CYTOTOXIC AND INFLAMMATORY EFFECTS OF CONTACT LENS MULTIPURPOSE SOLUTIONS ON HUMAN CORNEAL EPITHELIAL CELLS

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Multipurpose solutions (MPSs) are the leading method for cleaning and disinfecting soft contact lenses (CLs). During recent years, numerous clinical studies have evaluated the MPS damage to the ocular surface. This study examined the cytotoxic and the inflammatory effects of MPSs and hydrogen peroxide disinfection system (H202) compared to appropriate controls on human corneal epithelial (HCE) cells. Primary cultured HCE cells were exposed to eight different commercially available MPS products (MPS A, ReNu MultiPlus®; MPS B, Opti Free® EverMoist; MPS C, Solo-care Aqua®; MPS-D, Complete®; MPS-E, Unica Sensitive®; MPS-F, Options Multi®; MPS-G, Biotrue®; MPS-H, COMPLETE® RevitaLens). Morphological changes and cytotoxic effects were examined with FITC-Annexin V/ PI and MTT assays. The protein contents of the inflammatory cytokines interleukin (IL)-1β, TNF-α, IL-6 and IL-8 were examined by multiplex fluorescent bead immunoassay (FBI), and the mRNA expression was examined by real time PCR. Lipopolysaccharide (LPS) with 500 ng/ml CD14 and 500 ng/ml LBP (LPS complex), polyinosinic: polycytidylic acid (Poly I:C) and un-neutralized H202 served as positive controls, respectively. Phosphate-buffered saline (PBS) was added as a negative control. The study demonstrated that most of the MPSs induced varying degrees of cytotoxicity to HCE cells, and increased production of pro-inflammatory cytokines compared to the negative control. In addition, several MPS increased the mRNA level of inhibitory factor-κBα (I-κBα). Among the various MPSs, MPS-H induced the highest protein contents of the pro-inflammatory cytokines (14.37±2.2-fold for TNF-α, 41.39±2.5-fold for IL-1β and 5.24±0.6-fold for IL-6) compared to the negative control (p<0.05). In contrast, no significant differences were noted between the neutralized H202 and the negative control. We conclude that most of the currently used MPSs induce significant damage and inflammatory response in corneal epithelial cells. MPS-induced inflammation was mediated through NF-κB signal transduction. This study demonstrates for the first time inflammatory responses at the molecular level in primary HCE cells following exposure to a large series of commercially available and commonly used MPSs. These findings strongly suggest that certain MPSs may be partially involved in the pathogenesis of contact lens intolerance. Therefore, we recommended that practitioners advise patients as to the preferable disinfecting contact lens solutions, and to consider using the hydrogen peroxide disinfection systems instead.

Disinfection of contact lenses is one of the   crucial methods of preventing dangerous infections

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of the ocular surface. There are two main types of products currently available for disinfecting contact lenses (CLs) and eliminating pathogens; hydrogen peroxide CL disinfection systems (H2O2) and CL multipurpose solutions (MPSs).

In the past, H2O2 were used exclusively for the disinfection of contact lenses, by oxidizing the microbial pathogens on the lens surface. H202 were used for CL disinfection due to its broad antimicrobial activity (1-3).

H202 are normally formulated with a 3% hydrogen peroxide concentration. Because hydrogen peroxide is toxic to the cornea, following disinfection, hydrogen peroxide must be neutralized before inserting the CL to the eye. The neutralization can be divided into two main types: the one-step catalytic systems (platinum disc or a soluble catalase tablet); and the two-step system, which includes the addition of sodium pyruvate or catalase following the disinfection period (1, 3). Both processes decompose hydrogen peroxide into water and oxygen, thus catalytically turning the solutions preservative-free and providing additional advantage over the MPSs.

Currently multipurpose solutions (MPSs) are the leading method for cleaning and disinfecting soft CLs (4), estimated to be used by almost 90% of all soft CL users (5, 6). The MPSs are designed as a one-bottle system and contain a complex combination of ingredients. These solutions aim to provide a convenient method of disinfecting and rinsing contact lenses, with the use of only one step and in one bottle (4).

Although the MPSs are widely used by silicone hydrogel CL consumers (5), and serve as a useful technique for cleaning CL, they do represent a foreign solution to the eye, and are capable of producing clinically significant signs and symptoms of ocular surface inflammation by interfering with the normal corneal epithelial integrity (7).

Exposure of the ocular surface to the solutions is relatively brief and depends on the viscosity of the solution (8-9). However, despite this brief exposure, significant damage to the corneal epithelium can occur, as manifested by the asymptomatic corneal fluorescein staining and corneal infiltrative events, which have been commonly found in patients using MPSs (10-11).

Studies have been carried out comparing the level of corneal epithelial fluorescein staining with different MPSs in combination with different types of contact lens materials. For example, it was found that MPSs containing Polyhexamethylene Biguanide (PHMB) result in higher levels of asymptomatic corneal epithelial staining when used with silicone hydrogel and hydrogel contact lenses (12). The greater the toxic effect of the MPSs, the more likely the patient is to discontinue wearing contact lenses due to ocular discomfort. Studies have also shown that solutions preserved with Polyquaternium-1 (PQ-1) cause less staining than PHMB (13). Some MPSs and silicone hydrogel CL combinations may cause excessive corneal staining two hours after lens insertion (11).

The specific inflammatory responses at the molecular level and their correlation to the cytotoxic effects of the various MPSs in HCE cells have never been reported previously. Hence, in this study we investigated the inflammatory responses of a large series of commercially available and widely used MPSs, and compared their effects on the expression of the major inflammatory mediators and evaluated their cytotoxicity in HCE cells *in vitro*.

MATERIALS AND METHODS

This study followed the tenets of the Declaration of Helsinki. The Hadassah Medical Center Institutional Review Board (IRB) approval was obtained for this study (IRB protocol number: TVE-MPS-IV-01), and all of the study procedures were carried out in accordance with the IRB guidelines.

Culture of human corneal epithelial (HCE) Cells

Human HCE cells were cultured from human corneoscleral rim explants, taken from four different human donors, provided by the Department of Ophthalmology at the Hadassah Medical Center, using a previously described method (14).

Corneoscleral rim explants were placed into culture dishes with supplemented hormonal epithelial medium (SHEM). Cultures were incubated at 37°C under 95% humidity and 5% CO_2. The culture medium was replaced every other day. Cultures were kept for 10 to 14 days until a 90% confluence was observed. At this time, cells were passaged and seeded onto 6-well plates at a density of 2.0×10^5 cells/well. Cells were observed by phase-contrast microscopy to ensure uniformity of morphology. The purity of HCE cultures was confirmed by staining

for cytokeratin- 19 with the indirect immunoperoxidase procedure with monoclonal antibody to human cytokeratin-19 (Abcam, Cambridge, UK). Second generation cells were used in all experiments.

Experimental design

Eight commercially available and widely used MPSs (marked as MPS A through H, Table I), containing different components, were chosen for this study. The various MPSs were applied on HCE cells in 30% v/v and 50% v/v concentrations (diluted in culture medium) for 2-15 hours as previously described (7, 15).

HCE cells were counted and seeded 24 to 48 h before the experiment at density of 2.0×10^5/wells in 2 ml of medium. Culture medium was refreshed after the cells achieved adhesion, and exchanged with 2 ml of serum-free medium (SFM) before the experiments.

The morphological changes of HCE cells were observed under an inverted light microscope after the HCE cells achieved confluence. HCE cells were exposed to each

of the different MPSs or to neutralized hydrogen peroxide lens disinfection system (N-H2O2) in a concentration of 50% v/v for 2 h. Un-neutralized hydrogen peroxide lens disinfection system (UN-H2O2; 10% v/v) served as a positive control and PBS was added as a negative control.

In order to evaluate the cytotoxic effects, HCE cells were incubated with each one of the eight different MPSs (MPS A to H, Table I) at concentrations of either 30% v/v or 50% v/v for 12 h. In addition, 10% v/v UN-H2O2 or PBS were added as the positive and negative controls, respectively. HCE cell viability was assessed with FITC-Annexin V/PI assay and the MTT assay.

