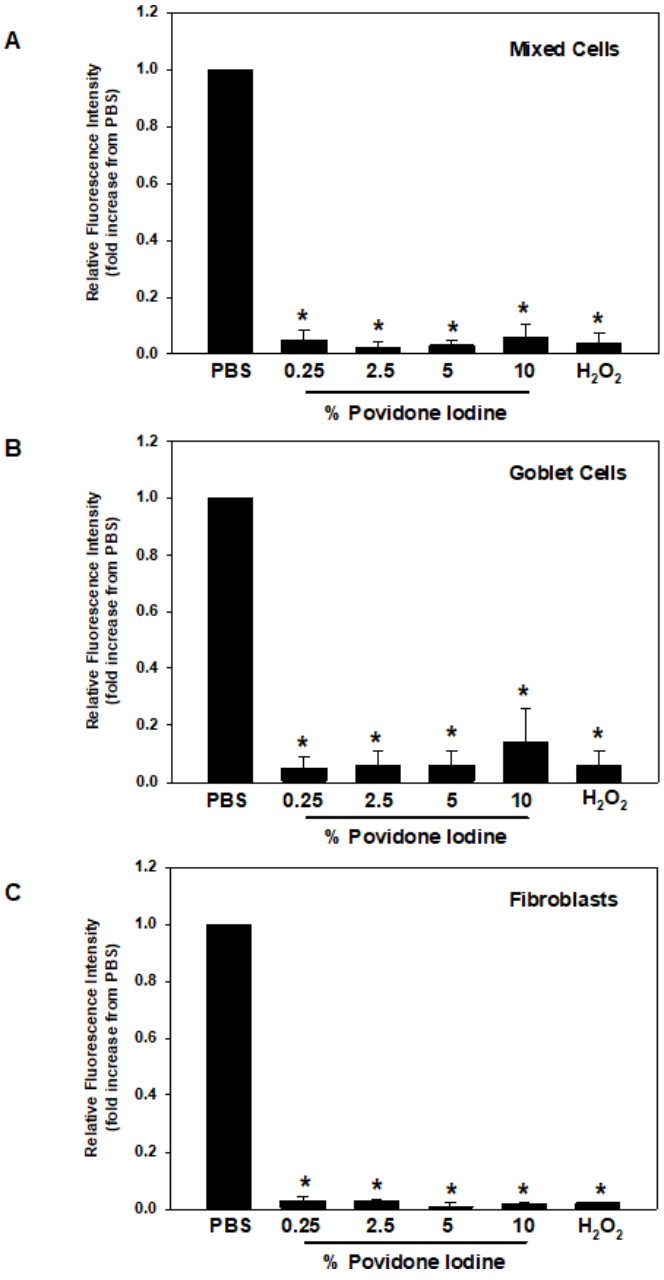

Figure 2 Viability of cells after treatment with PI. Cultured mixed cells (A), purified goblet cells (B) and purified fibroblasts (C) were treated with PBS, 0.25%–10% PI or 30% H_2O_2 for 5 min. Relative fluorescence intensity of Alamar Blue is expressed as fold change when intensity of PBS was set to 1. Data are mean±SEM from three individuals. Asterisk (*) indicates significance from PBS. PBS, phosphate-buffered saline; PI, povidone iodine.

These results demonstrate that all three types of cells responded similarly to PI application independent of the concentration used. They also show that a short PI treatment damages all these cells and the goblet cells, the only cell type tested for recovery, do not regain their proliferative function.

