行政院國家科學委員會專題研究計畫   成果報告

對眼部組織細胞毒性之實驗性研究

計畫類別：個別型計畫
計畫編號：
執行期間：年 月 日至 年 月 日
執行單位：國立成功大學醫學系眼科

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中華民國 年 月 日
Triamcinolone acetonide suspension toxicity to corneal endothelial cells

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PURPOSE: To investigate the cytotoxicity of triamcinolone acetonide (TA) suspensions to corneal endothelial cells (CECs).

SETTING: Department of Ophthalmology, Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan.

METHODS: New Zealand white rabbit CECs were exposed for 1 minute to balanced salt solution (BSS); commercial TA suspension (cTA); vehicle-removed TA (–vTA); pure vehicle (V); 1/10 dilutions of cTA, –vTA, or V in BSS; or benzyl alcohol (BA) (cTA preservative) 9 mg/mL. Corneal endothelial cell toxicity was assessed by light microscopy (trypan blue staining) and transmission electron microscopy. The effects of 3-, 10-, or 30-minute exposures to 1/10 cTA, 1/10 –vTA, or V were also investigated.

RESULTS: One-minute exposures to –vTA or 1/10 –vTA did not damage CECs; however, cTA, V, or 1/10 dilutions of cTA or V caused damage and cells exposed to BA showed severe ultrastructural damage/lysis. A 30-minute exposure to 1/10 –vTA did not cause significant cell damage, whereas 3- to 30-minute exposures to 1/10 cTA or V showed significant time-dependent cytotoxicity.

CONCLUSIONS: Commercial TA suspension was cytotoxic to cultured rabbit CECs because of the preservative, BA, in the vehicle. Because 1/10 –vTA appeared to be safe for up to 30 minutes of exposure, use of 1/10 dilutions of vehicle-removed TA is suggested to help surgeons visualize prolapsed vitreous during anterior vitrectomy in complicated cataract surgeries.

Table 1. Test solutions prepared from the commercial triamcinolone acetonide suspension (Kenacort-A) (cTA) or BA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Solution</th>
<th>TA (mg/mL)</th>
<th>Preservative (BA) (mg/mL)</th>
<th>Osmolality Measured* (mOsm/kg)</th>
<th>pH Measured†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>BSS</td>
<td>0</td>
<td>0</td>
<td>305</td>
<td>7.4</td>
</tr>
<tr>
<td>Solution 1</td>
<td>Commercial TA (cTA)</td>
<td>40</td>
<td>9</td>
<td>326</td>
<td>6.2</td>
</tr>
<tr>
<td>Solution 2</td>
<td>Vehicle-removed TA in BSS</td>
<td>~40</td>
<td>0</td>
<td>295</td>
<td>7.7</td>
</tr>
<tr>
<td>Solution 3</td>
<td>Pure vehicle (V)</td>
<td>0</td>
<td>9</td>
<td>321</td>
<td>6.1</td>
</tr>
<tr>
<td>Solution 4</td>
<td>1/10 dilution of cTA in BSS</td>
<td>4</td>
<td>0.9</td>
<td>306</td>
<td>7.5</td>
</tr>
<tr>
<td>Solution 5</td>
<td>1/10 dilution of –vTA in BSS</td>
<td>~4</td>
<td>0</td>
<td>298</td>
<td>7.3</td>
</tr>
<tr>
<td>Solution 6</td>
<td>1/10 dilution of V in BSS (1/10 V)</td>
<td>0</td>
<td>0.9</td>
<td>305</td>
<td>6.8</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>BA 9 mg/mL in BSS</td>
<td>0</td>
<td>306</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BA = benzyl alcohol; BSS = balanced salt solution

*Osmolality measured by Osmometer, model 3D3 (Advanced Instruments)
†pH measured by pH meter pH211 (Hanna Instruments)

salt solution (BSS) (~vTA). Solution 3 was the pure vehicle of cTA (V). Solutions 4, 5, and 6 were 1/10 dilutions of solutions 1, 2, and 3, respectively, in BSS. To prepare solutions 2 and 3, 12 mL of cTA was centrifuged (4000 rpm for 10 minutes) to separate the TA particles from the vehicle.12 The sediment of TA particles (about 1 mL) was resuspended in BSS to achieve the initial volume of 12 mL. Centrifugation and resuspension in BSS were repeated once to obtain solution 2 (~vTA). Then, the supernatant of vehicle (approximately 11 mL) from the first centrifugation of cTA was passed through 0.2 μm pore Millipore filters to obtain solution 3 (V), consisting of pure vehicle without TA particles. Diluting 1 mL of solution 1, 2, or 3 with 9 mL of BSS yielded solution 4 (1/10 cTA), solution 5 (1/10 ~vTA), and solution 6 (1/10 V), respectively.