To evaluate the inflammatory effects at the molecular level, the MPSs were incubated with HCE cells under the same conditions as mentioned above. In addition, cells were stimulated either with lipopolysaccharide (LPS) at a dose of 1000 ng/ml with 500 ng/ml CD14 and 500 ng/ml LBP (LPS complex) or with polyriboinosinic:polyribocyt idylic acid (Poly I:C) at a dose of 25 μg/ml.

LPS complex and Poly I:C served as positive controls

Table I. *The components of MPSs.*

	Solutions trade name	Manufacturer	Antimicrobial agent	Other components
MPS-A	ReNu MultiPlus®	Bausch + Lomb	PHMB 5^{-5}%	Boric Acid, Sodium Borate ,0.9%NaCl, Edetate disodium, Asorbic Acid, Hydroxyalkyl Phosphonate, Poloxamine,
MPS-B	Opti Free EverMoist®	Alcon	PQ-1 1^{-3}% MAPDA 6^{-4}%	sodium citrate, 0.9%NaCl, Boric Acid, aminomethylpropanol, Sorbitol, disodium EDTA, Tetronic 1304, Polyoxyethylene - polyoxybutylene
MPS-C	Solo-care Aqua®	CIBA Vision	PHMB 1×10^{-4}%	Tromethamine, Sodium Phosphate, 0.9% NaCl, Edetate disodium, Poloxamer 407, Dexpanthenol, Sorbitol
MPS-D	Complete®	Abbott Medical Optics (AMO)	PHMB 1×10^{-4}%	phosphate, Edetate Disodium , 0.9%NaCl, Potassium Chloride, Poloxamer 237
MPS-E	Unica Sensitive®	Avizor	PHMB 1×10^{-4}%	0.9%NaCl, EDTA, Poloxamer, Sodium Hyaluronate
MPS-F	Options Multi®	Cooper vision	PHMB 1×10^{-4}%	disodiumhydrogen phosphate dehydrate, 0.9%NaCl, Coopase 1 & 2, Disodium Edetate Dihydrate
MPS-G	Biotrue®	Bausch + Lomb	PQ-1 1×10^{-4}% PHMB 1.3×10^{-4} %	Hyaluronan, Sulfobetaine, Poloxamine, Edetate Disodium, Sodium Borate, 0.9%NaCl, Poloxamine
MPS-H	Complete RevitaLens®	Abbott Medical Optics (AMO)	PQ-1 3^{-4}% alexidine dihydrochloride 1.6×10^{-4}%	Boric Acid, Sodium Borate Decahydrate, 0.9% NaCl, Tetronic 904, EDTA, Trisodium Citrate Dehydrate
N- H2O2	EasySept®	Bausch + Lomb	Hydrogen Peroxide 3%	Phosphonic Acid, Sodium Chloride, Phosphate
PBS				
UN- H2O2	Aosept®	Ciba vision	Hydrogen Peroxide 3%	Phosphate, 0.9%NaCl, Poloxamer, Phosphonic Acid

PHMB: polyhexamethylenbiguanide HCL; MAPDA: myristamidopropyl dimethylamine; PQ-1 polyquaternium-1; BA: boric acid; EDTA: ethylenediaminetetraacetic acid; NaCl: Sodium chloride; N-H2O2: Neutralized hydrogen peroxide.

for this part of the study, while PBS was added as the negative control. Either MPSs or the controls were applied after the HCE cells achieved complete confluence in the 24-well plates. Exposure of cells to the various MPSs or controls lasted 15 h for protein content measurements, and 12 h for mRNA expression level measurements, of several inflammatory cytokines. For the measurement of the inflammatory mediators protein contents, culture supernatants were collected after 15 h, aliquoted and stored at -80ºC. For mRNA expression levels, total RNA was extracted from the cells after 12 h and stored at -80ºC until thawed for measurement. The effects of cytokine release were examined at the protein level by multiplex fluorescent bead immunoassay (FBI) and at the gene expression level by real-time polymerase chain reaction (RT-PCR).

All experiments were performed on HCE cells taken from four different human cornea donors.

Microscopic observation of HCE cells

The morphological changes of HCE cells were observed under an inverted light microscope after the HCE cells achieved confluence. HCE cells were exposed to each of the MPSs or to N-H2O2 at concentrations of 30% and 50% v/v for 2 h. UN-H2O2 (10 % v/v) served as a positive control and PBS was added as a negative control.

At the end of incubation the HCE cell morphology was observed and recorded by an inverted microscope model CKX41 (Olympus Optical, Tokyo, Japan), with mounted CCD camera model DP71 (Olympus Optical, Tokyo, Japan). Immediately after the incubation period, images were captured using the Olympus DP controller image software version 1.2.1.108 (Olympus Optical, Tokyo, Japan).

The images were analyzed and quantified by Image-Pro Plus version 7.0 software (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA). The amount of cellular damage was calculated as the ratio of abnormal cellular area to total field area for each of the fields examined. Images were obtained from 8 representative fields, each taken from four independent experiments, which were performed on cell cultures from four different human corneo-scleral rims.

HCE cells viability profile

The apoptosis assay was performed with FITC-Annexin V/PI assay (MBL, Nagoya, Japan) and analyzed by flow cytometry. Detached HCE cells (5.0×10^5) were analyzed after 12-h incubation with the MPSs and N-H2O2 at concentrations of 30% and 50% v/v. UN- H2O2 (10% v/v) served as a positive control and PBS was added as a negative control. At the end of the incubation,

adherent and floating cells were collected and resuspended in Annexin binding buffer (MBL, Nagoya, Japan).

One µg/ml of Annexin V-FITC and 1 µg/ml of propidium iodide (PI) were added and mixed with the cell suspension, and the mixture was incubated in the dark for 5 min at ambient temperature. Without washing, the cells were placed in 500 µl of Annexin V binding buffer and kept on ice, and within 10 min they were evaluated using a Coulter FC-500 flow cytometer (Beckman-Coulter, Fullerton, CA, USA). Flow cytometry data were analyzed using CXP Analysis 2.0 software (Beckman-Coulter, Miami, FL, USA).

Mitochondrial enzyme activity of HCE cells

HCE cell mitochondrial enzyme activity was assessed using the colorimetric MTT assay based on the tetrazolium salt MTT (Sigma-Aldrich, St. Louis, Missouri, USA), as previously described (16). Briefly, 200 µl suspension of HCE cells per well were seeded onto 96-well plates (Nunclon Surface, NUNC, Roskilde, Denmark) at a density of 10,000 cells/ml. HCE cells were kept in the culture medium for 24-36 h to ensure cell recovery and adherence. Next, the HCE cells were treated for 12 h with each of the various MPSs with concentrations of 30% or 50%v/v. Equal volumes of PBS or UN-H2O2 (10% v/v) served as the negative and positive controls, respectively. After the incubation time, a fresh 20 µl of MTT solution was applied to each of the wells and the cells were incubated for additional 3 h in 5% CO2 and at 37°C. The crystals were then dissolved with 220 µl of MTT solubilization solution (Sigma-Aldrich, St. Louis, Missouri, USA) and gently shaken for 5 min. The plates were rapidly read using a BMG Fluorostar Galaxy 403 plate reader (BMG Lab Technologies, Inc. Offenburg, Germany).

Protein contents of inflammatory cytokines - multiplex fluorescent bead immunoassay (FBI)

The cytokine protein content was measured using a multiplex fluorescent bead immunoassay (Cytometric bead array, CBA, Human Inflammatory Cytokines Kit, BD Biosciences, San Jose, CA, USA). This kit allows for simultaneous measurement of protein levels of Interleukin (IL)-1β (IL-1ß), IL-6, IL-8, IL-10, Tumor Necrosis Factor Alpha (TNF-α), and IL-12 (p70) in a single sample. The test was performed and analyzed according to the manufacturer's instructions and was performed as previously described (17). Briefly, 50 µl of six premixed capture bead populations (coated with capturing antibodies specific for different cytokines) were mixed with 50 µl of the provided standards. Eight point standard curves ranging from 20 to 5000pg/ml were obtained by serial dilutions of the reconstituted lyophilized standards.