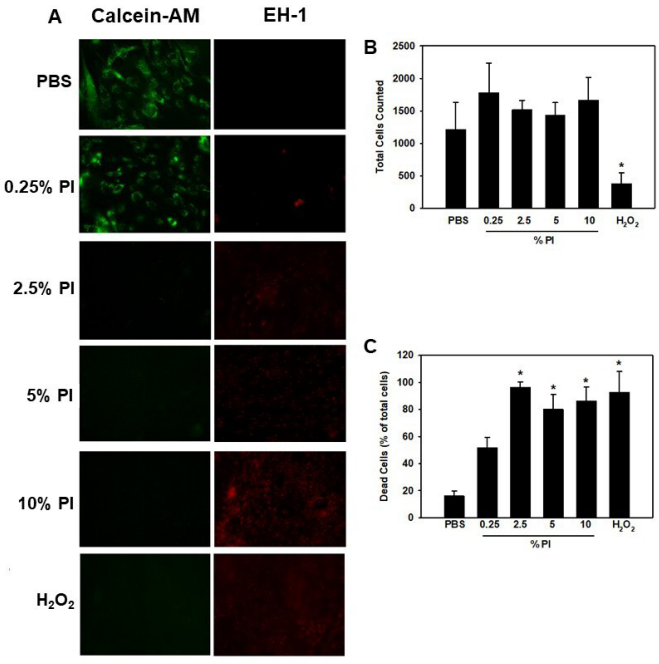

Figure 3 Viability of cultured mixed epithelial cells after treatment with PI. Cultured mixed epithelial cells were treated with PBS, 0.25%–10% PI or 30% H_2O_2 for 5 min. Calcein staining (green) indicates live cells, and EH-1 (red) indicates dead cells (A). The total number of cells counted for each group was recorded and analysed (B), and the percentage of dead cells as a per cent of total cells is indicated on the y-axis (C). Data are mean±SEM from three individuals. Asterisk (*) indicates significance from PBS. EH-1, ethidium homodimer-1; PBS, phosphate-buffered saline; PI, povidone iodine.

Effect of PI on conjunctival cell viability as determined by live/Ddead assay

A second viability test utilising calcein/AM and EH-1 was performed to confirm the effect of PI on different conjunctival cell types. Cultured cells were treated with either 0% (PBS control), 0.25%, 2.5%, 5% and 10% PI diluted in PBS or 30% H_2O_2 for 5 min. The total number of live cells as labelled green by calcein/AM, and number of dead cells as labelled red by EH-1 were counted and the total number of cells present were determined by counting the number of DAPI-positive cells. Live cells were present after treatment with PBS, viability control or 0.25% PI (figure 3A, n=3). Treating mixed cultures with concentrations of PI of 2.5% and above as well as 30% H_2O_2 killed more cells after 5 min than the PBS control. The mean number of cells counted was not different between PBS and each concentration of PI (figure 3B). Treatment with H_2O_2 significantly decreased the number of cells present, indicating cell death and loss of cell attachment to the tissue culture plate. When EH-1-stained dead cells were counted in the mixed cultures treated with 0.25% PI, the number of dead cells was not significantly increased compared with the number of dead cells present in the PBS control (51.7%±7.9% and 16.1%±3.8%, respectively, figure 3C). Following treatment with 2.5%, 5%

