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LABORATORY INVESTIGATION

Cytotoxicity of Triamcinolone on Cultured Human Retinal Pigment Epithelial Cells: Comparison with Dexamethasone and Hydrocortisone

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Abstract

Purpose: Triamcinolone acetonide (TA) is a corticosteroid that can be used in the treatment of cystoid macular edema (CME) and other ocular inflammatory conditions. This study aims to investigate the degree of cytotoxic effect of TA on human retinal pigment epithelium (ARPE19 cell line) and to compare the relative toxicity of TA with two other corticosteroids, hydrocortisone (HC) and dexamethasone (DEX), over a range of concentrations and durations of exposure.

Methods: The ARPE19 cell line was cultured and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and HAMS F12 medium containing 3 mM L-glutamine supplemented with 10% fetal bovine serum, penicillin G, and streptomycin sulfate. Following an initial overnight incubation, corticosteroids (0.01–1 mg/ml) or vehicle (benzyl alcohol, 0.025%), diluted in culture medium, was added to the ARPE19 culture (5000 cells/well) on Day 0. Subsequently the culture medium containing corticosteroid or vehicle was refreshed daily. After 1, 3, and 5 days, the proliferated amount of cells with and without corticosteroid treatment was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All samples were read in triplicate, with $n = 4$ in all cases. The final results were analyzed using analysis of variance.

Results: TA, DEX, and HC caused a significant reduction in cell numbers throughout the whole range of concentrations when cells were exposed to them for more than one day. The action of the corticosteroids, apart from TA, was biphasic. There was an initial rise in cell proliferation in the presence of DEX and HC at 0.01–0.1 mg/ml on Day 1. Log-linear plots of DEX and HC concentrations against percent viability (mean % ± SD) showed a significantly higher total viable cell percentage versus TA: 120.5 ± 1.8% and 134.9 ± 4.1% in the presence of DEX, and 110.0 ± 15.3% and 118.3 ± 9.0% in the presence of HC. The LD_{50} values of the three corticosteroids show that, regardless of the duration of exposure, TA was the most toxic, with relative toxicity of TA > DEX > HC, equivalent to a ratio of 1.0:1.6:1.8, after one day of incubation. The vehicle alone had no effect.

Conclusions: The present study demonstrated the degree of cytotoxicity of TA compared with DEX and HC. The results provide a profile of this drug relative to other common corticosteroids. Further studies are planned to characterize its effects and the degree of influence on cells of different ocular regions in order to show the full cytotoxicity of TA. **Jpn J Ophthalmol** 2004;48:236–242 © Japanese Ophthalmological Society 2004

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Introduction

Triamcinolone acetonide (9α-fluoro-16α-hydroxyprednisolone, TA) is an intermediate acting corticosteroid suspension that has been traditionally used in periocular injections for the treatment of ocular inflammatory diseases. More recently, it has been used intravitreally in small case series for the treatment of cystoid macular edema resulting from diabetic retinopathy, and for retinal vein occlusion, which has been resistant to laser photocoagulation.[1–4] The results have been promising, with improvements in visual acuity and in the degree of macular edema. It has been used in the treatment of exudative macular degeneration,[5–6] scleritis and uveitis,[7–8] and iris and choroidal neovascularisation,[9–11] and to reduce the incidence of proliferative vitreoretinopathy after complicated posterior segment surgeries.[11–13] Although TA is only intermediate in its anti-inflammatory action compared with other corticosteroids, it has the physical advantage of being in a sustained-release crystalline form. This makes it suitable as a local depot injection for chronic ocular conditions. Apart from triamcinolone, there are many other corticosteroids on the market that have a similar effect, although most of them are soluble. Clinically, dexamethasone has also been used intravitreally to reduce inflammatory damage in cases of endophthalmitis. The side effects of ocular corticosteroid therapy are well known, including intraocular pressure elevation,[3,14–15] cataractogenesis,[8] and potential cytotoxicity on ocular structures such as photoreceptors and retinal pigment epithelial (RPE) cells.[16]

Despite the encouraging clinical results of intravitreal TA, its cytotoxicity needs to be characterized if it is to be used with confidence. The present study aims to show the effect of TA on human retinal epithelial cells and to compare its relative toxicity with those of two other commonly used corticosteroids, hydrocortisone (HC) and dexamethasone (DEX). The present study also evaluates these corticosteroids in human retinal epithelial cells under different concentrations and durations of exposure.