Fifty microliters of capture bead populations were also mixed with culture supernatant samples, allowing for the different cytokines in the samples to be captured by their analogous beads. Afterwards, the cytokine capture beads were mixed with 50 µl of phycoerythrin-conjugated detection antibodies to form sandwich complexes. These sandwich complexes were successively incubated in the dark for 3 h at room temperature. After incubation the mixture was washed, centrifuged (at 200x g for 5 min) and the pellet was resuspended in 300 µl of wash buffer. The BD LSRII flow cytometer (BD Biosciences, San Diego, CA, USA) was calibrated with setup beads and 1,800 events were acquired for each sample. Results were generated in graphical format and analyzed with FCAP array software v1.0.1 (BD Biosciences, San Diego, CA, USA).

Real-time polymerase chain reaction (PCR)
RNA isolation

Total RNA was extracted from the HCE cell samples with RNAqueous Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Quantification of total RNA was performed in a NanoDrop spectrophotometer (ND-1000; Nano-Drop Technologies, Wilmington, DE, USA). RNAs were stored at -80°C until further utilization.

cDNA synthesis

cDNA was synthesized from purified concentrated 0.5 µg RNA from each sample, using a high capacity cDNA reverse transcription kit (Applied Biosystems, ABI, USA). A 20 µl total reaction volume was made with 10 µl RNA, 2 µl 10X RT buffer, 0.8 µl dNTP Mix (100 mM), 2 µl 10X RT random hexamer primers, 1 µl MultiScribe™ reverse transcriptase, 1 µl RNase inhibitor and 3.2 µl nuclease-free water. Synthesis was carried out in an ABI 7900 Thermo cycler (Applied Biosystems, Foster City, CA, USA) and reaction conditions were 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. cDNA samples were stored at -20°C.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously (18). Negative controls were included to evaluate DNA contamination of isolated RNA and reagents.

Real-time polymerase chain reaction assays for the selected cytokines were: TNF-α, IL-1β, IL-6 and IL-8 (Applied Biosystems, Foster City, CA, USA) and I-κBα (Applied Biosystems, Foster City, CA, USA). An amount of 1 µL cDNA was loaded in each total volume of 20 ml of reaction mixture and assays were performed in triplicates.

The fold changes of the gene expression in the samples were normalized to the endogenous gene hypoxanthine phosphoribosyltransferase 1 (HPRT1; Applied Biosystems, Foster City, CA, USA). Quantitative analysis was performed using the comparative ($\Delta\Delta C_T$) method, in which C_T value is defined as the cycle number in which the detected fluorescence exceeds the threshold value, ΔC_T is the difference between C_T of a target gene and the endogenous control, and $\Delta\Delta C_T$ is the difference between ΔC_T of the analyzed sample and the calibrator (control sample) (18). The results were analyzed by DataAssist™ Software Version 2.0 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

All tests were carried out on four independent cell cultures, derived from four different cornea donors, and performed in triplicate for each of the treatments. The levels of the cytokines protein contents and mRNA expression were calculated as a ratio relative to medium. Statistical analysis and multiple comparisons were performed by one-way ANOVA using the InStat software version 3.10 (GraphPad Software Inc, San Diego, CA, USA).

RESULTS

Morphological observations in HCE cells

The MPSs and the controls were applied after the cells achieved complete confluence in the wells. Exposure of the HCE cells to MPSs at concentrations of 30% v/v and 50% v/v for 0.5 h and 1 h, and to 30% v/v for 2 h had no effect on cell morphology and there was insignificant difference compared to UN-H2O2 and SFM (p>0.05; data not shown). However, exposure to 50% v/v MPSs for 2 h caused cell detachment and reduction in HCE area density.

After two hours, no apparent differences were noticed in the proportion of the damaged area following exposure to PBS (1.32±3.02%) compared to un-stimulated cells (SFM; 0.05%±0.006 p>0.05). In contrast, there was a highly significant difference in the damaged area between UN-H2O2 (98.46±3.07%) and SFM (0.05±0.006%; p<0.001; Fig. 1).

Exposure of HCE cells after two hours to 50% v/v MPSs A, B, E, F and H caused a statistically significant reduction in the cultured HCE area density and caused marked disruption of cell junctions
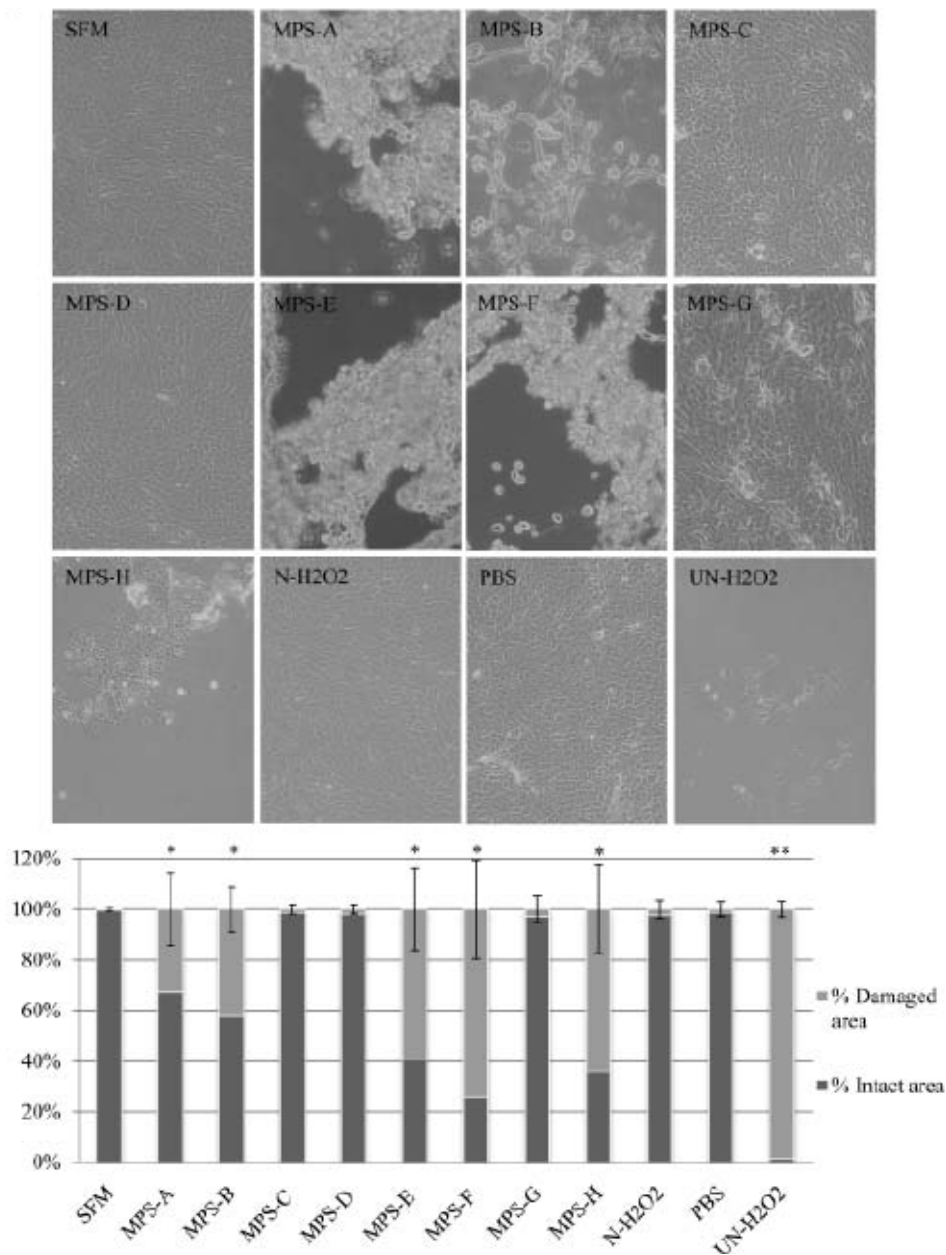
Fig. 1. *Morphological observation on HCE cells. The values for percent area densities were calculated by the ratio of damaged cells to the total field area. Images were obtained from 8 representative areas taken from four independent experiments. Results are shown as means ± SD. The asterisk represent significance (p<0.001) compared to SFM. MPS: Multipurpose Solutions; N-H2O2: Neutralized hydrogen peroxide lens disinfection system; UN-H2O2: un-neutralized hydrogen peroxide lens disinfection system; SFM: serum-free medium; PBS: phosphate-buffered saline*

compared to SFM (p<0.05; Fig. 1). The cells were irregular in shape, variable and altered in size, and the intercellular spaces increased with disruption of the cell-to-cell junctions after exposure to MPSs A, B, E, F and H compared to SFM (Fig. 1). Slight to moderate swelling was observed in the HCE

cells after exposure to MPSs A, E and F, moderate swelling was observed in MPS B, and reduction in cell size was observed in MPS H (Fig. 1).