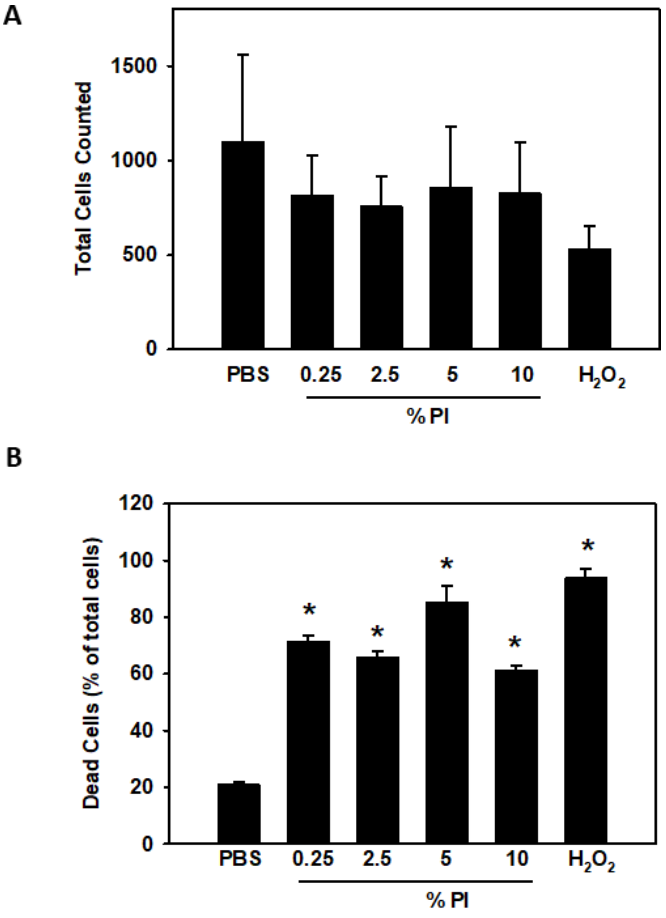

Figure 4 Viability of cultured goblet cells after treatment with PI. Cultured goblet cells were treated with PBS, 0.25%–10% PI or 30% H_2O_2 for 5 min. The total number of cells counted for each group was recorded and analysed (A), and the percentage of dead cells as a per cent of total cells is indicated on the y-axis (B). Data are mean±SEM from three individuals. Asterisk (*) indicates significance from PBS. PBS, phosphate-buffered saline; PI, povidone iodine.

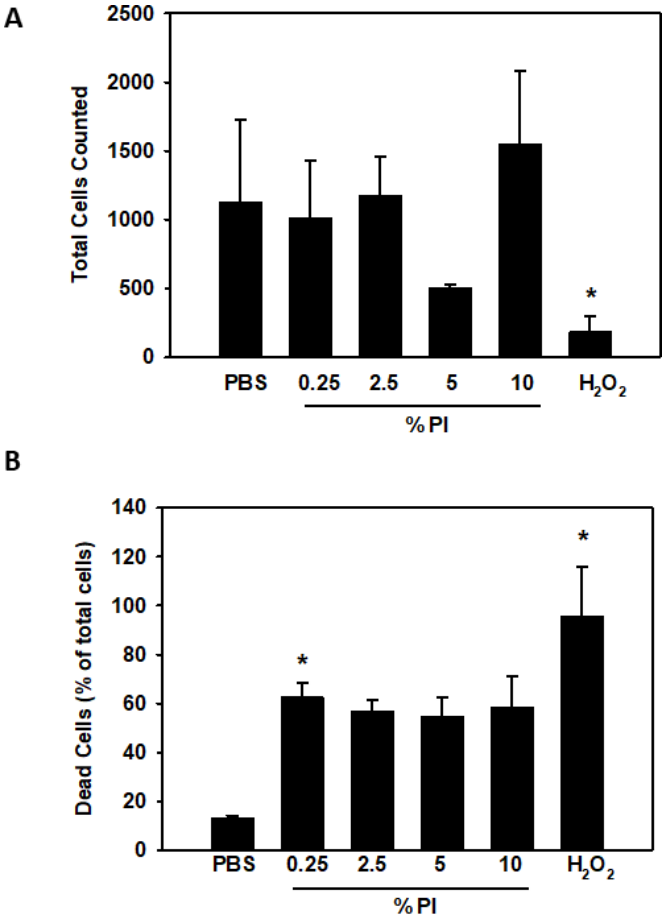

Figure 5 Viability of cultured fibroblasts after treatment with PI. Cultured fibroblasts were treated with PBS, 0.25%–10% PI, or 30% H_2O_2 for 5 min. The total number of cells counted for each group was recorded and analysed (A), and the percentage of dead cells as a per cent of total cells is indicated on the y-axis (B). Data are mean±SEM from three individuals. Asterisk (*) indicates significance from PBS. PBS, phosphate-buffered saline; PI, povidone iodine.

and 10% PI the number of dead cells was 96.4%±3.85%, 80.1%±11.0% and 86.3%±10.0%, respectively, each significantly increased over the number of dead cells in PBS. With the cell death control 92.7%±15.5% of cells died after treatment with 30% H_2O_2.

