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2   **Effect of Artificial Tear Formulations on the Metabolic Activity of Human Corneal Epithelial**
3   **Cells after Exposure to Desiccation**
4
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30 **SUMMARY:**
31 The purpose of this protocol is to evaluate if different artificial tear formulations can protect
32 human corneal epithelial cells from desiccation using an in vitro model. After exposure to artificial
33 tear formulations and desiccation, human corneal epithelial cells are assessed for metabolic
34 activity.
35

36 **ABSTRACT:**
37 Artificial lipid-containing tear formulations are developed to reduce tear evaporation by the
38 restoration of a deficient tear lipid layer. Artificial tear formulations that prevent cell desiccation
39 will result in ocular surface protection and the maintenance of cell metabolic activity. During
40 dehydration, cells undergo the process of loss of metabolic activity and subsequently cell death.
41 This work describes a method for assessing the efficacy of artificial tear formulations. The
42 metabolic dye (i.e., alamarBlue) changes from a low fluorescent molecule resazurin to a
43 fluorescent molecule resorufin in viable cells. The biological performance of an artificial tear
44 formulation is measured as the ability of the formulation to (a) maintain cell viability and (b)

45   provide cell protection from desiccation. Growth media and saline are used as controls for the
46   cell viability/desiccation tests. Cells are incubated with test solutions for 30 min and then
47   desiccated for 0 or 5 min at 37 °C and 45% relative humidity. Cell metabolic activity after initial
48   exposure and after cell desiccation is then determined. The results show the comparative effects
49   of eye drop formulations on cell metabolic activity and desiccation protection. This method can
50   be used to test dry eye formulations that are designed to treat individuals with evaporative dry
51   eye.
52
53   **INTRODUCTION:**
54   Many different ingredients in multiple-dose ophthalmic solutions are needed to maintain
55   stability, pH, osmolarity, and efficacy. However, some chemicals such as preservatives or
56   surfactants in ocular solutions can cause damage to the corneal epithelium[1,2]. Cell viability tests
57   using human corneal epithelial cells (HCEC) can be performed to assess the potential damage
58   caused by these various ingredients in ophthalmic formulations. One of the in vitro assays that is
59   particularly useful for assessing cell damage is the alamarBlue assay[3]. In this assay, the metabolic
60   dye (i.e., alamarBlue) contains resazurin, which functions as a cell viability indicator. During
61   cellular respiration, resazurin is reduced to resorufin by accepting electrons[4]. The reduction of
62   resorufin causes a change from a non-fluorescent molecule to a fluorescent molecule that can
63   be detected using a spectrofluorometer[5].
64
65   This investigation uses two different treatments of the corneal epithelial cells to determine how
66   dry eye products may perform in an in vitro model. In the first evaluation, the cells are treated
67   with these products for 30 min. After treatment, the cells are then evaluated for metabolic
68   activity immediately after exposure to the dry eye products and after a recovery time interval.
69   This assessment determines the effect of these products on cells prior to desiccation in a
70   humidity chamber. Solution preservatives, surfactants or buffers in these solutions may have
71   some cytotoxic effects on the cells that can be detected using the metabolic dye.
72
73   The second evaluation determines the metabolic activity of the cells after exposure to the dry
74   eye products and desiccation. In this assessment, if the product treatment offers protection to
75   the cells from desiccation damage, the metabolic activity of the cells will be maintained. Cell
76   protection from the harmful effects of desiccation may occur if the dry eye product can coat the
77   cells with lipids or the product may absorb liquid from the surroundings adding moisture to the
78   cells.
79
80   This protocol is designed to simulate severe conditions of environmental stress on cells. Other
81   studies have used various models to create this external stress. Some studies have dried the cells
82   in laminar flow hoods[6-8], whereas others have used room temperature and various relative
83   humidity (RH) values[9-11]. A method of simulating dry eye conditions is to increase the osmolarity
84   of the incubating solution[12,13]. Another in vitro method for simulating adverse environmental
85   conditions is to add cytokines to the cell media to simulate the tear fluid of dry eye patients[14].
86   Although these tests are interesting models that can evaluate how chemicals reduce osmotic
87   stress or stimulate the immune system, these models do not directly show how chemical
88   formulations can protect cells from desiccation stress.