MPS-F exhibited the greatest reduction in the HCE area density (74.05±19.54%, p<0.001) followed by MPS-H (63.93%±17.42, p<0.001) and
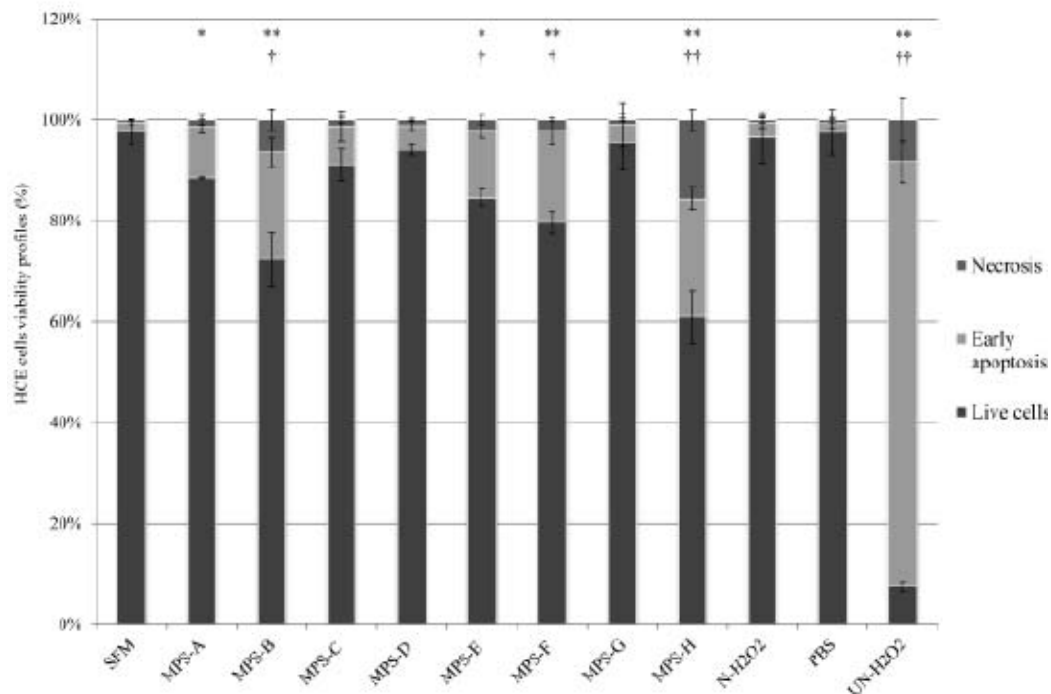
Fig. 2. *Annexin V-FITC Apoptosis Assay Analysis of MPSs. Flow cytometry profiles represent un-staining (live cells), early apoptosis (Annexin-V-FITC positive staining and PI negative staining) and late apoptosis/necrosis (Annexin V positive staining and PI positive staining). Results are shown as means ± SD (n=4). The asterisk (p<0.01) and double asterisk (p<0.001) represent statistical significance for early apoptosis as compared to SFM. The dagger (p<0.01) and double dagger (p<0.001) represent statistical significance for late apoptosis/necrosis as compared to SFM. MPSs: Multipurpose solutions; N-H2O2: Neutralized hydrogen peroxide lens disinfection system; UN-H2O2: un-neutralized hydrogen peroxide lens disinfection system; PBS: phosphate-buffered saline*

MPS-E (58.93±16.12%, p<0.001) compared to SFM (Fig. 1). Insignificant differences in the proportion of damaged areas between MPSs and SFM were observed when the HCE cells were incubated with the various MPSs at concentrations of 30% v/v.

Effects of MPSs on HCE cells viability profiles (FITC-Annexin V/PI assay)

Both concentrations tested demonstrated a significant reduction in HCE cell viability profiles after the cells were exposed to the positive control (UN-H2O2) (84.26%±6.68 for early apoptotic cells; 8.26%±3.85 for necrosis; p<0.001) as compared to SFM.

Almost all the MPSs showed a significant reduction of the viability profiles at both concentrations compared to the SFM (p<0.05), except for MPS-C and MPS-D (Fig. 2; data not shown for 50% v/v).

MPS-H exhibited the greatest negative effect on the viability profiles (23.36%±2.23 for early apoptotic cells; 15.65±2.11 for necrosis; p<0.001), followed by MPS-B (21.12%±2.98 for early apoptotic cells; 6.52%±2.21 for necrosis; p<0.001) as compared to SFM (Fig.2).

Effects of MPSs on HCE mitochondrial enzyme activity (MTT assay)

All the MPSs showed a significant loss of mitochondrial activity at both concentrations compared to SFM (p<0.05; Fig. 3). MPS-H exhibited the greatest inhibitory effect on mitochondrial activity (0.42±0.035 for 30% v/v, p<0.05; 0.263±0.041 for 50% v/v, p<0.05) followed by MPS-B (0.42±0.041 for 30% v/v; p<0.05; 0.28±0.02 for 50% v/v; p<0.05) and MPS-F 50% concentration (0.34±0.035, p<0.05) as compared to SFM (Fig. 3).

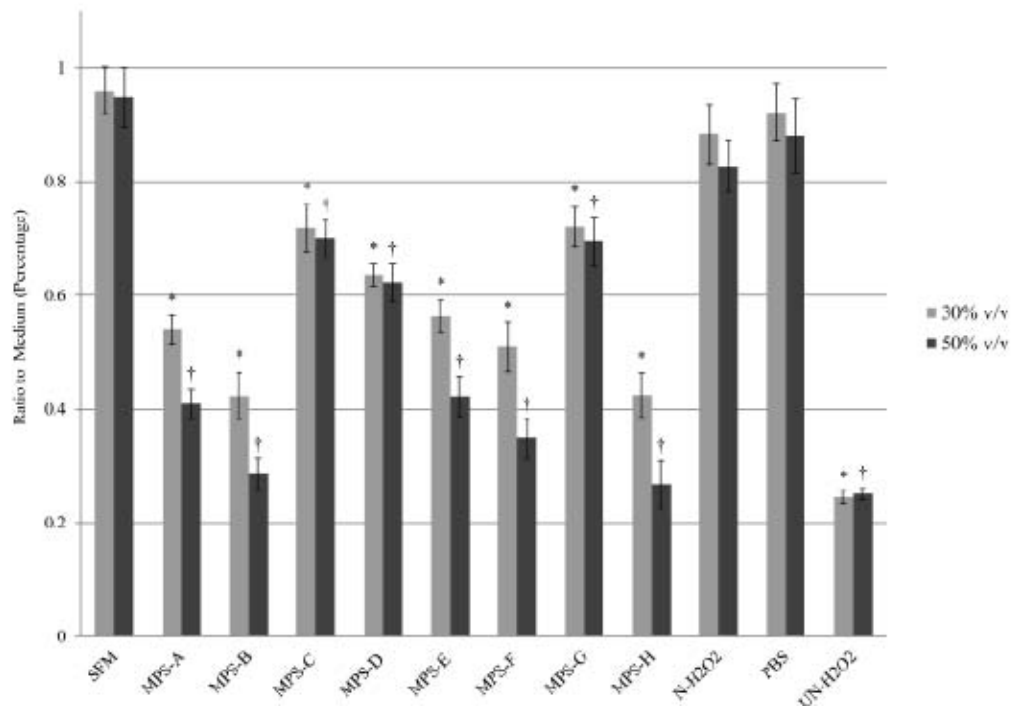An insignificant difference in the mitochondrial activity was observed after the HCE cells were

Fig. 3. *Mitochondrial enzyme activity of MPSs. The results were expressed as percent in relation to the medium. All experiments were performed in three independent cell cultures taken from 4 different cornea donors, with 8 replications for each treatment. Results are shown as means ± SD. The asterisk and dagger represent statistical significance (p<0.01) for 30% and 50 % v/v MPSs concentration respectively as compare to SFM. MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); MPS: Multipurpose Solutions; N-H2O2: Neutralized hydrogen peroxide lens disinfection system; UN-H2O2: un-neutralized hydrogen peroxide lens disinfection system; SFM: serum-free medium; PBS: phosphate-buffered saline*

incubated with the negative control (PBS) as compared to the SFM (0.92±0.05 for 30% v/v, p>0.05; 0.88±0.065 for 50% v/v, p>0.05). In contrast, the positive control (UN-H2O2) significantly reduced the mitochondrial activity as compared to SFM (0.24±0.01for 30% v/v, p<0.05; 0.25±0.01 for 50% v/v, p<0.05; Fig. 3).