When purified goblet cells were treated with PBS, any concentration of PI or H_2O_2, the number of cells present was not altered (figure 4A, n=7). When compared with the PBS control, all concentrations of PI tested as well as 30% H_2O_2 killed significantly more cells in goblet cell preparations (figure 4B). The number of dead cells was measured as 21.0%±0.957% of total cells in PBS. Treatment with 0.25%, 2.5%, 5% and 10% PI and 30% H_2O_2 killed 71.4%±1.91%, 65.8%±2.21%, 85.26%±5.69%, 61.36%±1.69% and 93.83%±3.28% of total cells, respectively.

When purified fibroblasts were treated with PBS or any concentration of PI, the number of cells present was not altered (figure 5A, n=3). Treatment with H_2O_2 decreased the number of cells present compared with PBS. When compared with the number of dead cells treated with PBS, purified fibroblast cultures were only killed in statistically increased numbers by treatment with 0.25% PI and 30% H_2O_2 (62.6%±6.03% and 95.9%±19.9% of total cells, figure 5B). Treatment with 2.5%, 5% and 10% PI did not kill (57.0%±4.48%, 54.8%±7.65% and 58.5%±12.4%) a significantly greater number of cells than the PBS control (13.1%±1.10%).

Each type of conjunctival cell population used was killed by several concentrations of PI. All three types of conjunctival cells whether in mixed populations or purified were sensitive to PI treatment.

DISCUSSION

Using two different methods to measure cell viability, we demonstrated that a short treatment with the antiseptic PI, commonly used prior to ocular surgeries, can irreversibly damage and kill the three main cell types of the conjunctiva: stratified squamous cells, goblet cells and fibroblasts. The effect of PI was comparable to that of

the positive control of 30% H_2O_2 known to damage cells. One or more concentrations of PI and 30% H_2O_2 significantly decreased cell viability according to both tests, and all concentrations of PI significantly reduced viability across all preparation types according to the Alamar Blue assay. Fibroblasts, however, were significantly killed by fewer concentrations of PI according to the calcein/AM and EH-1 test suggesting a potential fibroblastic resistance to PI. A clinical investigation into a link between PI treatment and postsurgery vernal conjunctivitis caused by an increase in conjunctival fibroblast growth may be warranted. Our results suggest that care must be taken of the conjunctiva after surgical PI treatment to allow it to heal and repopulate the different cell types. The time needed for the cell numbers and proportions to return to pretreatment levels is unknown and likely varies from type to type and individual to individual. This may be particularly deleterious to patients undergoing repeated procedures.

Low concentrations of PI (0.25%) administered repeatedly throughout cataract surgery does significantly reduce ocular bacterial loads without significantly changing corneal endothelial cell densities.[22] The current study demonstrates that a single 0.25% PI application for 5 min does not kill a statistically significant number of fibroblasts in vitro but does significantly affect the viability of both goblet cells and mixed populations of epithelial cells suggesting that while the corneal endothelium probably remains intact, the conjunctival epithelium is likely damaged. A single application of 0.05% PI for only 30 s can reduce ocular bacterial load while minimising damage to the ocular surface in humans[35] and concentrations of 0.5% and 1% PI applied only once demonstrate significantly less corneal damage than higher PI concentrations in rabbits as measured by fluorescein stain. The effect of repeated low duration, low concentration PI was not evaluated in this study and may provide an alternative to single-application, high-concentration applications that last for minutes.

It should be noted that repeated applications of 5% PI have been demonstrated to be deleterious to the ocular surface as measured by the Schein dry eye questionnaire and measurements of tear osmolarity.[36] Indeed, even a single application of 5% PI can significantly decrease the integrity of the corneal epithelium as measured by a sodium fluorescein stain and increase subjective vision complaints as measured by the Schien dry eye test.[37]

In the present study mixed cultures were composed of on average 46.5%±0.57% goblet cells, 32.1%±0.30% stratified squamous cells, while the remaining cells are believed to be a combination of fibroblasts and undifferentiated cells. The conjunctiva is generally considered to have more stratified squamous cells than goblet cells. The relative percentage of these cells, however, differs depending on the area of the conjunctiva examined. There is a higher stratified squamous cell to goblet cell ratio in the bulbar conjunctiva than in the forniceal conjunctiva which contains more goblet cells and undifferentiated progenitor cells.[38 39] Undifferentiated cells form a small population in the conjunctiva with these cells distributed randomly throughout the conjunctiva. The relative population of goblet cells used herein can thus be used to mimic the conjunctival epithelium.