89

90 The desiccation model uses a temperature/humidity chamber 37 °C and 45% RH. It simulates
91 environmental conditions that can cause evaporation from the ocular surface. Simulations of
92 other extreme environmental conditions such as the low humidity found in airplane cabins[15] or
93 in indoor environments during the winter months[16] can also be performed using this method.

94

95 Cell culture modeling is used to simulate the effect of desiccation stress on the human cornea. In
96 order to detect cell stress, cell viability testing is undertaken to determine the impact on cell
97 health. Several different physiological tests can be performed on cells to determine if they are
98 stressed[17]. The analysis performed in this test procedure uses the commercially available
99 metabolic dye (**Table of Materials**). It has the advantage over many other cell stress tests in that
100 it is non-toxic, soluble in growth media and can evaluate cells without cell fixation[18]. In this
101 procedure the cells are tested for metabolic activity immediately after each treatment and a
102 second set of cells are placed back into growth media and evaluated after 18 h recovery. The
103 reason for testing immediately and then after a recovery period is to identify cell damage that
104 causes immediate effects on metabolic activity and cell damage that causes delayed effects. Also,
105 it provides an opportunity to evaluate the impact of these formulations on short-term versus
106 long-term damage and the ability of the formulations to recover/not recover from the initial
107 insult.

108

109 This investigation is used to compare various lipid-containing dry eye products for their effect on
110 HCEC metabolic activity with and without desiccation. The ingredients in the dry eye products
111 tested are described in **Table of Materials**. The ingredients in solution #1 are
112 carboxymethylcellulose sodium (0.5%), glycerin (1%), and polysorbate 80 (0.5%), in solution #2
113 they are light mineral oil (1.0%) and mineral oil (4.5%), and in solution #3 the lipid is propylene
114 glycol 0.6%. These lipids are the formulation components that are expected to provide protection
115 of the HCEC from desiccation.

116

117 **PROTOCOL:**

118

119 **1. Human corneal epithelial cell culture**

120

121 1.1. Grow immortalized HCEC[19] in collagen-coated 75 cm^2 flasks with 20 mL of Dulbecco's
122 modified Eagle medium/nutrient mixture F-12 (DMEM/F12) containing 10% fetal bovine serum
123 (FBS) and 1% penicillin/streptomycin at 37 °C with 5% CO_2.

124

125 NOTE: Shaking is not required.

126

127 1.2. Change the media every 2−3 days.

128

129 **2. Preparation of cells for testing**

130

131 2.1. After the cells are almost confluent remove culture media.

132

133 2.2. Add 4–6 mL of cell disassociation solution to each 75 cm^2 flasks. Incubate the cells at 37 °C
134 until the cells detach (approximately 20–30 min), checking under the microscope periodically.

136 2.3. Add 2–6 mL of DMEM/F12 containing 10% FBS to each 75 cm^2 flask.

138 2.4. Transfer the contents of the flask to a 50 mL centrifuge tube.

140 2.5. Centrifuge the cells at 450–500 x g for 5 min. Pipet out the supernatant and resuspend the
141 cells in pre-warmed media.

143 2.6. Count the cells using a hemocytometer. Calculate the volume of media that contains a total
144 of 1 x 10^5 cells.

146 2.7. Add 1 x 10^5 cells to each well of a 48 well collagen-1 coated culture plate. Add enough media
147 to the well so that the final volume of media in the well is 0.5 mL of culture DMEM/F12 with 10%
148 FBS.

150 NOTE: An artificial tear formulation may cause cytotoxicity that can be measured by a decrease
151 in the metabolic activity of HCEC. The consistency of the data is dependent on adding the same
152 number of HCEC to each well when seeding. The culture concentration that is recommended for
153 48 well plates is 1 x 10^5. Because mammalian cells can settle in the centrifuge tube after
154 resuspending, it is recommended to resuspend the cells by agitation frequently while seeding the
155 plates with cells so that the cell suspension has the HCEC equally distributed.

157 2.8. Incubate the cells at 37 °C and 5% CO_2 for 24 h.

159 **3. No desiccation protocol**

161 3.1. Control procedure

163 3.1.1. After the 24 h incubation remove the culture media from the plate.

165 3.1.2. Immediately treat with 150 µL of a test formulation and media control solution.