Effects of MPSs on cytokines protein production

Multiplex FBI results demonstrated that all five MPSs: A, B, E, F and H, stimulated significantly higher levels of pro-inflammatory cytokines in HCE cells compared to the SFM. MPS-H induced the highest amount of pro-inflammatory cytokine production (p<0.001; Figs. 4 and 5, Table II).

MPS-H elicited up to 14.37±2.2 (p<0.001) fold higher levels of TNF-α, up to 41.39± 2.5 (p<0.001) fold higher levels of IL-1β, up to 5.24±0.6 (p<0.001) fold higher levels of IL-6 and up to 2.54±0.2

(p<0.001) fold higher levels of IL-8 compared to the SFM. This was followed by MPSs B, F, A and E. MPS-B elicited up to 8.94±1.49(p<0.001) fold higher levels of TNF-α, up to 27.4±3.4 (p<0.001) fold higher levels of IL-1β, up to 3.99±0.5 (p<0.001) fold higher levels of IL-6 and up to 1.84±0.26 (p<0.01) fold higher levels of IL-8 compared to the SFM (Figs. 4-5, Table II).

In contrast, there was no significant difference (p>0.05) between SFM as compared to N-H2O2 in the protein content level of all the cytokines. An insignificant difference was noticed in all the cytokine protein content between the negative control (PBS) and SFM (p>0.05). However, the positive control (poly I:C) demonstrated a significant difference in all cytokine protein content compared to SFM (p<0.001, Figs. 4 and 5). The other positive control (LPS complex) demonstrated a significant difference in TNF-α, (p<0.001), IL-6 (p<0.001), IL-8 (p<0.05)

Table II. *Protein contents of cytokines in HCE cells following MPSs 30% and 50% v/v incubation.*

	TNF-α Fold to SFM		IL1-β Fold to SFM		IL-6 Fold to SFM		IL-8 Fold to SFM	
	30% v/v	50% v/v	30% v/v	50% v/v	30% v/v	50% v/v	30% v/v	50% v/v
SFM	1±0.23	1±0.17	1±0.2	1±0.29	1±0.21	1±0.2	1±0.25	1±0.15
MPS-A	3.1±1.78*	5.27±1.3*	16.34±4.54**	25.34±3.32**	2.23±0.98*	3.59±0.3*	1.52±0.34	1.75±0.16*
MPS-B	5.23±1.89*	8.94±1.49**	21.35±3.24**	27.4±3.41**	3.15±1.56*	3.99±0.5**	1.68±0.45	1.83±0.26*
MPS-C	1.56±0.21	1.25±0.3	2.65±0.26	4.86±0.75	1.1±0.25	1.13±0.17	1.25±0.34	1.83±0.2
MPS-D	1.65±0.15	2.15±0.41	2.64±0.32	5.24±1.02	1.09±0.23	1.21±0.31	1.11±0.35	1.62±0.32
MPS-E	2.65±1.55	5.55±0.97**	11.9±2.98**	22.94±3.24**	2.9±1.85*	3.51±0.57*	1.88±0.25*	2.32±0.18**
MPS-F	3.24±0.97	5.69±0.95**	15.26±1.54**	22.55±3.16**	2.4±1.56	3.17±0.58	2.02±0.15*	2.16±0.26*
MPS-G	1.87±0.56	3.13±0.87	4.1±0.57	5.31±1.92	1.89±0.56	3.31±0.6	1.45±0.31	1.60±0.26
MPS-H	9.12±3.29**	14.8±2.23**	24.7±5.64**	41.39±2.54**	4.98±2.56**	5.24±0.6**	2.35±0.42**	2.54±0.24**
N-H2O2	1.19±0.12	1.34±0.27	2.31±0.47	3.36±0.47	1.2±0.1	1.09±0.15	1.09±0.15	1.10±0.26
PBS	1.12±0.19	1.08±0.11	1.21±0.19	1.39±0.19	1.07±0.13	0.96±0.21	1.08±0.23	1.02±0.06
LPS complex	29.35±2.3**	29.35±2.3**	4.35±1.12	4.35±1.12	4.51±0.85**	4.5±0.85**	2.5±0.36*	2.5±0.36*
Poly I:C	86.28±9.1**	86.28±9.1**	14.68±1.65**	14.68±1.65**	34.31±4.2**	34.3±4.2**	4±0.5**	4±0.36**

Protein secretion levels of TNF-α, IL-1β, IL-6 and IL-8 were examined by multiplex FBI after the HCE cells were exposed to various MPSs. Data shown is the ratio to SFM (mean ± SD) from four independent experiments (n=4). The asterisk (p<0.01) and double asterisk (p<0.001) represent statistical significance as compared to SFM. LPS: Lipopolysaccharide; LBP: Lipopolysaccharide binding protein; CD14: Cluster of differentiation 14; Poly I:C: Polyinosinic:polycytidylic acid; MPSs: Multipurpose Solutions; N-H2O2: Neutralized hydrogen peroxide lens disinfection; PBS: phosphate-buffered saline

protein content compared to the SFM (Figs. 4 and 5, Table II). There were insignificant differences observed in the levels of cytokine production of IL-10 and IL-12 (p70) between the various MPSs compared to SFM.

Similar to the results of the multiplex fluorescent bead immunoassay, MPS-H 50% v/v elicited highly significant mRNA expression of TNF-α of up to 15.32±2.58 (p<0.001) fold, IL-1β up to 15.17±2.64 (p<0.001) fold, IL-6 up to 6.45±0.78 (p<0.001) fold and IL-8 up to 8.98±1.56 (p<0.001) compared to SFM (Fig. 6). Likewise, highly significant increased mRNA expression of all 4 cytokines was noted for MPS-B and MPS-F. Exposure to MPS-E induced highly significant increased mRNA expression of IL-6, IL-8 and IL1-β (Fig. 6).

In contrast, there were insignificant differences (p>0.05) between N-H2O2 and SFM in the mRNA expression levels of all cytokines tested (Fig. 6A-D).

Effects of MPSs on IκB mRNA expression

The mRNA expression level of I-κBα was significantly increased (8.26±2.43 fold, P<0.005) in HCE cells upon stimulation with MPS-H 30% v/v (Fig. 7A) compared to SFM, followed by MPS-B, F, A and E (which increased the I-κBα expression by 4.19±1.5 fold, 3.08±1.61 fold, 2.74±1.81 fold and 2.49±1.76 fold, respectively). Similar results were demonstrated when the HCE cells were exposed to 50% v/v MPSs (Fig. 7B).

There was an insignificant difference in I-κBα mRNA expression levels between the negative control (PBS) and SFM (p>0.05). However, the positive controls (Poly I:C, p<0.001; LPS complex, p<0.05) demonstrated a significant difference in the I-κBα mRNA expression levels as compared to SFM with either 30% v/v or 50% v/v concentrations) Fig. 7, A and B).