The two types of viability assays used measure different cellular properties. The Alamar Blue assay measures cell metabolic activity and is often used to determine cell number. The live–dead assay in contrast directly measures cell death. When cell number is compared by the two assays, the three different types of cultures were similarly affected by all concentrations of PI. When cell death was measured, the mixed cultures of epithelial cells were similarly resistant to PI treatment when compared with the purified goblet cell and fibroblast cultures. As the mixed culture used in the present study more closely mimics the ocular surface and contains several cell types, the mixture of cells may have different types of cell–cell interactions than either of the purified cultures. The different cell–cell interactions in the mixed cultures do not appear to provide protection to PI treatment.

The use of PI to prevent infections is well established. Several studies have demonstrated the efficacy of PI to reduce ocular infections. In healthy newborns, 1.25% PI was as effective as 2.5% in reducing the number of colony-forming units[40] and 2.5% PI decreased adenoviral conjunctivitis.[41] Carrim *et al* demonstrated that a 3 min treatment with PI decreased the number of positive cultures in patients[42] and a 10% treatment reduced the number of colony-forming units in patients undergoing cataract surgery.[43] The optimum time and concentration of PI to prevent infections is unknown, however, 5% PI decreases bacterial load significantly more than 1% PI in vivo, while in vitro studies indicate the opposite is true with lower PI concentrations appearing to decrease bacterial load more than higher concentrations.[44 45] The finding that even 0.25% PI damages and kills conjunctival cells in the present study highlights the need to determine a minimal, but effective amount and time of contact of PI with the ocular surface to prevent infection with the least amount of cell damage. The negative effect of PI on the ocular surface should be included in that determination. Shimada *et al* demonstrated that repeated irrigation with 0.025% PI is effective at reducing the bacterial infections after cataract surgery.[46] It is possible that this technique, which is the only technique known to sterilise the anterior chamber at the conclusion of cataract surgery, may be less toxic to the conjunctiva, while still protecting the eye from bacterial infection.

Conjunctival goblet cells secrete the mucin MUC5AC into the tear film.[5] Stratified squamous cells express the mucins MUC4, 16 and 20 that are the constituents of the apically located glycocalyx which lubricates and protects the ocular surface.[2] As PI at all concentrations tested in this study is detrimental to the stratified squamous and goblet cells, it is possible that preoperative treatment with PI could lead to the development of dry eye. Indeed, dry eye syndrome post cataract surgery or post LASIK has

not received sufficient attention. It is possible that the surgery prep contributes significantly to this condition.

In patients with an iodine allergy, an alternative preoperative antiseptic, chlorhexidine, is available. Like PI, chlorhexidine is used over a wide range of concentrations (0.05%–4%) and has been demonstrated to significantly reduce ocular surface bacterial load.[47] While very few patients display an allergy to chlorhexidine, ocular topical use is specifically contraindicated by the manufacturer (https://www.pfizer.com.au/products/chlorhexidine-irrigation-solution). Chlorhexidine concentrations above 2% are known to be toxic to the corneal epithelium and conjunctiva of rabbits.[48] However, in humans, a much lower concentration of chlorhexidine (0.05%) has been successfully used as a single-use ocular antiseptic prior to vitrectomy, although in a limited sample.[49] The demonstrated toxicity of PI and lack of a demonstrably reliable alternative highlights the importance of including epithelial toxicity as a criterion in the evaluation of ocular antiseptics.

We conclude that the commonly used antiseptic PI significantly reduces the viability and kills conjunctival cells in culture including goblet cells, fibroblasts and mixed cultures of goblet cells and stratified squamous cells. Our findings suggest that ophthalmologists should consider pursuing preoperative antisepsis techniques that decrease conjunctival toxicity. This would not only be less toxic but would be more effective at decreasing bacteria counts, thus lessening the risk of endophthalmitis.

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