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Comparative *In vitro* Cytotoxicity of Artificial Tears

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Abstract

Artificial tears are some of the most commonly used ocular medications. The relative cytotoxicity of the most popular brands is not well established. In this study corneal epithelial cells were cultured in the presence of popular brands of artificial tears of varying concentrations. The cellular metabolic activity was then assessed with MTT ((3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) colorimetric cytotoxicity assay and the results compared. We found preservative-free artificial tears were significantly less cytotoxic than their preservative containing counterparts. Products based on carboxymethylcellulose tended to be less cytotoxic than those based on polyethylene glycol. Among non-preserved artificial tears Refresh® Preservative-Free was found to be least cytotoxic to cultured epithelial cells. Among artificial tears with preservatives Systane®, Blink®, and Genteal® were found to have the most *in vitro* cytotoxicity. This study demonstrates significant differences in the *in vitro* toxicity of popular artificial tears.

INTRODUCTION

Dry eye is one of the most common ocular conditions in the world with prevalence of over 10 million in the United States alone [1]. It can result in severe ocular symptoms and visual disability. The primary treatment for dry eyes is instillation of artificial tears over the course of the day [2]. The role of artificial tears is to either replace or augment the native tear film, with the goal of maintaining ocular surface lubrication.

While the drop-wise concentration of the ingredients in artificial tears is relatively low, the cumulative dose can be high when taken over many years, as is often the case. This is especially important in the context of the ancillary ingredients, such as preservatives and stabilizing agents, some of which are known to be cytotoxic [3-8]. In fact, it is their cytotoxicity that is leveraged to inhibit microbial overgrowth and thus improve shelf life and promotes sterility. Thus, there is a risk of toxicity that must be balanced against the benefit of ocular surface lubrication. Recently, there has been a surge of "preservative-free" formulations that have been manufactured without the addition of potentially cytotoxic agents, though at the expense of shelf life and relative sterility.

In this study, a number of commercially available artificial tear preparations were compared in an *in vitro* cytotoxicity study on cultured human corneal epithelial cells. The products included: Blink®, Blink® Preservative-Free, Refresh®, Refresh® Preservative-Free, Systane®, Systane® Preservative-Free, Thera-Tears®, and Genteal®. Each product was tested alongside the commonly added preservative benzalkonium chloride (BAK; Alfa Aesar, Ward Hill, MA). Lastly, the cytotoxicity of these artificial tears was also studied as a function of concentration by dilution of the full strength product.

MATERIALS AND METHODS

Cell Line

Human corneal epithelial cells immortalized with Adenovirus 12-SV40 hybrid virus (HCE-2 [50.B1]) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured on polystyrene plates coated with 0.01 mg/mL bovine fibronectin (Sigma-Aldrich, St. Louis, MO) and 0.03 mg/mL collagen type I (PureCol, Advanced Bio Matrix, San Diego, CA), and maintained at 37°C with 5% CO_2 in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) supplemented with 0.05 mg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, 0.005 mg/mL human insulin, 500 ng/mL hydrocortisone, and 1% antibiotics/antimycotics (Invitrogen, Carlsbad, CA). Cells from passages 5 to 10 were used for experiments.

Preparation of Artificial Tears and Reagents

Three brands of lubricant eye drops, both in preserved and preservative-free forms, were obtained over-the-counter: (1) Blink® Tears Lubricating Eye Drops and (2) Blink® Tears Preservative-Free Lubricating Eye Drops (Abbott Laboratories Inc., Abbott Park, IL); (3) Systane® Ultra Lubricant Eye Drops and (4) Systane® Ultra Lubricant Eye Drops Preservative-Free Formula (Alcon Laboratories, Inc., Fort Worth, TX), (5)

Refresh® Plus® Moisture drops for dry eyes and (6) Refresh® Tears® Moisture drops for dry eyes, sensitive, preservative-free (Allergan, Inc., Irvine, CA). Two other lubricant drops with preservatives were also tested: (7) Genteal® Lubricant Eye Drops (Alcon), and (8) TheraTears® Lubricant Eye Drops (Akorn, Inc., Lake Forest, IL) (Table 1).