DISCUSSION

Our study examined the cytotoxic and inflammatory effects following exposure of cultured HCE cells to a battery of commonly used contact lens MPSs.
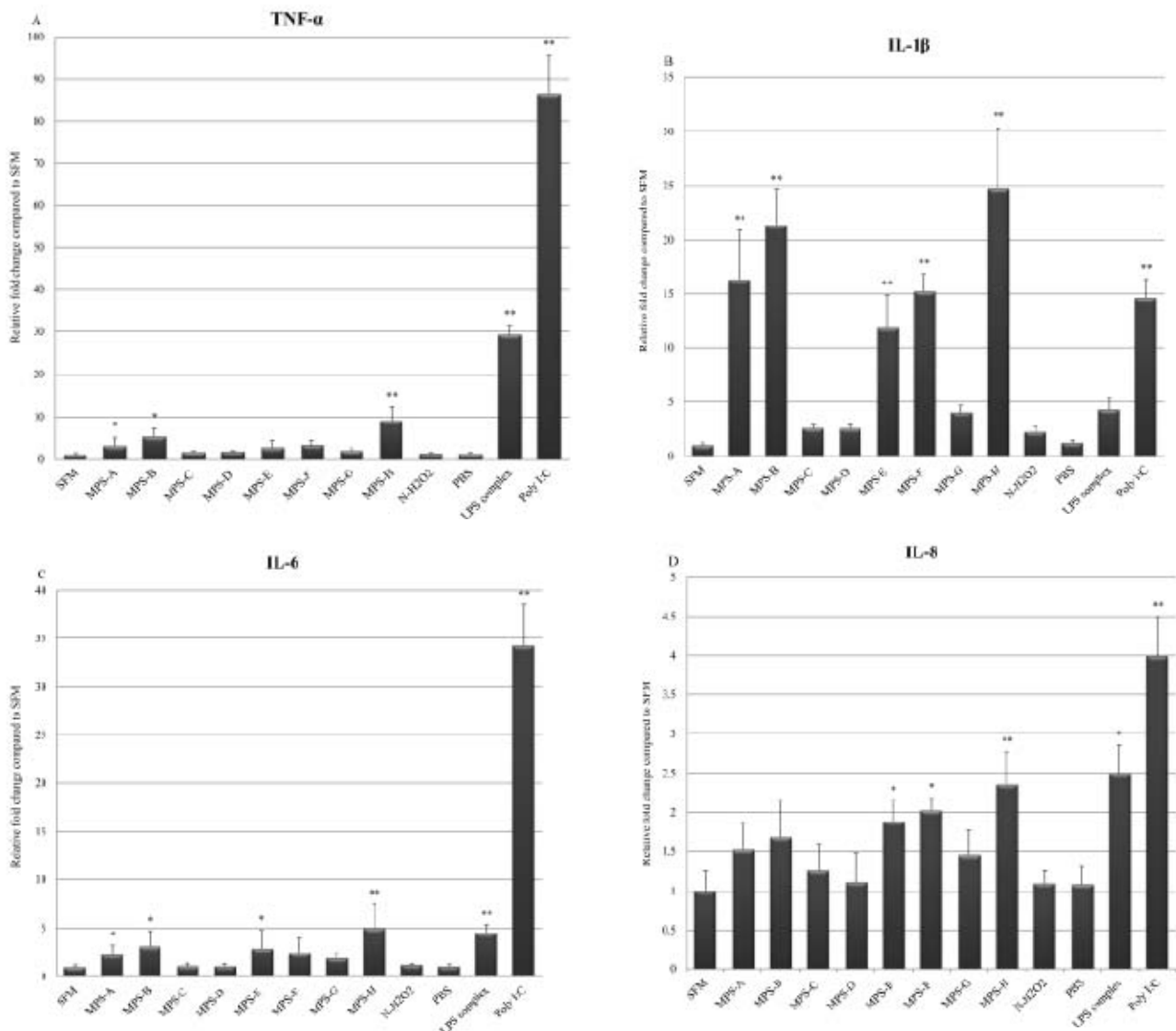
Fig. 4. *Cytokine protein contents in HCE cells following MPSs (30% v/v) incubation. Protein secretion levels of TNF-α (A), IL-1β (B), IL-6 (C) and IL-8 (D) were examined by multiplex FBI. Data shown is the ratio to SFM (mean ± SD) from four independent experiments (n=4). The asterisk (p<0.01) and double asterisk (p<0.001) represent statistical significance for experiments as compare to SFM. LPS: Lipopolysaccharide; LBP: Lipopolysaccharide binding protein; CD14: Cluster of differentiation 14; Poly I:C: Polyinosinic:polycytidylic acid; MPSs: Multipurpose Solutions; N-H2O2: Neutralized hydrogen peroxide lens disinfection system; PBS: phosphate-buffered saline*

We have shown that most of the MPSs induced varying degrees of cytotoxicity to the HCE cells, and increased production of pro-inflammatory cytokines. Almost all of the MPSs induced increased levels of IL-1β, TNF-α, IL-6 and IL-8 in HCE cells. MPS-H, followed by MPS-B, stimulated the highest levels of pro-inflammatory cytokines. In addition, several MPSs increased the mRNA level of I-κBα. Of all of the various MPSs, MPS-H was significantly the most cytotoxic when compared to the negative control, followed by MPS-B. In contrast, no significant differences were noted between the N-H2O2 and the negative control in cell toxicity, in the viability profiles, and in the expression of the inflammatory cytokines.
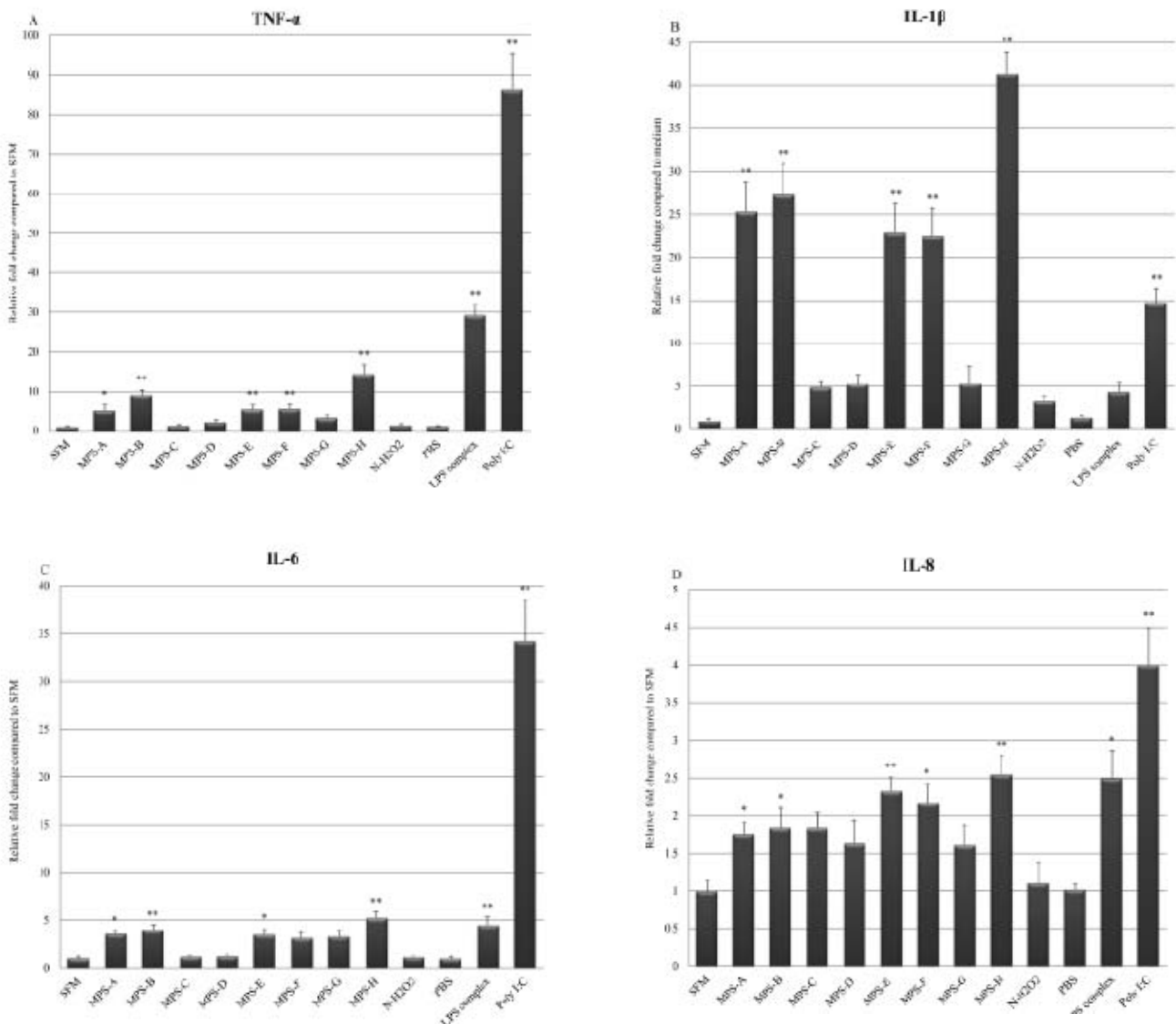
The MPS concentrations of 30-50% v/v which