167 3.1.3. Incubate the cells at 37 °C and 5% CO_2 for 30 min.

169 3.1.4. Remove the test solutions from the cells.

171 3.1.5. Add 0.5 mL of 10% metabolic dye solution to test for metabolic activity. Incubate the cells
172 for another 4 h at 37 °C and 5% CO_2.

174 3.1.6. After the 4 h incubation period, remove 100 µL of metabolic dye solution from each well
175 and place each 100 µL into a well of a 96 well plate.

176

177 3.1.7. Measure the fluorescence of each well using a fluorescent plate reader (**Table of**
178 **Materials**). Set the excitation wavelength at 540 nm and the emission wavelength at 590 nm.
179
180 3.2. No desiccation protocol (recovery procedure)
181
182 3.2.1. Repeat steps 3.1.1–3.1.4.
183
184 3.2.2. Add 0.5 mL of DMEM/F12 media to each well.
185
186 3.2.3. Incubate one set of cultures for 18 h at 37 °C and 5% CO_2.
187
188 3.2.4. Remove the media and add 0.5 mL of 10% metabolic dye solution. Repeat steps 3.1.6 and
189 3.1.7.
190
191 **4. Desiccation protocol**
192
193 4.1. Control procedure
194
195 4.1.1. After the 24 h incubation (step 2.8), remove the culture media from the plate.
196
197 4.1.2. Immediately treat with 150 µL of a test formulation and media control solution.
198
199 4.1.3. Incubate the cells at 37 °C and 5% CO_2 for 30 min.
200
201 4.1.4. Remove the test solutions from the cells.
202
203 4.1.5. Place the cells in a 37 °C and 45% RH chamber for 5 min to desiccate the cells.
204
205 4.1.6. Add 0.5 mL of 10% metabolic dye solution to test metabolic activity. Incubate the cells for
206 another 4 h at 37 °C and 5% CO_2.
207
208 4.1.7. After the 4 h incubation period, remove 100 µL of metabolic dye solution from each well
209 and place each 100 µL into a well of a 96 well plate.
210
211 4.1.8. Measure the fluorescence of each well using a fluorescent plate reader. Set the excitation
212 wavelength at 540 nm and the emission wavelength at 590 nm.
213
214 4.2. Recovery procedure
215
216 4.2.1. Repeat steps 4.1.1-4.1.5.
217
218 4.2.2. Add 0.5 mL of DMEM/F12 media to each well.
219
220 4.2.3. Incubate one set of cultures for 18 h at 37 °C and 5% CO_2.

221

222   4.2.4. Remove the media and add 0.5 mL of 10% metabolic dye solution. Repeat steps 4.1.7 and
223   4.1.8.

224

225   **5. Data analysis**

226

227   5.1. Calculate the average fluorescence of the media control. Calculate the percent viability of
228   the sample using the follow formula:

229

230   Percent viability of the sample = test sample fluorescence/media control fluorescence x 100

231

232   NOTE: The media control average is 100% viability. The metabolic dye by itself has a small amount
233   of fluorescence associated with it. The fluorescence of the metabolic dye reagent can be
234   subtracted from the control and test sample fluorescence prior to calculating % viability of the
235   test samples to the media control.

236

237   5.2. Perform a normality test and a test for equal variances on the control and test samples.

238

239   5.3. If the normality test passes and the variances are equal then perform a one-way ANOVA. To
240   identify significant differences between specific groups perform a pairwise comparisons post hoc
241   test (i.e., Tukey or Dunnett's multiple comparisons tests).

242

243   5.4. If the normality test passes and the variances are different then perform the Welch's ANOVA.
244   To identify significant differences between specific groups perform a pairwise comparisons post
245   hoc test (i.e., Dunnett's or Games-Howell multiple comparisons tests).

246

247   5.5. If the normality test fails use a nonparametric test (i.e., Kruskal-Wallis test). To identify
248   significant differences between specific groups perform a pairwise comparisons post hoc test
249   (i.e., Dunn's test).