Dilutions

Dilutions of artificial tears ranging from a concentration of 100% to 12.5% were prepared with phosphate buffered saline pH 7.4 (PBS, Invitrogen, Carlsbad, CA).

MTT Assay

MTT ((3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay (ATCC, Manassas, VA) was used to quantify viable cells after exposure to artificial tear solutions. The tetrazolium salt MTT is reduced by metabolically active cells to generate intracellular purple formazan that can be solubilized and quantified. Previously, length of the MTT incubation period had been optimized to 2 hours for this cell line to produce spectrophotometric readings that fell within a linear range for the relevant cell counts.

Five thousand corneal epithelial cells were seeded into pre-coated 96-well tissue culture polystyrene plates (Cellstar; Greiner Bio-One, Monroe, NC) and cultured for 3 days to reach confluence. Prior to incubation in artificial tear and control solutions, wells were washed with PBS pH 7.4. Corneal epithelial cells were incubated in 100 µL of artificial tear or control solutions for 1 hour at 37˚C and 5% CO_2. All wells were washed three times with PBS to remove remnant artificial tear solution.

Wells were incubated in 100 µL of complete culture medium containing 10% MTT and incubated for 2 hours at 37˚C and 5% CO_2. The same volume of detergent reagent (MTT Cell Proliferation Assay; ATCC, Manassas, VA) was used to dissolve the intracellular formazan at room temperature in the dark for 2 hours. Absorbance readings were measured at 570 nm with a microtiter plate reader (BioTek Instruments, Inc., Winooski, VT).

Controls

All solutions were tested five times in parallel with positive and negative controls. Negative controls consisted of formalin at 10% and 0.01% w/v BAK, and these were used to establish the cytotoxic baseline MTT absorbance. Positive control was cell culture media, and this was used to established the signal strength of what is to be considered non-toxic "fully" biocompatible. For the dilutions 0.01% BAK was also similarly diluted and used as negative control for the assay. All solutions except 10% formalin were warmed in a 37˚C, 5% CO_2 incubator.

Statistical Analysis

Statistical analyses were performed with commercially available software (SPSS for PC, Version 20.0, SPSS, Chicago, Illinois). Comparisons of the means between two sets of data were formed using unpaired test students T tests. Comparisons of means for more than two sets of data were performed using one way ANOVA. All p-values were 2-sided and considered significant if <0.05.

RESULTS AND DISCUSSION

The results of the MTT assay yielded consistent results. We here describe the assay results as a percentage of the metabolic activity in control media (positive control). As expected the preservative BAK (18.5%, p=.006) and formalin (21.1%, p=0.007) yielded the lowest assay results as compared to positive control media solution. Among the tested artificial tears, Systane® with preservative (30.5%, p=0.006) and Blink® with preservative (34.4%, p=0.007) yielded the lowest assay results. Genteal® (39.3%, p=0.006) was next, followed by Systane® Preservative-Free (66.0%, p=0.01), Thera-tears® (67.4%, p=0.009), and Blink® Preservative-Free (74.2%, p=0.009). Refresh® (92.6%, p=0.012) and Refresh® Preservative-Free (104.4% p=0.01) performed the best on the assay and showed little to no toxicity (Figure 1).

Using ANOVA testing we can divide the artificial tears into groups. Blink® (34.4%), Systane® with preservative (30.5%),

Table 1: Composition of Artificial Tear Products Tested. Active ingredient and preservatives of the tested products.