Fig. 5. *Cytokine protein contents in HCE cells following MPSs (50% v/v) incubation. Protein secretion levels of TNF-α (A), IL-1β (B),IL-6 (C) and IL-8 (D) were examined by multiplex FBI. Data shown is the ratio to SFM (mean ± SD) from four independent experiments (n=4). The asterisk (p<0.01) and double asterisk (p<0.001) represent statistical significance for experiments as compare to SFM. LPS: Lipopolysaccharide; LBP: Lipopolysaccharide binding protein; CD14: Cluster of differentiation 14; Poly I:C: Polyinosinic:polycytidylic acid; MPSs: Multipurpose Solutions; N-H2O2: Neutralized hydrogen peroxide lens disinfection system; PBS: phosphate-buffered saline*

were used in our study are partially based on previous studies which investigated the residence time and concentrations of solutions and artificial tears on the ocular surface (8-9, 19). Specifically, it was shown that after 25 minutes, a drop of solution applied to the eye would contain half of its original concentration, and after an hour the concentration will decrease to 20% (8). However, these concentrations and

exposure periods may be higher in the setting of contact lens use, as contact lenses may absorb the solutions, and slowly release them as a sustained release system. A study demonstrated that polyvinyl alcohol, a component of artificial tears, when added to contact lens matrix for later release into the tears, had a consistent near zero order release over a 20-hour period (20). Since our study relates
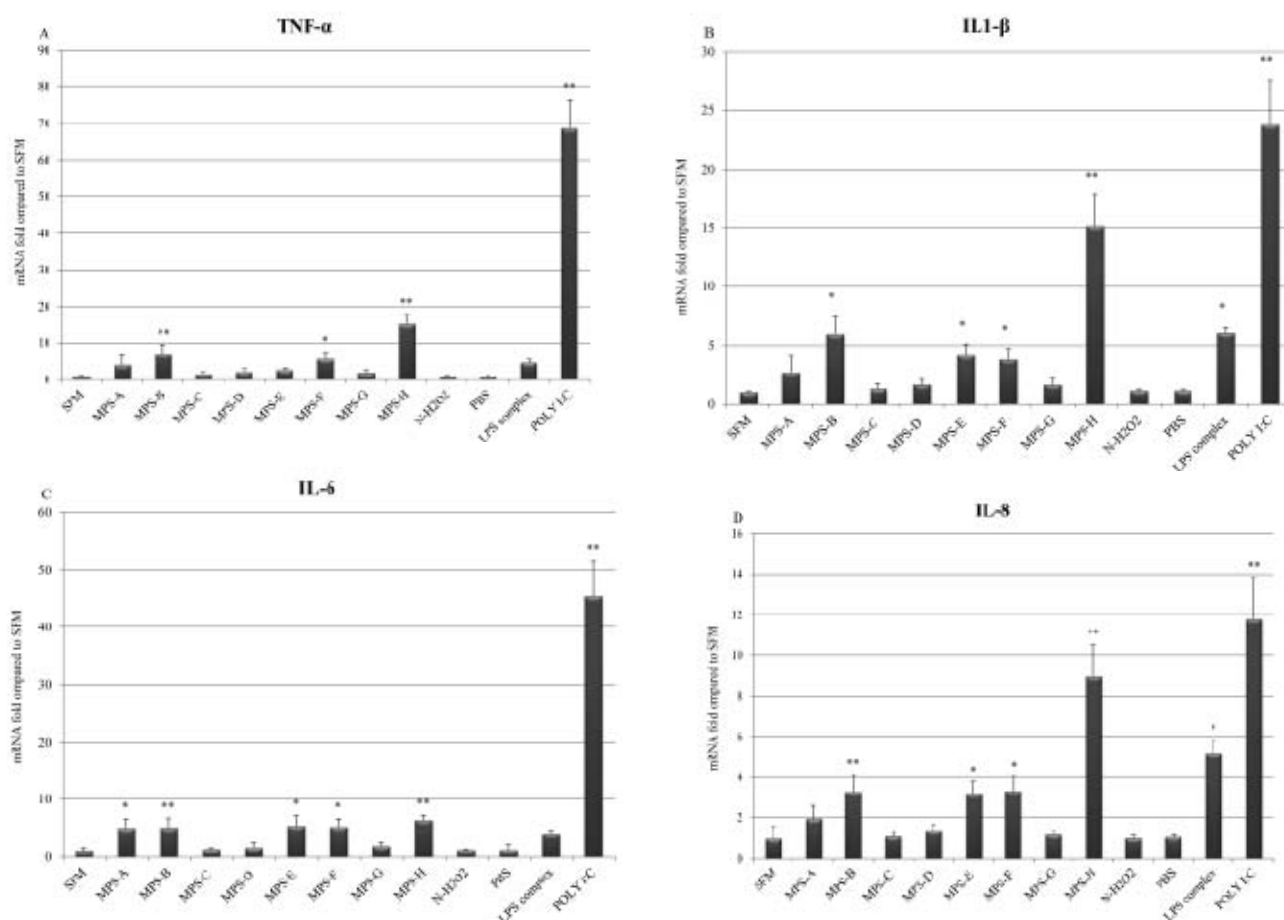
Fig. 6. *Cytokine mRNA expression in HCE cells following MPSs (50% v/v) incubation. mRNA expression levels of TNF-α (A), IL-1β (B),IL-6 (C) and IL-8 (D) were analyzed by real time PCR. mRNA cytokines mRNA expression levels are presented in ratio fold compare to SFM which normalized to 1.0. Data derived from four independent experiments (n=4) and shown as mean ± SD. Data shown is the ratio to SFM. The asterisk (p<0.01) and double asterisk (p<0.001) represent statistical significance for experiments as compare to SFM. LPS: Lipopolysaccharide; LBP: Lipopolysaccharide binding protein; CD14: Cluster of differentiation 14; Poly I:C: Polyinosinic:polycytidylic acid; MPSs: Multipurpose Solutions; N-H2O2: Neutralized hydrogen peroxide lens disinfection system.*

to contact lens solutions, the actual concentrations and exposure times of MPSs in the ocular surface are higher than that of regular topical drug solutions, as a result of a continuous slow release mechanism (19, 21) of the solutions that are secreted from the contact lenses to the tears. Indeed, similar MPS concentrations and incubation periods were used in previous cytotoxicity studies of MPSs (15, 22).