Materials and Methods

Cell Culture

The ARPE19 cell line[17] was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture reagents, fetal bovine serum, and chemicals were purchased from GIBCO (Rockville, MD, USA), and containers were purchased from Corning (Acton, MA, USA). Human ARPE19 cells, within five passages from the time of purchase, were maintained in 1:1 mixture of Dulbecco's modified Eagle's medium and HAMS F12 medium (Gibco, Cat No. 12400-024, Grand Island, NY, USA) containing 3 mM L-glutamine supplemented with 10% fetal bovine serum and antibiotic mixtures of 100 units/ml penicillin G and 100 μg/ml streptomycin sulphate. Cell suspension volume of 5000 cells/ml was seeded onto 24-well tissue culture plastics. After overnight incubation, the seeded cells were washed gently with phosphate-buffered saline to remove cell debris before fresh culture medium was reintroduced.

Corticosteroid Preparations

Triamcinolone acetonide (9α-fluoro-16α-hydroxyprednisolone, Kenacort-A, Bristol-Myers-Squibb, Baar, Switzerland), hydrocortisone sodium succinate (Solu-Cortef, Pharmacia Upjohn, Uppsala, Sweden), and dexamethasone sodium phosphate (9α-fluoro-16α-methylprednisolone, Weimer Pharma, Rastatt, Germany) were serially diluted to appropriate concentrations directly from the original vials. All these compounds were obtained from the clinic in pure, ready-to-use forms.

Concentration Selection and Reconstitution

The concentrations used were derived from the known concentrations that have been used previously in experimental and clinical settings. Table 1 shows the dosages, equivalent vitreous concentrations, and the route of administration used in these studies. As the present study was in vitro and the stock of corticosteroids was diluted in culture medium directly, lower concentrations were employed. Dexamethasone came in a 5 mg/ml glass ampoule. Hydrocortisone, 100 mg of sterile powder, was mixed with 2 ml of benzyl alcohol prior to adding it to the culture medium. Triamcinolone was supplied in suspension in benzyl alcohol. In order to ensure even dispersion of TA (40 mg/ml) particles, the contents of the entire vial (1 ml) was added to 39 ml of culture medium to make a stock concentration of 1 mg/ml. This stock was then vortex mixed, and, before the suspended TA particles settled, serial dilutions were performed to obtain concentrations of 0.1 and 0.01 mg/ml of TA-containing culture medium. Diluted TA was well mixed before adding to the ARPE19 cells. Both DEX and HC were prepared in the same manner and were already contained within the culture medium prior to adding to the ARPE19 cells. As these corticosteroids have similar molecular weights, the concentrations (0.01–1 mg/ml) chosen in this study translate to approximately 30–3000 μM.

The Cytotoxic Effect of Corticosteroids

After an initial overnight incubation, corticosteroids (0.01–1 mg/ml) at the corresponding concentrations or the highest concentration of vehicle (benzyl alcohol, 0.025%) were added to the ARPE19 cells (Day 0). After 24 h, the ARPE19 cells were washed and the amount of cell proliferation under normal growth conditions or under corticosteroid treatment was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were washed with phosphate-buffered saline (PBS), and

Table 1. Dosages of triamcinolone and equivalent vitreous concentrations that have been used previously in experimental and clinical research

Site of injection	Dosage of triamcinolone	Equivalent vitreous concentration[a]	Reference
Sub-Tenon	40 mg	Not applicable	Jaffe et al.[25]
Subconjunctival	2–8 mg	Not applicable	Zamir et al.[7]
Intravitreal	0.8 mg	0.2 mg/ml	Ciulla et al.[9]
Intravitreal	4 mg	1 mg/ml	Danis et al.[5]
Intravitreal	4 mg	1 mg/ml	Kivilcim et al.[21]
Intravitreal	20 mg	5 mg/ml	Jonas et al.[1,10]
Intravitreal	25 mg	6.25 mg/ml	Jonas et al.[3,4]
In vitro cell culture	—	0.01–1 mg/ml culture medium	Present Study

[a]The vitreous volume is about 4 ml in the human eye; the equivalent vitreous concentrations used in the various studies therefore correspond to 0.2 to 6.25 mg/ml of vitreous. For this reason, the concentration chosen in the present study was within 0.01–1 mg/ml.