250

251   **REPRESENTATIVE RESULTS:**
252   Results from a study comparing three dry eye formulations are shown in **Figure 1**. This figure
253   shows that there are differences in the effect the three products, Solution #1, solution #2 and
254   solution #3 (**Table of Materials**) have on the viability of HCEC. Solution #1 and solution #2 had a
255   significant effect on the metabolic activity of HCEC before desiccation. This means that there are
256   formulation components in these products that can disrupt the metabolic activity of the HCEC.
257   The additional drop in cell metabolic activity that occurred after 18 h recovery of cells exposed
258   to solution #1 shows that HCEC were injured initially and after 18 h the injury was not repaired.
259   In comparison, solution #3 had only a mild effect on the metabolic activity of the HCEC.

260

261   **Figure 2** shows the effect of these lipid-containing dry eye formulations on cell protection. With
262   a near 0% HCEC metabolic activity at the 18 h recovery interval, solution #1 and solution #2 did
263   not protect cells from desiccation stress. Solution #3, however, offered some protection against
264   desiccation stress.

265

FIGURE LEGENDS:

Figure 1: Effect of dry eye lipid enhanced products on cell viability. Relative viability measured by HCEC metabolic activity after treatment with lipid-containing dry eye products. * = statistical significance ($p < 0.05$) relative to solution #3, Δ = statistical significance ($p < 0.05$) relative to the media control for the appropriate recovery period. The height of the bar represents the mean value, and the error bars are the SD.

Figure 2: Effect of dry eye lipid enhanced products on desiccation protection. Effect of lipid-containing dry eye formulations on cell protection. Solution #1 and solution #2 did not protect cells from desiccation stress, and they did not recover from desiccation effects over time. In contrast, solution #3 offered some protection against desiccation stress. * = $p < 0.05$ relative to solution #3, and Δ = $p < 0.05$ relative to untreated (control) media for the appropriate recovery time. The height of the bar represents the mean value, and the error bars are the SD.

DISCUSSION:

This protocol is designed to determine the protective effects of artificial tear solutions against cell desiccation. Initially, the cells are exposed to the dry eye drops to coat the cells. Next, the cells are dried, and the metabolic activity is monitored to assess whether the formulations can mitigate the detrimental effects of desiccation stress. These steps are critical for understanding the overall impact the dry eye formulations could have on the overall viability of corneal epithelial cells.

After most of the dry eye formulations are removed from the culture wells, the chemistry of the remaining molecules that are in contact with the cells will impact cell desiccation. Some products contain micro-emulsions of oils that minimize cell evaporation[20]. A study that evaluated friction analysis after rinsing showed maintenance of low friction for some dry eye formulations which may be an indicator of the enhanced residence time of the dry eye formulations[21]. One of the limitations of this assay is that although desiccation protection can be evaluated in comparison to other eye drop formulations the duration of this protection cannot be determined due to the short drying time assessed. Longer drying times in this in vitro assay may require the use of multilayer cultures that have overall more moisture content than a cell monolayer.

The specific incubation times for the formulations on the cells and the duration of drying time may need to be adjusted if temperature and humidity values are changed. Selecting a temperature, RH and air flow for simulating environmental stress conditions that can contribute to dry eye can be difficult as the seasonal and local environmental conditions are quite variable[21]. Modern-day environment chambers for human studies can vary the temperature between 5 °C and 35 °C, and RH between 5% and 95%[22]. Using an evaporimeter and tightly fitted goggles it was determined that at RH of 40–45% the evaporation rate from the cornea was 0.037 $\mu L/cm^2/min$ and at RH of 20–25% the evaporation rate was 0.065 $\mu L/cm^2/min$[23]. If the in vitro model is looking at the protective effect of formulations under extremely low RH conditions such as in airplanes[15], deserts, or dry indoor environments[16], incubation times may need to be reduced to accommodate the enhanced evaporation rates from the cells. In the human tear fluid, a variety

309 of lipids derived from meibomian and other glands are present that protect the tears from
310 evaporation. These lipids have different melting points[24]. Temperature changes could impact the
311 fluidity of the tear fluid so tests performed at ambient temperature could have significantly
312 different evaporation rates than tests conducted at 37 °C. Since the average ocular surface
313 temperature range is from 32.9 to 36 °C[25], performing the test near this temperature range is
314 recommended.