Brand	Preservative	Active Ingredient
Blink® Tears Lubricating Eye Drops[1]	OcuPure®(Sodium chlorite; stabilized oxychloro complex 0.005% m/v)	Polyethylene glycol 400 0.25%
Blink® Tears Preservative Free Lubricating Eye Drops[1]	none	Polyethylene glycol 400 0.25%
Refresh Tears® Moisture drops for dry eyes[2]	Purite™ (Stabilized oxychloro complex 0.005% m/v)	Sodium carboxymethylcellulose 0.5%
Refresh Plus® Moisture drops for dry eyes, Sensitive, preservative-free[2]	none	Sodium carboxymethylcellulose 0.5%
Systane® Ultra Lubricant Eye Drops[3]	PolyQuad® (polyquaternium-1) 0.001%	Polyethylene glycol 400 0.4%, Propylene glycol 0.3%
Systane® Ultra Lubricant Eye Drops Preservative-Free Formula[3]	none	Polyethylene glycol 400 0.4%, Propylene glycol 0.3%
Genteal® Lubricant Eye Drops[3]	GenAqua™ (Sodium perborate)	Hypromellose 0.3%
TheraTears® Lubricant Eye Drops[4]	Sodium perborate	Sodium carboxymethylcellulose 0.25%
[1]Abbott Laboratories Inc., Abbott Park, IL. [2]Allergan, Inc., Irvine, CA. [3]Alcon Laboratories, Inc., Fort Worth, TX. [4]Akorn, Inc., Lake Forest, IL.		

and Genteal® (39.3%) were significantly more cytotoxic than the other brands but were not significantly different from each other. Blink® Preservative-Free (74.2%), Thera-Tears® (67.4%) and Systane® Preservative-Free (66.0%) were the next group. They were less cytotoxic than the first group, but not significantly different from each other. Refresh® (92.6%) and Refresh® Preservative-Free (104.4%) demonstrated the least cytotoxicity but were not significantly different from each other.

Preservative-free formulations of artificial tears were significantly less toxic than their preservative-containing counterparts for all brands. This was seen most in Systane® and Blink® with Systane® Preservative-Free yielding a MTT assay result 2.16 times higher than Systane® with preservative ($p<0.001$) and Blink® Preservative-Free yielding an assay result 2.16 times higher than Blink® with preservative ($p<0.001$). The difference was less dramatic but still present for Refresh® Preservative-Free, with its assay result being only 1.13 times higher in that of Refresh® with preservative ($p<0.001$) (Figure 1).

In a comparison of the different dilutions and thus concentration-dependent toxicity, there appears to be change in toxicity with diluted test samples. For the products containing polyethylene glycol as their active ingredient, being Blink®, Systane® and their preservative-free counterparts, there appeared to be less toxicity as the product became more dilute. This was especially true for the preservative-containing formulations, with 12.5% dilution of Blink® having 3.00 times more metabolic activity than undiluted Blink® ($p=0.004$) and 12.5% dilution of Systane® having 2.90 times more metabolic activity than undiluted Systane® ($p=0.009$). The effect was not as dramatic in the preservative-free formulations: the 12.5% dilutions of those had 1.22 and 1.26 times more metabolic activity than the original concentration in preservative-free Blink® and Systane®, respectively. Also for Genteal®, in which the active ingredient is hypromellose, there was a mild dilution-dependent increase in metabolic activity (Figure 2).

In contrast, dilution in the carboxymethylcellulose- containing products (Refresh®, Refresh® Preservative-Free) was associated with somewhat lower metabolic activity. The 12.5% dilutions of each of these had 0.93, 0.83, and 0.86 times as much metabolic activity as their undiluted counterparts, respectively. It is interesting that the metabolic activity decreases with dilution of these compounds and we speculate there could be an ingredient