To further clarify the effects cTA might have on CECs, a laboratory preparation of the main ingredient of the vehicle, BA, was diluted with BSS to achieve a concentration, 9 mg/mL, that was identical to the concentration of BA in cTA.

Cell Culture

New Zealand white rabbits weighing 1.0 to 1.5 kg were killed by intravenous injection of phenobarbital, and CECs were obtained from the excised corneas using a method that has been described.10,11 The cells from 8 animals (16 corneas) were combined to inoculate 8, 3 cm culture dishes and were incubated in a 5% carbon dioxide–humidified atmosphere at 37°C. On day 3, the medium was changed and Descemet’s membrane fragments were discarded. When cells reached confluence (about day 7), cells in each dish were divided into 4 or 6 portions (depending on the size of the cell monolayer) and subcultured in the same medium in 3 cm dishes. Subcultures (passage 1) grew to confluence in about 4 days and were subcultured again in the same medium in 3 cm culture dishes (passage 2). The cell monolayers that grew from passage 2 were used for experiments.

Exposure to Solutions

In the first experiment, cultures of CECs were exposed to the control (BSS) or test solution by removing the culture medium from the dish and adding 0.5 mL of the solution to overlay the monolayer of endothelial cells. After 1 minute, the solution was carefully washed from the cell culture and the procedure to determine percentage of cells damaged was begun. Each exposure was performed in quadruplicate and the mean used for analysis.

In the second experiment, the cytotoxicity of each solution was evaluated over time by exposing cell cultures to BSS, V, 1/10 cTA, or 1/10 ~vTA for 1 (control) for 3, 10, or 30 minutes. Each exposure was performed in quadruplicate and the mean used for analysis.

Determination of Percentage of Damaged Cells

The trypan blue staining method of Chang et al.10,11 was used to determine the percentage of cells in each CEC culture that were damaged by exposure to solutions. After CEC exposure, the confluent layer of cells was washed with phosphate-buffered saline (PBS) and trypsinized. Trypsinization was halted by addition of fetal bovine serum to the cell suspension to a final concentration of 10%. Trypan blue 0.2% was added for 5 minutes to stain dead and dying cells.

The numbers of stain-positive and stain-negative cells in each culture were counted using a hemocytometer chamber. The mean of the percentages of stain-positive cells in cultures in each experimental group was compared to the mean in the control cultures for that experiment. Differences were evaluated using a 2-tailed Student t test. A P value less than 0.05 was considered statistically significant.
Structural Changes in Rabbit Corneal Endothelial Cells

When counted in the hemocytometer chamber, cells were also evaluated by light microscopy (LM) for their size and shape and for the integrity of the cell membrane, cytoplasm, and nucleus. Ultrastructural changes were evaluated by transmission electron microscopy (TEM) (JEM-1200EX, JEOL). The CECs were cultured in 4-slide chambers and exposed for 1 minute to BSS, cTA, V, or BA. The cell monolayer was washed with PBS and, without trypsinization, was fixed with 2.5% glutaraldehyde–0.5% paraformaldehyde for 4 hours. After they were rinsed in 0.1 M PBS, cells were osmicated, dehydrated, and embedded in epoxy resin and examined by TEM.

RESULTS

Damage to Corneal Endothelial Cells from 1-Minute Exposures

In the control group, 5.9% of cells stained with trypan blue and stained cells had the same size and shape as unstained cells (Figure 1, A). Cultures exposed for 1 minute to –vTA or 1/10 –vTA showed relatively intact cellular morphology (Figure 1, C and F). In contrast, the trypan blue-stained cells in cultures exposed for 1 minute to cTA or V showed serious abnormalities (variation in size, irregular cell membranes, cell swelling, and cell lysis) (Figure 1, B, D, E, and G). The CECs exposed to BA 9 mg/mL were so extensively damaged that the percentage of cells damaged could not be determined by viewing in the hemocytometer (Figure 1, H).

The percentages of damaged cells were similar in control cultures and cultures exposed to –vTA (6.9%; P = .23) or 1/10 –vTA (6.4%; P = .54) (Figure 2). In contrast, cultures exposed to solutions with full-strength or diluted vehicle had significantly higher percentages of damaged cells (cTA, 16.4%; V, 13.7%; 1/10 cTA, 9.5%; 1/10 V, 8.6%) (P<.05).