316 In addition to the metabolic dye (alamarBlue) used in this protocol, there are other chemical
317 reagents that can be used to assess the effects of product formulations on cell metabolic activity.
318 The tetrazolium salts (MTT, XTT, MTS, WST) are alternative chemicals that can be used. The
319 difference in the tetrazolium salts is that MTT is a positively charged molecule so it can enter the
320 cytoplasm of living cells[26]. MTT becomes insoluble after its reduction by NAD(P)H[27]. It must be
321 solubilized prior to reading absorbance on a plate reader. The other tetrazolium salts are
322 negatively charged[28]. They must be reduced by secondary molecules outside the living cell
323 because they cannot enter the cell and interact directly with intracellular molecules[29]. For the
324 XTT, MTS, and WST-1 assays, the addition of an electron coupling agent 1-methoxy phenazine
325 methosulfate (PMS) can improve the performance of the assays[30]. In contrast to the metabolic
326 assays that use tetrazolium salts, alamarBlue does not require an additional solubilizing or
327 electron coupling agents. Also, unlike XTT, MTS or WST-1, alamarBlue penetrates cell membranes
328 and can be reduced directly by cellular enzymes[27,29]. Direct comparison of alamarBlue to
329 tetrazolium salts have shown that alamarBlue is more sensitive for evaluating the metabolic
330 activity of various cell lines including HCEC[3,27,31,32].

332 Other biochemical endpoints can also be considered for evaluating the protection of human
333 corneal epithelial cells by desiccation. Fluorescent dyes can be used to detect cell membrane
334 permeability and cell esterase activity[1]. Also, apoptosis can be detected by staining for apoptotic
335 markers on the cell surface or by measuring for caspase activity[1,33]. Other assays have determined
336 the effects of ophthalmic products on HCEC tight junctions[34] . These assays could be incorporated
337 in future assessments utilizing the desiccation procedures described in this article.

339 There are many causes of dry eye. Dry eye can be caused by decreased secretion of tears,
340 increased ocular surface inflammation or increased dehydration at the ocular surface. For the
341 individuals that have evaporative dry eye use of a dry eye drop that prevents their cells from
342 drying during desiccation conditions may alleviate the symptoms of dry eye. One of the problems
343 in some dry eye formulations is the presence of preservatives that may harm the ocular surface.
344 Injuring cells is not helpful to dry eye patients as it can cause more cells to die as injured cells are
345 more susceptible to desiccation stress[35].

347 A recent review article by Garrigue et al.[36] provides an assessment of the current knowledge of
348 how lipid-based products act to alleviate evaporative dry eye. Adding lipids to lipid deficient tears
349 can prevent evaporation, thus protecting the cells from desiccation. In addition, humectants are
350 molecules that attract and retain water at the ocular surface[37]. Both glycerin in solution #1 and
351 propylene glycol in solution #3 are humectants. Solution #3 showed a greater protective effect
352 of the HCEC that may have been due to the lipid and humectant properties of the formulation

ingredients.

This protocol is designed to evaluate the metabolic activity of HCEC after exposure to the dry eye formulations and to desiccation stress. By using the methods described in this article, new nonirritating and protective eye drops can be developed for people that suffer from evaporative dry eye.