All the clinical studies to date have examined the cytotoxicity effects of MPSs using the contact lenses as carriers for delivery of the MPSs. As the contact lens by itself can be a causal factor for significant damage to the ocular surface (i.e. poorly fitted contact lenses, debris on contact lenses, or defective contact lenses), the isolated effects of the MPSs were thus not adequately evaluated in these various clinical reports. Among the methods used in *in vitro* studies on MPSs, the cytotoxicity effects of MPSs were examined after contact lenses were soaked in multipurpose solution, and then were laid on cultured cell lines (23). In our study, the MPSs were diluted in the culture medium at concentrations which are similar to those that are expected following dilution by the tears after several hours of contact lens wear.
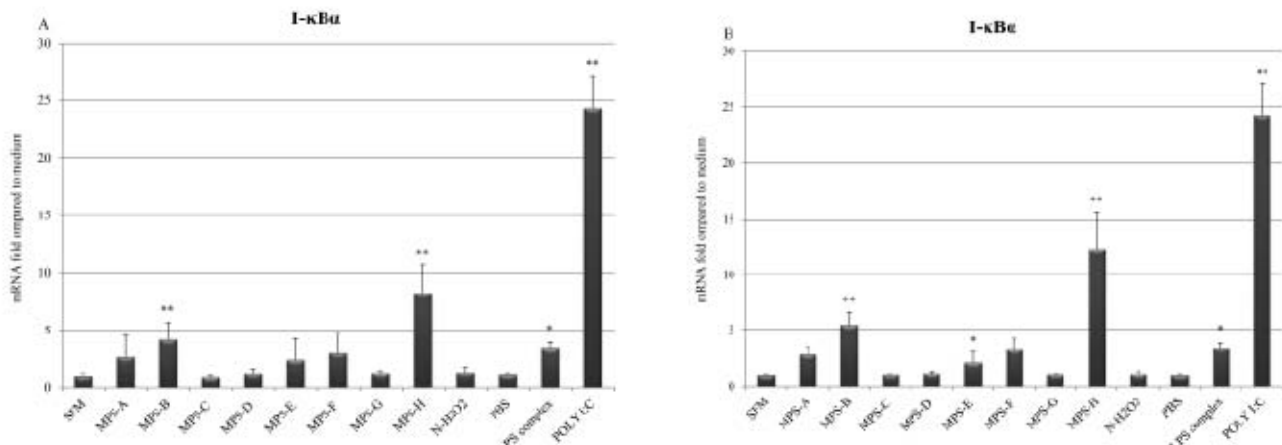
Fig. 7. *Inhibitory factor-κBα (I-κBα) mRNA expression in HCE cells following MPSs (30% v/v and 50% v/v). mRNA expression levels of I-κBα (**A** and **B**) were analyzed by real time PCR. mRNA expression levels of I-κBα are presented in ratio fold compare to SFM which normalized to 1.0. Data derived from four independent experiments (n=4) and shown as mean ± SD. Data shown is the ratio to SFM. The asterisk (p<0.01) and double asterisk (p<0.001) represent statistical significance for experiments as compare to SFM. LPS: Lipopolysaccharide; LBP: Lipopolysaccharide binding protein; CD14: Cluster of differentiation 14; Poly I:C: Polyinosinic:polycytidylic acid; MPSs: Multipurpose Solutions; N-H2O2: Neutralized hydrogen peroxide lens disinfection system.*

Therefore it supplies a more accurate method for the evaluation of the isolated effects of the MPSs on the cells.

In addition, nearly all the previous *in vitro* studies had used non-corneal human or animal cell lines to study the cytotoxic effects of MPSs. These cell lines are generated following a process of immortalization, and may not accurately present with the multiple cellular and molecular functions of HCE cells *in vivo*. Hence, our model using primary cultured HCE cells more closely resembles the *in vivo* state in the corneal epithelium.

Preservatives used in ophthalmic eye drops may induce significant cytotoxic and inflammatory changes, including inflammatory infiltrates, epithelial alterations and various degrees of keratinization in the ocular surface after short term use (24). *In vitro* cytotoxicity was demonstrated by CLs soaked in benzalkonium chloride (25), and low concentrations of benzalkonium chloride induced significant amounts of inflammatory cytokines (26). Likewise, clinical performance of various MPSs with different CL has been associated with adverse effects as shown in the corneal and conjunctival fluorescein staining, damaged epithelial barrier function and low grade infiltrative responses (10-11, 15).

Many ocular surface disorders can interrupt the homeostasis and cause an inflammatory response. This inflammatory response is characterized by the production of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8 and TNF-α. These cytokines have been demonstrated in the tear film and the ocular surface epithelia in dry eye and other ocular surface inflammatory conditions (27). We have therefore attempted to examine these cytokines in response to exposure of cultured corneal epithelial cells to the various MPSs. These cytokines may damage the barrier function of the HCE cells. TNF-α was found to disrupt the epithelial cell tight junctions, by affecting the localization of ZO-1 at tight junctions (28). Similarly, IL-1β can induce the redistribution of ZO-1, thereby contributing to the reduced barrier function of the corneal epithelium (29). Our data suggest that the MPS-induced IL-1β and TNF-α production may lead to epithelial barrier dysfunction in the corneal epithelium, as found in our cytotoxicity data. Notably, the MPSs that induced the highest levels of IL-1β production also showed the most cytotoxic effects in HCE cells.

Our finding demonstrated that five MPSs (A, B, E, F and H) stimulated significant higher levels of pro-inflammatory cytokines and cytotoxicity effects in HCE cells, whereas MPS-C, -D and -G induced considerably lesser effects. The reason for these

differences is not entirely clear. There are no distinctive difference in the preservative compositions between MPS-A, -B, -E, -F and -H (MPS-H contains PQ-1 and Alexidine-Dihydrochloride; MPS-B contains PQ-1 and Myristamidopropyldimethylamine; MPS-A, -E and -F contain PHMB) compared to MPS, C, D and G (MPS-C and MPS-D contains PHMB; MPS-G contains PHMB and PQ-1) (Table II).

A clinical study which compared the corneal fluorescein staining of different MPSs found that solutions containing PQ-1 were the most damaging (30). A similar result was found in an *in vitro* study (22). However, other *in vivo* studies found that solutions containing PQ-1 caused less staining than PHMB (13, 31). At the same time, it was claimed that fluorescein staining correlated poorly with corneal epithelial cytotoxicity, which may explain the variable results between these studies (32).

MPSs contain a highly complex combination of ingredients, including preservatives, surface-active agents, chelating agents, wetting agents, buffered solutions and protein removers, some of which have been shown to be cytotoxic. For example, boric acid and 2-amino-2-methyl-1-propanol which are buffering agents have been shown to be cytotoxic at concentrations of 0.1%, while Citric acid and sodium chloride are not likely to be cytotoxic (33). There is also a possibility that the combination of PQ-1 or PHMB with some of the other components in the solutions may cause damage to the cells (22).

Interestingly, we found that previous methods of contact lens disinfection with N- H2O2 were safer to the corneal epithelium compared to the currently used MPSs. H2O2 is used for CL disinfection due to its broad antimicrobial activity. Using this system, CLs required a neutralization process prior to insertion into the eye to avoid possible corneal damage. Neutralization was achieved either during the disinfection process (one-step type), or afterwards (two-step type). After neutralization, the hydrogen peroxide solution is completely converted into a preservative free saline solution. The main limitation of the H2O2 is the significant inconvenience using a two-step procedure. Based on our results we are strongly convinced that H2O2 is preferable as a disinfecting and sterilizing system for CLs. Our *in vitro* results support the clinical studies which reported that the H2O2 achieved a longer comfortable

wearing time (34), lower incidence of corneal infiltrative events (35) and lesser solution-induced corneal staining compared to the commercially available MPSs (11, 35). These data, combined with the fact that one-step systems are less effective against Acanthamoeba cysts (1), demonstrate the use of H_2O_2 as being both safer and more effective.

In conclusion, this is the first comprehensive study that comparatively evaluates a large series of commercially available and commonly used MPSs at the molecular level, investigating the inflammatory responses in HCE cells. Our study found that several MPSs induced significant cytotoxic and inflammatory effects in human corneal epithelial cells. In contrast, the hydrogen peroxide lens disinfection system did not induce any cytotoxic or inflammatory effects in human corneal epithelial cells. These findings suggest that certain MPSs may play a partial role in the etiology of contact lens intolerance. CL intolerance is associated with multiple factors, including dry eye disease, poor contact lens fitting, and prolonged daily use. The toxic and inflammatory effects of MPS may be add additional factors to the many potential causes for CL intolerance. MPSs could thus enhance CL complications and contribute to the drop-out rate for contact lens wearers.

We recommend that practitioners advise patients as to the preferable disinfecting contact lens solutions, and to consider using the hydrogen peroxide disinfection systems instead, or alternatively use daily disposable contact lenses where these solutions are not needed.

ACKNOWLEDGEMENTS

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