MTT was diluted using serum-free medium to obtain a concentration of 0.5 mg/ml. This concentration was added to the culture and incubated for 3 h. Formazan extraction was performed using isopropanol, and the quantity determined colorimetrically using NanoDrop at $\lambda = 570$ nm with the correction of interference at 690 nm in triplicate with four individual samples per group. The effect of corticosteroids on the degree of ARPE19 cell viability and proliferation was determined again after another 2 and 4 days (Day 3 and Day 5 after the initial corticosteroid exposure at Day 0). The highest concentration of benzyl alcohol (0.025%) was used to test its effect alone on ARPE19 cells over this 5-day period.

Expression of Results and Statistics

The results are expressed as units of absorbance of MTT at 570 nm ± SD. The absorbance readings under different treatments are further converted to relative percent cell viability for comparative analysis. As each group originated from a single pool of ARPE19, the changes in absorbance reflect the changes in the total numbers of viable cells of the same population over time and in relation to the concentrations of corticosteroids or vehicle used. The relative difference between controls (drug-free and vehicle) and corticosteroid-treated groups was analyzed using analysis of variance (ANOVA). In order to show the nature of the anti-proliferative action of corticosteroids and the extent of cytotoxicity, log concentrations of corticosteroids were plotted against the total percent viability of ARPE19 cells. The percent viability in the presence of corticosteroids was calculated by dividing the absorbance reading of cells under different concentrations of corticosteroids by the absorbance reading of cells under normal growth (assumed 100% viability on Day 0) in the absence of drugs or vehicle. Comparisons between corticosteroids were expressed as LD_{50} values, that is, the concentration of agent required to kill 50% of a population of cells. $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

The Effect of the Corticosteroids on ARPE19 Cells

One fundamental difference in exposing cells to these corticosteroids was that TA came in a suspension while DEX and HC were in complete solution. In the presence of 0.1 mg/ml TA, a large number of randomly distributed TA particles could be seen on the tops of the ARPE19 cells (Fig. 1). When the concentration was increased to 1 mg/ml, no usable image could be obtained because the entire field of view was virtually covered by TA particles.

The present experiment showed that TA, DEX, and HC caused a significant reduction in cell numbers across the whole range of concentrations (0.01–1 mg/ml) as long as the cells had been exposed to these agents for more than one day (Fig. 2). Although the vehicle contributed to a certain degree of cytotoxicity after 5 days of incubation, this effect was not significant. This result suggests that the greater part of the cytotoxicity action came from direct TA, DEX, and HC exposure. However, apart from TA, the action of the other corticosteroids was biphasic. There was an initial increase in cell proliferation in the presence of DEX and HC at the two lower concentrations (0.01 and 0.1 mg/ml) on Day 1. The presence of the benzyl alcohol vehicle did not produce a significant effect on ARPE19 cell viability. A log-linear plot of these corticosteroid concentrations against the viability (mean % ± SD) shows that the total number of viable cells increased to 120.5 ± 1.8% and 134.9 ± 4.1% in the presence of DEX, and to 110.0 ± 15.3 and 118.3 ± 9.0% in the presence of HC on Day 1 (Fig. 3a). The degree of cell proliferation began to decline to a similar level in the presence of these corticosteroids as the concentration was increased to 1 mg/ml. The percentage of viable cells was reduced to 63.2 ± 12.4% and 68.4 ± 6.9% in the presence of 1 mg/ml DEX and HC, respectively, at the end of Day 1. However, by Day 3 (Fig. 3b) and Day 5 (Fig. 3c), no initial cell proliferation was observed at low concentrations of DEX and HC. In fact, the damage to ARPE19 cells on Day 3 and Day 5 at 1 mg/ml was so severe that the viability was reduced to 5.0 ± 1.6% and 1.0 ± 0.3% under DEX