Among cultures exposed to undiluted solutions, cTA damaged more cells than –vTA (P<.01), but the percentages were similar for V and cTA (P = .21). Similarly, among diluted solutions, 1/10 cTA damaged more cells than 1/10 –vTA (P = .02), but 1/10 V damage was similar to 1/10 cTA damage (P = .29).

Regarding the dose effect, the percentage of damaged cells was significantly higher in cultures exposed to undiluted cTA than to 1/10 cTA (P = .03), and the percentage was also higher in cultures exposed to undiluted V (vehicle alone) than to 1/10 V (P<.01). However, there was no dose response for cultures exposed to vehicle-removed solutions (–vTA and 1/10 –vTA) (P = .59). These results show a dose-dependent cytotoxic response to solutions containing cTA or its vehicle, but not to TA alone in BSS.

Ultrastructural Changes After 1-Minute Exposures

On TEM, CECs in control cultures had intact cellular membrane, nucleus, and organelles; however, CECs exposed to cTA, V, or BA 9 mg/mL had various degrees of morphologic damage including significant swelling and disorganization of organelles, disruption of the cell membrane, cell lysis, and release of intracellular organelles extracellularly (Figure 3).

Damage to Corneal Endothelial Cells with Longer Exposures

Figure 4 shows the percentages of cells damaged in cultures exposed for 1, 3, 10, or 30 minutes to BSS, V, 1/10 cTA, or 1/10 –vTA. The percentage was similar for cultures exposed to BSS or 1/10 –vTA, even after 30 minutes
of exposure \((P > 0.05)\), whereas exposure to 1/10 cTA for 10 minutes or longer resulted in greater cell damage \((10 \text{ minutes}, \ P = 0.01; 30 \text{ minutes}, \ P = 0.003)\). Exposure to V for even 3 minutes caused significant damage \((P = 0.04, \ P = 0.04, \text{ and } \ P = 0.03 \text{ for } 3, 10, \text{ and } 30 \text{ minutes, respectively})\).

Under LM, trypan blue–positive cells in cultures exposed to 1/10 cTA or V for longer periods showed various cell sizes and irregular shapes; some of them appeared bizarrely pleomorphic, swollen, or even lysed \((\text{Figure 5})\). In general, the longer the cells were exposed to solutions containing the vehicle for cTA, the more severe the morphologic damage.

**DISCUSSION**

Because corneal endothelium plays a crucial role in maintaining corneal deturgescence, any trauma to this monolayer, such as intracameral injection of a cytotoxic agent, poses the risk for corneal decompensation. To our knowledge, this in vitro study is the first to investigate the risk for CEC damage from contact with the commercially available TA suspension used in a variety of ophthalmic surgical procedures.

Our study found that control and vehicle-removed test solutions had similar lack of effect on CECs, vehicle-containing solutions caused significantly more cell damage than control/vehicle-removed solutions, solutions containing vehicle caused similar damage, and the damage caused by vehicle-containing solutions was dose dependent, regardless of the concentrations of TA in the solutions. Collectively, these results indicate that the crystalline TA particles did not play a role in cytotoxicity. Rather, the cytotoxic effects of cTA and 1/10 cTA were mainly attributable to the vehicle. This conjecture was supported by the LM and ultrastructural findings of extensive lysis of CECs exposed to BA, the vehicle in cTA. Moreover, we found that cytotoxicity of the vehicle is time dependent, increasing significantly and progressively with 3-, 10-, and 30-minute exposures to 1/10 cTA or V. These findings imply that during anterior vitrectomy, even a 1/10 dilution of cTA can cause corneal endothelial damage and that only vehicle-removed solutions should be used.

In an experimental study similar to ours, Hida et al.\(^6\) found that preservatives in the vehicle for suspension of crystalline cortisone, rather than cortisone itself, could be toxic to the rabbit retina and lens. In addition, preservatives, such as benzalkonium chloride, or vehicles in the irrigation or other surgical solutions can be implicated in the recent outbreaks of toxic anterior segment inflammation and corneal endothelial destruction after uneventful cataract surgery.\(^{13,14}\) Benzyl alcohol is rarely used as the preservative in topical ophthalmic solutions because it is irritating, has slow activity, and can dissolve polystyrene.\(^{15}\) However, intravenous use of solutions containing BA has caused a fetal
toxic syndrome in premature infants.\textsuperscript{16} Although ocular toxicity of BA has not been proved in humans, these findings support our finding that BA is toxic to CECs.