DISCLOSURES:
Three authors (Rekha Rangarajan, Howard A. Ketelson, Lakshman Subbaraman) are employees of Alcon. Alcon is the sole source of funding and sponsor of this project. Lyndon W. Jones, over the past 3 years, has received research support or lectureship honoraria from the following companies: Alcon, Allergan, Contamac, CooperVision, GL Chemtec, Inflamax Research, J&J Vision, Menicon, Nature's Way, Novartis, PS Therapy, Santen, Shire, SightGlass and Visioneering. Lyndon Jones is also a consultant and/or serves on an advisory board for Alcon, CooperVision, J&J Vision, Novartis and Ophtecs. The following list of authors have nothing to disclose: Richard Do, David J. McCanna, Adeline Suko, Daryl Enstone, Jaya Dantam.
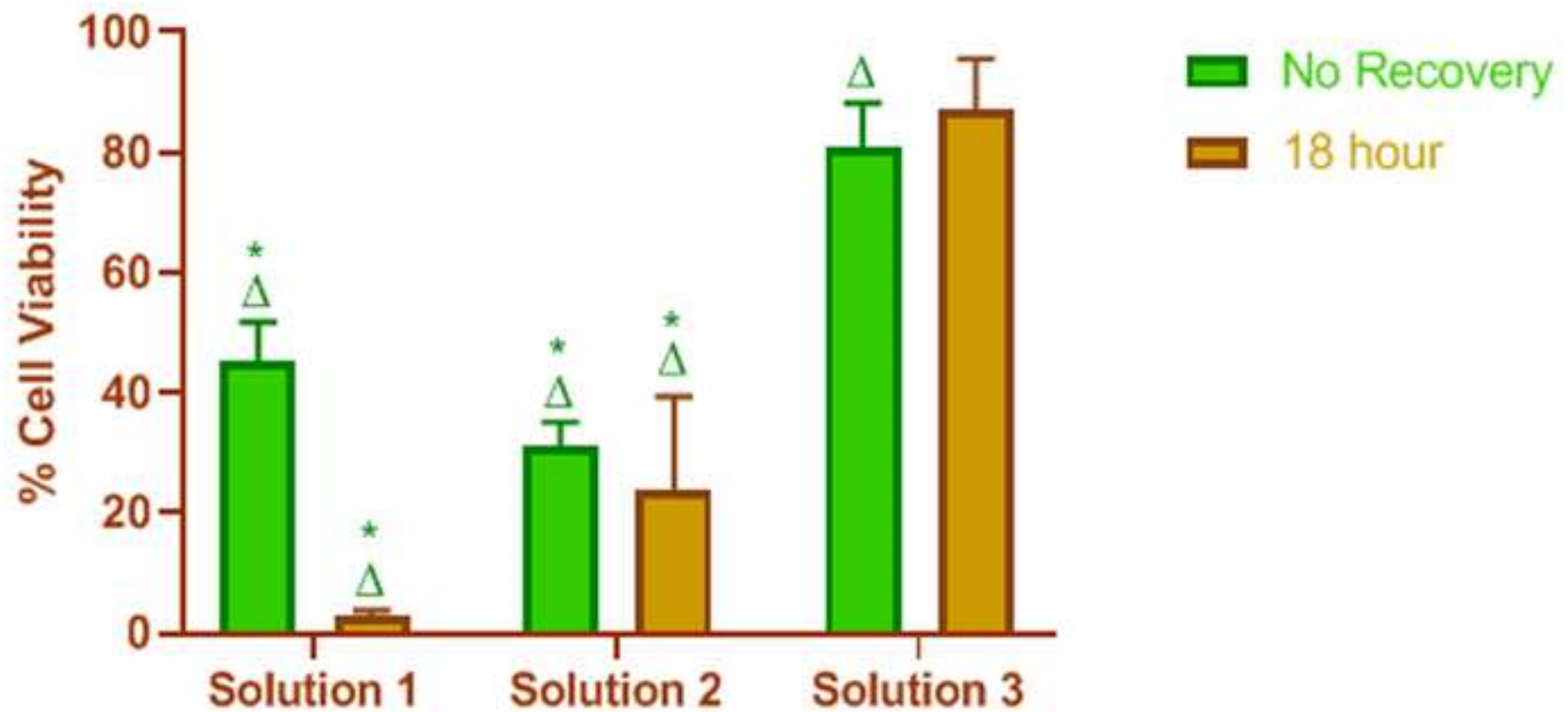
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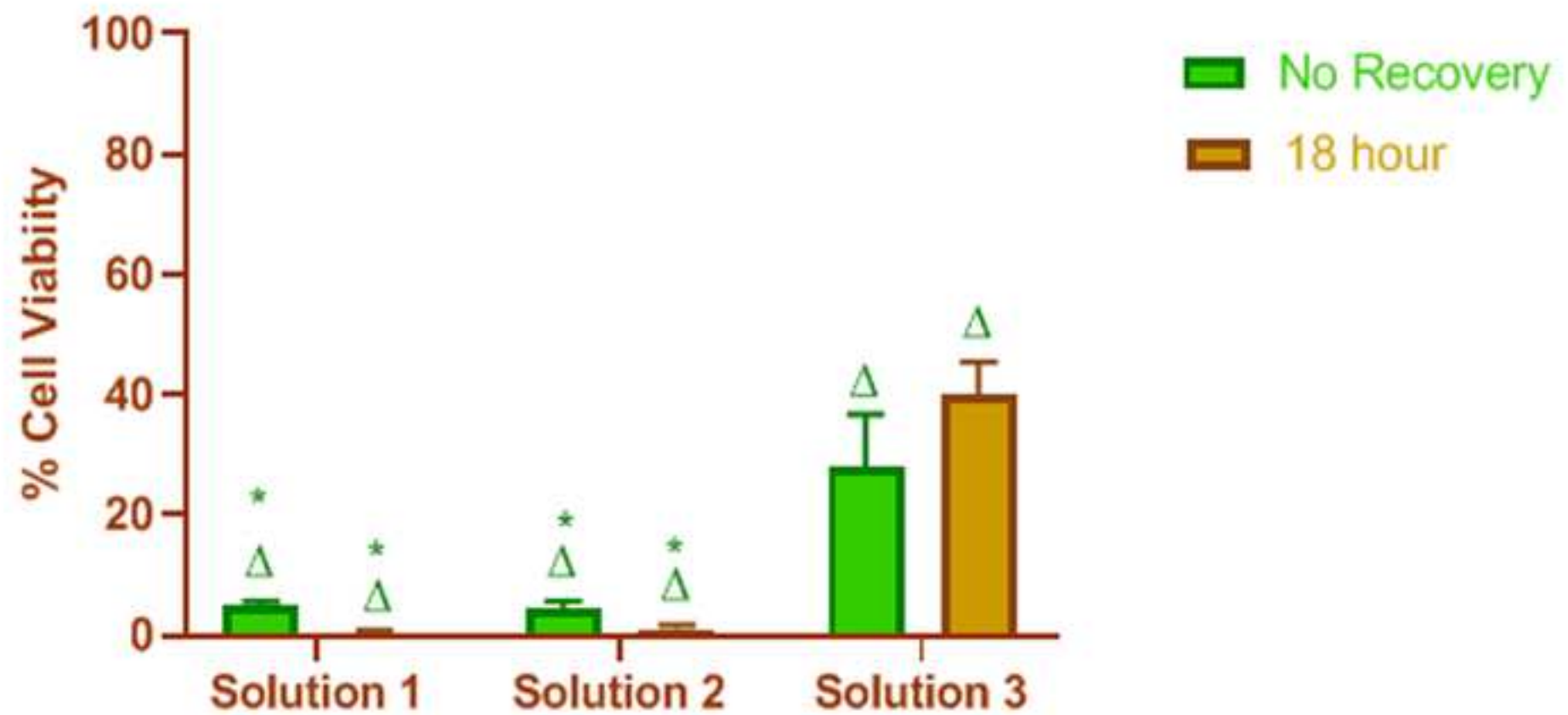
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Figure 1