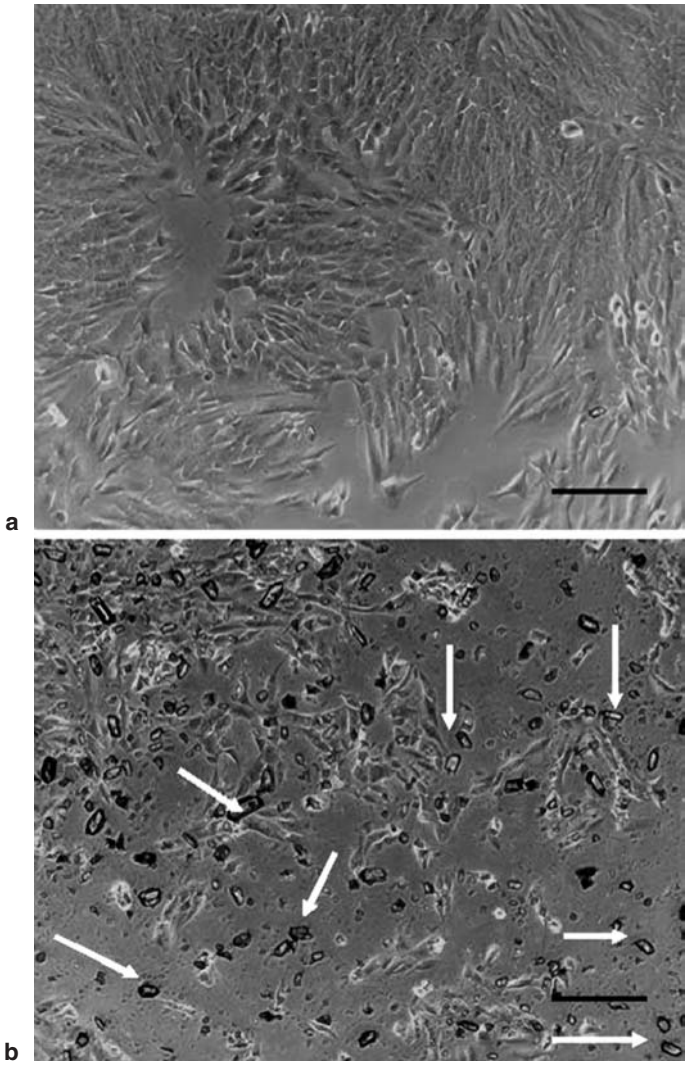


Figure 1a,b. Phase contrast images showing the presence of triamcinolone (TA) suspended particles adhering to the top of the cultured ARPE19 cells on day 3. **a** Concentration was 0.01 mg/ml; **b** 0.1 mg/ml. Note that there were many more TA particles on cells treated with 0.1 mg/ml. No image of the TA 1 mg/ml treatment could be taken, as nearly the entire field of view was covered with TA particles. The *white arrows* indicate the presence of TA particles on cultured ARPE19 cell surfaces. Bar = 50 μm.

treatment, and 1.1 ± 0.2% and 0.6 ± 0.2% in the presence of HC.

The LD_{50} values of the corticosteroids were calculated from Fig. 3. Table 2 shows the relationship between LD_{50} doses and the effect of these three corticosteroids on ARPE19 cells over 5 days. The data suggest that, regardless of the duration of exposure, TA was the most toxic of the three in terms of its cytotoxicity, with HC being the least potent. The relative toxicity was identical throughout the 5 days of corticosteroid treatment, that is, TA > DEX > HC. The present results show that, despite an initial rise in cell number with DEX and HC at low concentrations, the number of remaining viable cells was reduced as the duration of exposure increased. Furthermore, upon close examination of the data, it is clear that even with the highest relative toxicity ratio observed on Day 3 (1.0:4.4:8.6, TA, DEX, and HC, respectively), the total number of viable cells at 1 mg/ml was nearly the same among all corticosteroid-treated groups and throughout all 5 days of treatment.

Discussion

A clinician has to decide which corticosteroid to use and how much to use. This decision will be governed by the relative benefit-to-risk ratio of each corticosteroid preparation. The present study shows the comparative cytotoxicity of TA and two other commonly available soluble corticosteroids, DEX and HC, on the growth of ARPE19 cells. DEX was used for comparison because it is a soluble steroid that has been used intravitreally, while HC served as a unit reference. The retinal pigment epithelium is in close proximity to the vitreous space, separated from it by only the neuroretina. The ARPE19 cell line has morphological and functional properties similar to retinal pigment epithelial cells in vivo, and is capable of differentiating and proliferating in vitro.[17–18] In this study, ARPE19 cells served as a tool to demonstrate the effect of corticosteroids on cells within the posterior segment of the eye. Other ocular cells and tissues can also be used to provide a more comprehensive profile of TA as well as of other corticosteroids.