Even though cTA, V, and 1/10 V (solutions 1, 3 and 6, respectively in Table 1) contained a slightly lower pH (6.1 to 6.8) than BSS, the time of exposure was as short as 1 minute. Another study\textsuperscript{17} showed the pH tolerance for rabbit corneal endothelium was between 6.5 and 8.5 with the corneas perfused in an in vitro perfusion specular microscope up to 3 hours. We believe that the mildly lower pH in our study does not play a crucial role in CEC cytotoxicity.

The limitation of this study is that we did not perform the experiment in vivo or on excised corneal endothelial cells, which would provide an alternative assessment for such a study. Nevertheless, based on the current model, we evaluated the effect of the dyes\textsuperscript{10} and intracameral anesthetic agents\textsuperscript{11} on corneal endothelial cells, which yielded valid results that are valuable to cataract surgeons. Another limitation of this study is that it may overestimate the clinical toxicity of cTA. Toxicity may be less in eyes than in cells in culture because of the actions of proteins and buffered ions in the aqueous humor. Also, TA suspension injected into the anterior chamber for an anterior vitrectomy procedure would tend to become diluted by continuous irrigation. In addition, the use of an ophthalmic viscosurgical device (OVD) tends to protect the corneal endothelium in vivo.

Triamcinolone acetonide suspensions are used extensively for ocular conditions because their long-lasting effects can be useful in treating a range of ocular conditions including CME,\textsuperscript{18} central retinal vein occlusion,\textsuperscript{19} diabetic retinopathy,\textsuperscript{20,21} and choroidal neovascularization.\textsuperscript{22} They are also helpful in visualizing the vitreous during ocular surgeries.\textsuperscript{3–5,23} Much of the work in this area used TA 40 mg/mL (Kenalog), which also contains BA 9 mg/mL as its preservative.\textsuperscript{3,9,18} However, the TA suspensions Kenacort-A and Kenalog, which were developed for intra-articular or intramuscular injection, were only recently formulated for ocular off-label use and there are clinical observations that these solutions cause harm.\textsuperscript{6} For example, residual preservative can cause complications\textsuperscript{7–9} and residual BA might lead to postoperative sterile or toxic endophthalmitis. This hypothesis is supported by the finding that of 520 eyes that had an intravitreal injection of TA,
none that received vehicle-removed TA developed intraocular inflammation.\textsuperscript{24} Thus, we and others urge removal of the vehicle as completely as possible before the TA suspension is used intracameral.\textsuperscript{2,3,5,21}

Three methods to remove the vehicle from cTA have been proposed. Jonas et al.\textsuperscript{21} describe a “standstill” method in which TA suspension is drawn into a syringe that is held vertically for 30 minutes to allow sedimentation of TA crystals; the upper layer is ejected, and the settling procedure is repeated twice more before the suspension is used. Because this procedure takes hours, it is not practical when TA suspension is needed unexpectedly intraoperatively. Hernaez-Ortega and Soto-Pedre\textsuperscript{12} separated the TA particles from the vehicle by density-gradient centrifugation at 3000 rpm for 5 minutes. This method is simple and rapid, with little loss of TA particles; however, almost no operating rooms are equipped with density-gradient centrifuges. The third method for removing vehicle from cTA, introduced by Burk et al.\textsuperscript{3} and Kumagai,\textsuperscript{5} involves repeatedly passing the TA suspension through Millipore filters and resuspending the TA particles in BSS. Millipore filters are available in most ophthalmic operating rooms, so this method seems the most practical for routine clinical use. It is, however, necessary to titrate the solution to determine the exact concentration,\textsuperscript{25} particularly for intravitreal injection of TA to treat various retinal diseases.\textsuperscript{10,22}

Additional safety measures should be considered when using TA suspensions during ophthalmic surgery. First, even though there was no dose-response for cultures exposed to vehicle-removed solutions, we recommend using a 1/10 dilution of vehicle-removed TA because TA used clinically in anterior segment surgeries, the vehicle in the TA suspension should be removed. A 1/10 dilution of vehicle-removed TA appears safe for cultured rabbit CECs, and we recommend this preparation for intracameral injection into human eyes. We also recommend removing the TA as completely and quickly as possible. However, more clinical studies are needed to clarify the long-term safety of TA suspensions on human corneal endothelium and the retina.

REFERENCES