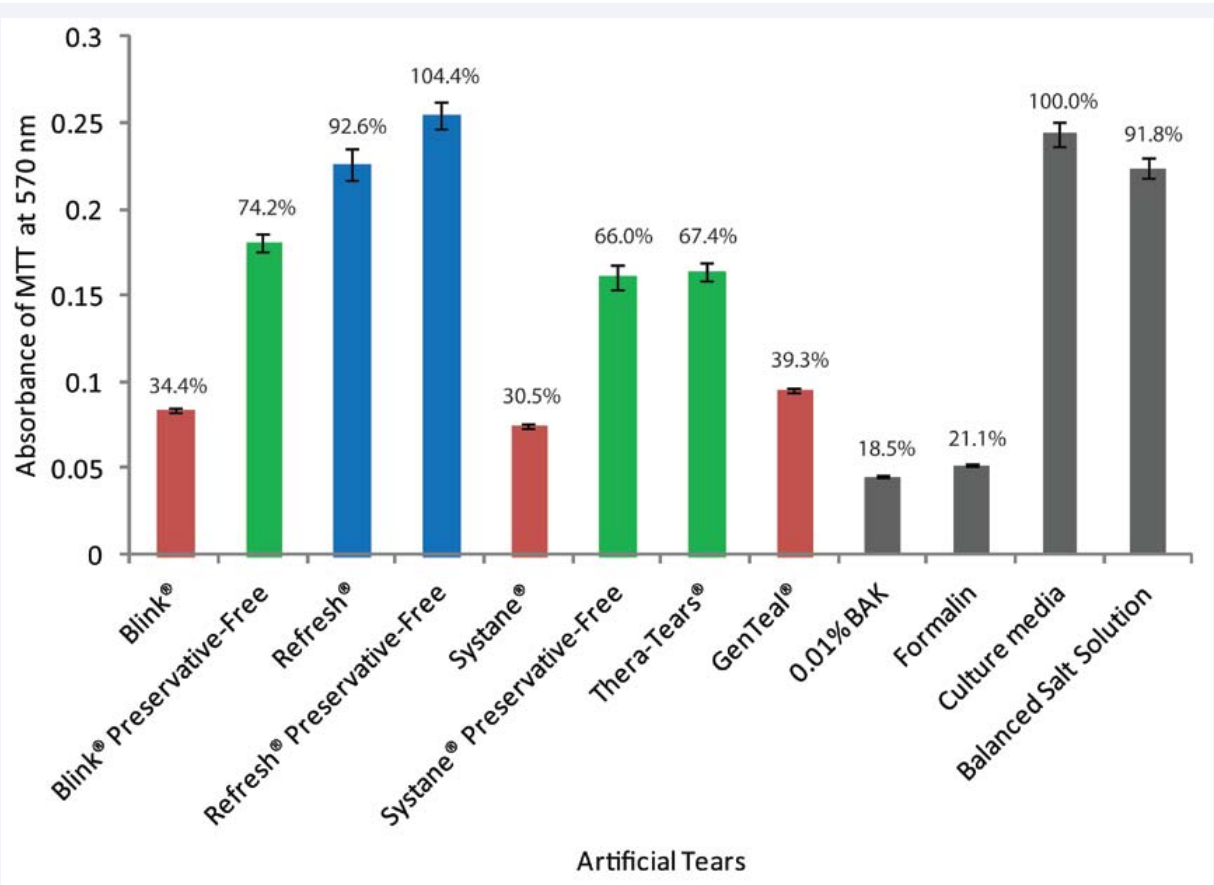

Figure 1 MTT assay results, 100% concentration of test products and controls. The absorbance of MTT at 570 nm, a measure of cellular metabolic activity, is plotted for each of the tested products. The percent activity as compared to control (culture media) is listed above each measurement. All values were significantly different from culture media control by T test. ANOVA testing reveals no significant differences between brands labeled with the same color in this chart (i.e. Blink®, Systane®, and Genteal® are not significant different from each other). The gray bars represent the negative controls (0.01% BAK and formalin) as well as positive controls (culture media and balanced salt solution).