It was apparent that the cytotoxic effects of TA and the other two corticosteroids were concentration-dependent. In the present in vitro study, we chose to use 0.01, 0.1, and 1 mg/ml, and this range of concentrations provided an

Table 2. The LD_{50} concentrations (mg/ml) of triamcinolone, dexamethasone, and hydrocortisone relating to the viability of ARPE19 cells

Duration of Exposure	Triamcinolone	Dexamethasone	Hydrocortisone
Day 1	0.74 (1.00)	1.15 (1.55)	1.36 (1.84)
Day 3	0.05 (1.00)	0.22 (4.40)	0.43 (8.60)
Day 5	0.04 (1.00)	0.16 (2.50)	0.16 (2.50)

The LD_{50} values (the concentration of an agent that is required to kill 50% of a population of cells) show that triamcinolone was more potent than dexamethasone and hydrocortisone. Figures in parentheses show the relative potency ratios of corticosteroids relative to triamcinolone throughout the 5-day treatment period, with triamcinolone being the most potent throughout.

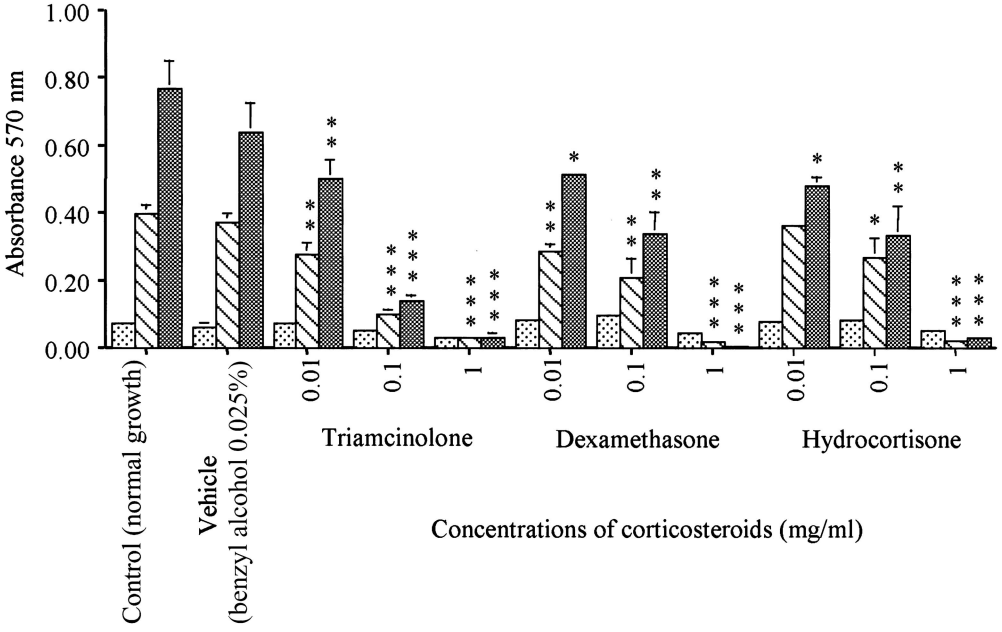
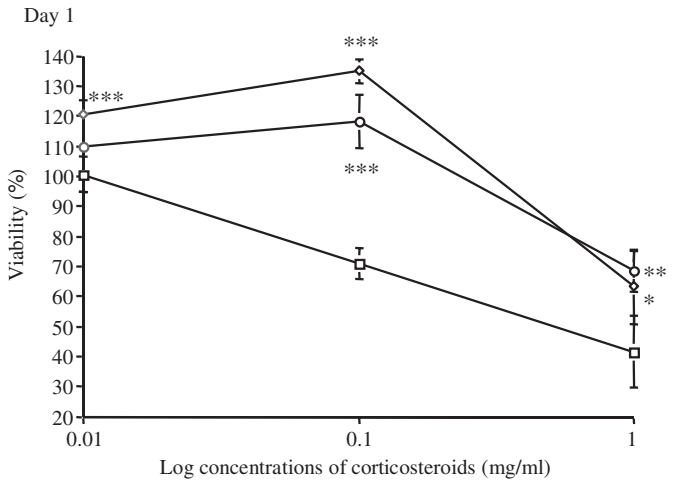