Effect of Dry Eye Products on Cell Viabilty

Figure 2

Desiccation Protection

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
75 cm^2 Vented Flask	Corning	354485	This is the BioCoat brand collagen coated
96 well plate	costar	3370	
alamarBlue	Fisher Scientific	dal 1025	
Corning 48 Well plates	Corning	354505	This is the BioCoat brand collagen coated
Cytation 5	BioTek	CYT5MPV	Can read fluorescence from 280 - 700 nm (for assay 540/590)
DMEM/F12 with L-glutamine and 15 mM HEPES	Gibco	11039-021	There is No Phenol Red in this media
Fetal Bovine Serum	Hycone	SH30396.03	
Human Corneal Epithelial Cells	University of Ottawa	N/A	SV40-immortalized human corneal epithelial cells from Dr. M. Griffith (Ottawa Eye Research Institute, Ottawa, Canada) and have been characterized Griffith M, Osborne R, Munger R, Xiong X, Doillon CJ, Laycock NL, Hakim M, Song Y, Watsky MA. Functional human corneal equivalents constructed from cell lines. Science. 1999;286:2169–72.
Humidity/Temperature Chamber	Associated Environmental Systems	LH-1.5	Economy Line Humidity Chamber
Penicillin Streptomycin	Gibco	15140-122	100X concentration so add 1ml to each 99ml of media
Refresh Optive Advanced (solution #1)	Allergan, Inc		Carboxymethylcellulose Sodium (0.5%),Glycerin (1%),polysorbate 80 (0.5%),-Lubricants- Inactive ingredients-boric acid; carbomer copolymer type A; castor oil; erythritol; levocarnitine; purified water; Purite (stablized oxychloro complex)and may contain sodium hydroxide to adjust pH.