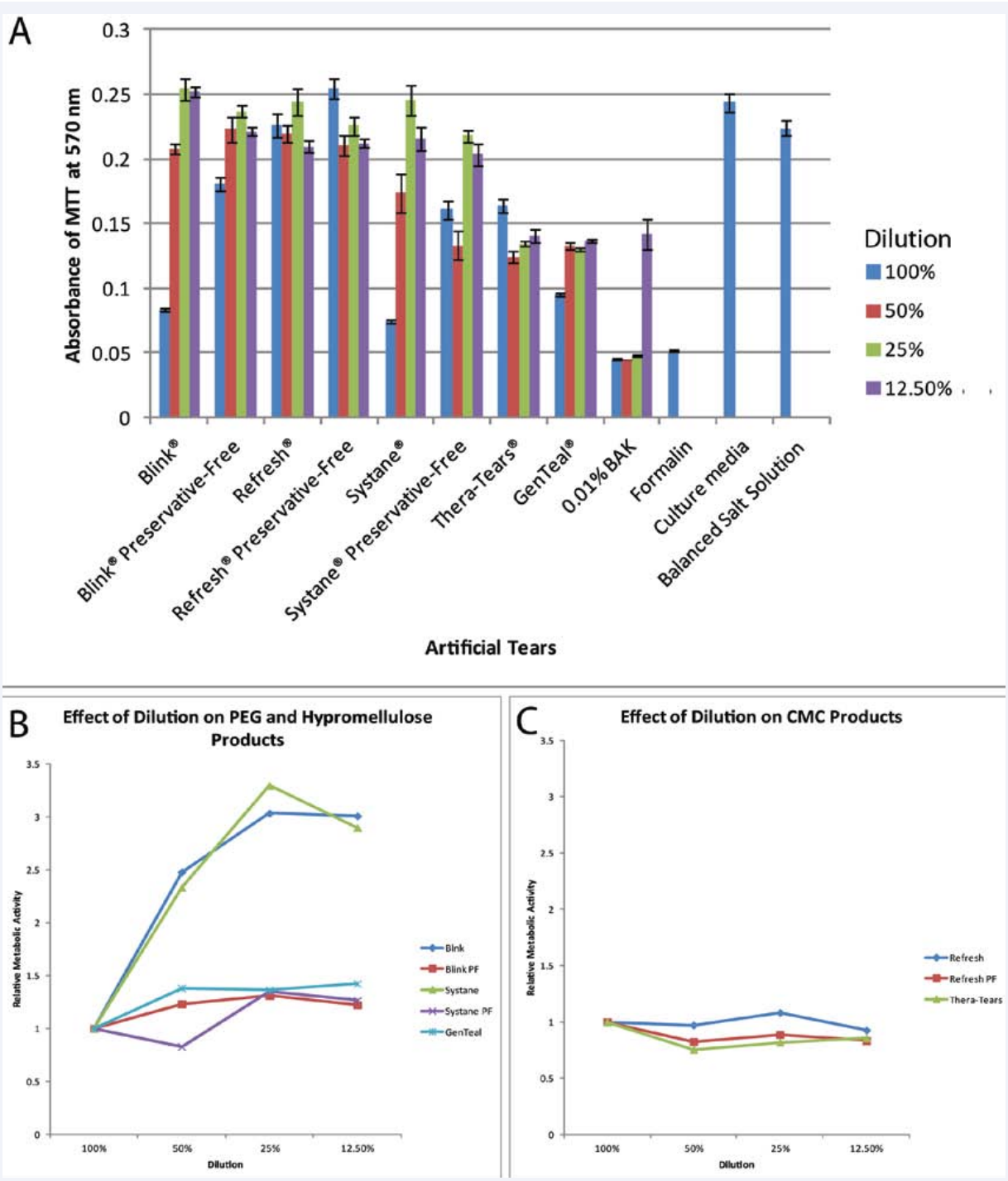

Figure 2 (A) MTT assay results, serial dilutions. The absorbance of MTT at 570 nm, a measure of cellular metabolic activity, is plotted for each dilution of the tested products. (B, C) Relative metabolic activity after dilution plot. These two charts reflect the effect of dilution on the relative metabolic activity of the tested products. (B) PEG active ingredient products, Blink®, Blink® PF, Systane®, and Systane® PF along with hypromellose active ingredient product Genteal®. (C) CMC active ingredient products, Refresh®, Refresh PF®, and Thera-tears®.

in their formulations, possibly carboxymethylcellulose, which promotes there *in vitro* metabolic activity and that this effect decreases with dilution (Figure 2).