Figure 2. The proliferation of ARPE19 cells in the presence of varying concentrations of corticosteroids and over a 5-day period. A significant reduction in cell numbers was observed for all concentrations of corticosteroids except on Day 1 at 0.01 mg/ml. No significant difference in cytotoxic effect was observed until the corticosteroids had been in contact with the cells for 3 days ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control or benzyl alcohol only cultures, ANOVA). ⊡ Day 1; ◪ Day 3; ▩ Day 5.



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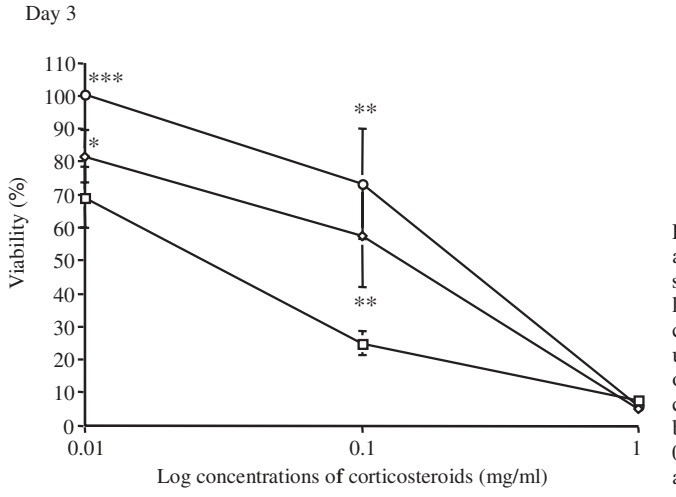

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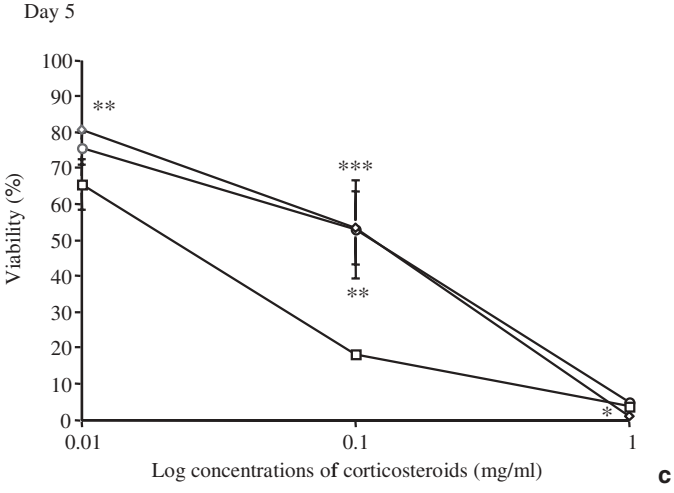

c

Figure 3a–c. Log-Linear LD_{50} plots of concentrations of corticosteroids against cell viability after 1 day (**a**), 3 days (**b**), and 5 days (**c**) of exposure. The percent viability in the presence of corticosteroids was calculated by dividing the absorbance reading of cells under different concentrations of corticosteroids by the absorbance reading of cells under normal growth (assuming 100% viability on Day 0) in the absence of drugs or vehicle. Differences between the percent viability of ARPE19 cells at a particular concentration and on a particular day were compared between corticosteroids using ANOVA. ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, dexamethasone or hydrocortisone vs. triamcinolone). —□— triamcinolone; —◇— dexamethasone; —○— hydrocortisone.

adequate profile of the cytotoxic effect of these corticosteriods on the ARPE19 cells, considering that these represent higher concentrations than those used in in vivo models, owing to the smaller cell numbers in culture. Toxicological studies seem to suggest that TA is relatively safe and nontoxic up to about 1 mg/ml when administered intraocularly or under in vitro conditions.[14,19–21] While most studies in human subjects have used up to 4 mg of intravitreal triamcinolone (equivalent to about 1 mg/ml of vitreous),[2,5,21] some recent case reports have used intravitreal injections of 20–25 mg of triamcinolone (up to 6.25 mg/ml of vitreous),[4,22–23] the safety of which has not been demonstrated in previous toxicology studies. The current in vitro study suggests that at these higher concentrations, all three corticosteroids studied may be toxic to ARPE19 cells, which may have implications for the upper limit of intravitreal corticosteroids used. This will need to be verified in animal models.