Soothe XP (solution #2)	Bausch & Lomb	Light mineral oil (1.0%), Mineral Oil (4.5%)-emolients-Inactive ingredients-boric acid, edetate disodium, octoxynol-40, polyquaternium-1 (preservative), polysorbate 80, purified water, sodium borate decahydrate. May contain hydrochloric acid and/or sodium hydroxide (to adjust pH).
Systane Complete (solution #3)	Alcon Inc.	Propylene Glycol 0.6% Lubricant Inactive ingredients-boric acid, dimyristoyl phosphatidylglycerol, edetate disodium, hydroxypropyl guar, mineral oil, polyoxl 40 stearate, POLYQUAD® (polyquaternium-1) 0.001% preservative, sorbitan tristearate, sorbitol and purified water. May contain hydrochloric acid and/or sodium hydroxide to adjust pH.
TrypLE Express (cell disassociation ș	Fisher Scientific	12605036

Xiaoyan Cao, Ph.D. Review Editor
Journal of Visualized Experiments (JoVE)
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Cambridge, MA 02140

Subject: Submission of revised manuscript

Revision Submission Date: January 2, 2020

Dear Dr. Cao,

Thank you for reviewing our paper. Please find below our comments on the few remaining
questions from Reviewer #2 on this manuscript.

Reviewer #2:

Thank you for your helpful comments. We have addressed your remaining questions on this
manuscript below.

<u>Question:</u> *the authors described, they found a new system to evaluate cellular metabolism,
could this method also useful in a dry eye mouse model?*

<u>Response:</u> This method may be useful in a dry eye mouse model that utilizes mouse *ex-vivo*
corneas in a controlled environmental chamber. In our *in-vitro* dry eye model, human corneal
epithelial cells were grown and then exposed to treatment and desiccation stress. AlamarBlue
was then added to the cells to measure for cell metabolic activity. AlamarBlue could also be
added to mouse *ex-vivo* corneas that are exposed to eye drops and desiccation stress to assess for
the metabolic activity of the corneal epithelial cells.

<u>Question:</u> *Although the authors discussed some other chemical reagents to assess cell
viability, but they did not provide direct data to compare them.*

<u>Response:</u> The chemical in alamarBlue that detects cell viability is resazurin whereas tetrazolium
salts are in MTT, XTT and WST-1. Non-fluorescent resazurin is reduced by viable cells to
fluorescent resorufin. Colorless tetrazolium salts are reduced by viable cells to colored
formazans[1]. These viability reagents have been directly compared for their use in detecting cell
metabolic activity. AlamarBlue showed greater sensitivity than MTT for measuring the
metabolic activity of HCEC[2]. In a study that evaluated cell viability of 6 carcinoma cell lines
after toxic chemical exposure, alamarBlue was more sensitive and showed less intra-assay
variability than XTT[3]. A resazurin based assay was also shown to be significantly more sensitive
than WST-1 when assessing the viability of immune cells.[4]

We have added the following to the discussion section of this article on lines 334.

Direct comparison of alamarBlue to tetrazolium salts have shown that alamarBlue is more sensitive for evaluating the metabolic activity of various cell lines including HCEC.[1-4]

Question: *As the metabolic dye alamarBlue, is it a commercial reagent or the authors first developed it? This needs further explanation in INTRODUCTION part.*

Response: Line 110 We added that alamarBlue is a commercially available viability reagent.

Question: *According to the results, it seems that solution #3 is protective in desiccation model. Why? What is the reason for the differences in the protection role between 3 solutions? This needs further explanation.*

Response: A recent review paper by Garrigue et al. provides an assessment of the current knowledge of how lipid-based products act to alleviate evaporative dry eye[5]. Adding lipids to lipid deficient tears can prevent evaporation, thus protecting the cells from desiccation. In addition, humectants are molecules that attract and retain water at the ocular surface[6]. Both glycerin in solution #1 and propylene glycol in solution #3 are humectants. Solution #3 showed a greater protective effect of the HCEC that may have been due to the lipid and humectant properties of the formulation ingredients.

The paragraph above has been added to line 352 of this manuscript.

Sincerely,

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