Our results show that Refresh® and Refresh® Preservative-Free had the least *in vitro* cytotoxicity among the different solutions tested. The active ingredient of both brands is sodium carboxymethylcellulose (CMC). This suggests that CMC is perhaps the least toxic active ingredient. The relatively good performance of the only other product containing CMC, Thera-Tears®, is also supportive of this, as it outperformed all non-CMC products; except for Blink® Preservative-Free. The products with the most cytotoxicity were Blink® and Systane® in their preservative-containing formulations. The active ingredient in these products

is polyethylene glycol (PEG). However, because of the relatively good performance of the preservative-free formulations of these two brands, PEG is not likely the cause of the relative cytotoxicity of Blink® and Systane®. Rather, the cause is likely the preservatives in these two products.

Blink® uses the preservative OcuPure® (Sodium chlorite; stabilized oxychloro complex 0.005% m/v) and Systane® contains the preservative PolyQuad® (polyquaternium-1) 0.001%. If we examine the dilution-dependent performance of these two products, we note that there is dramatic increase in metabolic activity as we dilute the products to 50% and then 25%. This suggests that as the concentration of preservative decreases, the toxicity of these two products decreases dramatically as well. This agrees with the results from the preservative-free formulations of Blink® and Systane®, for which we find that there is little change in cytotoxicity as the products are diluted.

Interestingly, there is minimal decrease in cytotoxicity as the preservative in Refresh® is diluted. Refresh® uses Purite™ (stabilized oxychloro complex 0.005% m/v). Given the relatively low cytotoxicity of Refresh®, even at its full concentration, and the lack of change with dilution, it appears that Purite™ is, *in vitro* at least, possibly a less toxic preservative than those found in Systane® and Blink®. In addition, though to a greater degree with Systane® and Blink® than Refresh®, our results align with previous reports of preservative-free ocular solutions being generally less cytotoxic than preservative-containing ones. [6,8-11] Though clearly the preservative containing formulations performed worse in our study, the advantages of having preservative in regard to cost and shelf life justify their availability.

Examining the formulations without preservatives, we still observe that the CMC-containing products tend to be less cytotoxic than the PEG-containing products. The reasons for this are open to speculation. We do know that the mechanisms by which PEG and CMC lubricate the ocular surface are likely to be quite different. PEG is a charge-neutral polymer that is hydrophilic by virtue of the oxide moieties along its chain. By contrast, CMC is a derivative of cellulose that contains carboxymethyl groups (-CH_2-COOH) bound to some of the hydroxyl groups on its backbone. It is most often used as a sodium salt, in which case the polymer is negatively charged. This renders the material highly hydrophilic. By virtue of its charge, it mostly likely interacts in a different way from PEG with both the tear film and the ocular surface.

Prior work in corneal epithelial cytotoxicity has been generally focused on glaucoma medications.[6-8,10,11] Other studies have focused on the preservatives used in solutions. Polyquaternium, the preservative in Systane®, was found to not inhibit the cytokinetic movement or mitotic activity epithelial cells [9]. However another study showed polyquaternium increased inflammatory marker secretion *in vitro* [12]. Perborate, the preservative in Genteal®, was found to have similar toxicity to EDTA 0.01% [5]. Our study is the first to examine *in vitro* cytoxicity in a range of popularly used artificial tear medications.

We must be careful, however, to not equate the *in vitro* conditions of our study with the *in vivo* conditions of the human eye. Indeed some recent *in vivo* studies have not found products containing CMC to be superior to PEG [13,14]. The most that can be concluded from our study is that one particular formulation—that is, its ingredients in concert—is more or less cytotoxic than another in this particular *in vitro* setting. The application of these drops directly over a monolayer of cells is not entirely reflective of what happens *in vivo*. Finally, all of these formulations contain variations of buffering solutions that may influence the performance of the products in these assays, making it difficult to elucidate the exact cytotoxic profiles of the active ingredients themselves.

CONCLUSION

This *in vitro* comparison of popular artificial tears shows that preservative-free formulations are less cytotoxic to cultured corneal epithelial cells than their preservative-containing counterparts. Refresh® and Refresh® Preservative-Free were the least cytotoxic. Purite™ seems to be least cytotoxic preservative.

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