The side effects of ocular corticosteroid therapy are well known. They include intraocular pressure elevation,[24–26] cataractogenesis,[8] and potential cytotoxicity on ocular structures such as photoreceptors and retinal pigment epithelial cells.[27] Kong et al.[28] demonstrated that chemical-induced stress can lead to apoptosis with the activation of the caspase-3 pathway. Indeed, it is known that activation of caspase-3 is one of the downstream events from the initial stimulation of the c-Jun NH_2-terminal kinase (JKN) pathway (one of the mitogen-activated protein kinase cascades). The stressful stimulus can cause calcium overload, and this induces mitochondrial depolarization, swelling, and cytochrome c release. Thus, there is subsequent activation of caspase 3.[29,30] In fact, there is evidence showing that the inhibition of caspase 3 can increase the survival of injured retinal ganglion cells.[31] Further studies are being carried out in our department to investigate the likely toxic mechanisms involved in TA-induced cytotoxicity.

One noteworthy finding was the cell proliferative effect observed with DEX and HC at 0.01 and 0.1 mg/ml; the effect was cytotoxic once the concentration exceeded 0.1 mg/ml. However, this particular proliferative effect was observed only on Day 1 after which, despite the consistently higher cell viability of DEX and HC compared to TA throughout all 5 days, the amount of viable cells declined as the concentrations increased. Blumenkranz et al.[32] showed that both DEX and TA, at 0.2 and 0.15 mg/ml, respectively, inhibited rabbit dermal and conjunctival fibroblast proliferation. However, paradoxically, there was a two fold increase in cell proliferation at concentrations between 0.001 and 0.030 mg/ml. Their findings are apparently consistent with our observations.

Results of the present study show that there was a slight reduction, albeit insignificant ($P > 0.05$, $n = 4$), in cell viability in the presence of the vehicle alone at a concentration equivalent to 1 mg/ml of corticosteroid (0.025% benzyl alcohol) by Day 5 (Fig. 2). However, had the concentration of the vehicle been increased even further, a significant change in viability as a result of the vehicle alone might have occurred after a few days of exposure. Care must therefore be taken to select an appropriate level of the vehicle for clinical use.

The present cytotoxic potency is TA > DEX > HC. The ratios were the same throughout all 5 days of treatment indicating that the relative potency of these corticosteroids was independent of time and concentration, and dependent only on each vehicle's characteristic efficacy. It is interesting to note that although the order of potency in terms of antiinflammatory action is DEX > TA > HC, TA was more cytotoxic than DEX. TA was actually more toxic to the cells it was in contact with than DEX, and this difference may be in part due to the difference in formulation. TA was in particle suspension, which means the drug was in contact with cells and maintained this contact at a particular concentration for a longer period of time than DEX. This cell–drug contact may also have significant implications in terms of localized retinal toxicity. DEX once injected can be rapidly eliminated or hydrolyzed, thus reducing its pharmacological effect. For chronic conditions such as cystoid macular edema secondary to diabetic maculopathy, retinal vein occlusion, or uveitis, it is the sustained-release property of TA that makes it an attractive therapeutic option, even if it means higher cytotoxicity. Indeed, sustained-release devices containing steroids such as fluocinolone[33] and dexamethasone[34] have been made, but the surgical insertion of these devices is not without complications.

Although it is not possible to directly compare the present in vitro findings to an in vivo situation, and it is difficult to draw a conclusion as to the equivalent clinical dosage that can be used safely, the results in this study provide a comparison between different corticosteroids and shed light on the pattern of cellular responses of the retinal pigment epithelium. Further studies are planned to characterize the effects of TA and the degree of influence on cells of different ocular regions, both in vitro and in animal models, in order to show the full cytotoxicity of TA.

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