

The Toxic and Stress Responses of Cultured Human Retinal Pigment Epithelium (ARPE19) and Human Glial Cells (SVG) in the Presence of Triamcinolone

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PURPOSE. To compare the cytotoxic effect of TA on human retinal pigment epithelium (ARPE19) and human glial (SVG) cells over a range of concentrations and durations of exposure.

METHODS. TA (0.01–1 mg/mL) or vehicle (benzyl alcohol, 0.025%) was added to the ARPE19 and SVG cultures on day 0 and then subsequently for 1, 3, or 5 days. The amount of cell proliferations with or without TA treatment was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All samples were read in triplicate ($n = 4$ in all cases). c-Fos, c-jun, caspase-3, c-myc, and p53 expression was determined after TA treatments after 0, 10, 20, 30, 40, 50, 60, and 90 minutes. All results were analyzed with ANOVA.

RESULTS. TA (0.01–1 mg/mL) caused a significant reduction in ARPE19 cells that had been exposed to it for more than 1 day. Significant reductions in the number of SVG cells were observed as early as day 1 at 0.1 and 1 mg/mL TA. In general, the level of remaining SVG cells was less than that of the ARPE19 cells over the 5 days. SVG cells appeared more susceptible to TA. Caspase-3 was elevated in both ARPE19 and SVG cells after TA treatment. c-Fos and c-jun expression was also increased in ARPE19 cells but not in SVG. The vehicle of TA had no effect, and there was no change in p53 or c-myc expression.

CONCLUSIONS TA was cytotoxic to both SVG and ARPE19 cells, with higher efficacy on SVG. TA caused the activation of the caspase-3 pathway more readily than the cell-protective c-fos and c-jun pathways in SVG cells, making those cells more vulnerable than the ARPE19 cells. The results suggest that TA toxicity in one cell type may not reliably indicate its toxicity in other cells. Different cells within the retina may react to TA differently, or TA may cause changes in the gene expressions differentially with different concentrations of the same stimulus. (*Invest Ophthalmol Vis Sci.* 2003;44:5293–5300) DOI:10.1167/iovs.03-0490

Triamcinolone acetonide (9 α -fluoro-16 α -hydroxyprednisolone, TA) is an intermediate-acting corticosteroid suspension that has been administered traditionally 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 that has been resistant to laser photocoagulation.^{1–4} The results have been promising, with an improvement in visual acuity and the degree of macular edema. TA has been used in the treatment of exudative macular degeneration,^{5,6} scleritis and uveitis,^{7,8} and iris and choroidal neovascularization^{9–11} and in reducing the incidence of proliferative vitreoretinopathy after complicated posterior segment surgery.^{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 class effect, although most of them are soluble. Clinically, dexamethasone has also been used intravitreally to reduce postoperative inflammatory damage in cases of endophthalmitis. The side effects of ocular corticosteroid therapy are well known, including elevation of intraocular pressure,^{3,14–15} cataractogenesis,⁸ and potential cytotoxicity on ocular structures, such as photoreceptors and retinal pigment epithelial cells.¹⁶

Although it is generally believed in toxicology that TA is relatively safe and nontoxic up to approximately 1 mg/mL when administered intraocularly or in *in vitro* conditions,^{14,17–19} the cytotoxicity should to be characterized if TA is to be used with confidence clinically. Furthermore, TA may influence different cells within the eye to different extents and the mechanism by which it induces cytotoxicity may be also different. The purpose of this study was to compare the effect of TA on two related cell types, both of which are morphologically similar to structures that can be found in the eye, which are likely to be influenced by the clinical application of TA. Because intravitreal injection seems to be the most common route of administration, human retinal epithelial cells (ARPE19) and human glial cells (SVG) were chosen to demonstrate the possible difference in cytotoxicity in the presence of TA under different concentrations and durations of exposure. The retinal epithelial cell was chosen because it is a major layer within the retina separating the retina from the remaining posterior ocular tissue layers. As the retina is of neuronal origin and is made up of several nerve cell layers, the SVG was chosen to reflect its response in the presence of TA. It might be useful to determine the level of cytotoxicity and the degree of stress induction of TA on ocularly related cells to compare and contrast its pharmacological profile. The cells being used in this study served merely as the tools in which TA was tested.

METHODS

Cell Culture

Human retinal pigment epithelial cell (ARPE19)²⁰ and human glial cell (SVG)²¹ lines were purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents, fetal bovine serum and chemicals were purchased from Invitrogen-Gibco (Rockville, MD), and containers were purchased from Corning Glass, Co. (Acton, MA).

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TABLE 1. The Dosage of Triamcinolone and Equivalent Vitreous Concentration That Have Been Used in Experimental and Clinical Research

Site of Injection	Dosage of Triamcinolone (mg)	Equivalent Vitreous Concentration* (mg/mL)	Reference
Sub-Tenon	40	Not applicable	Jaffe et al. ^{3,4}
Subconjunctival	2–8	Not applicable	Zamir et al. ⁷
Intravitreal	0.8	0.2	Ciulla et al. ⁹
Intravitreal	4	1	Danis et al. ⁵
Intravitreal	4	1	Kivilcim et al. ¹⁹
Intravitreal	20	5	Jonas et al. ^{1,10}
Intravitreal	25	6.25	Jonas et al. ^{3,4}
In vitro cell culture	—	0.01–1 mg/mL culture Medium	Present Study

* The vitreous volume is about 4 mL in humans, the equivalent vitreous concentrations used in various studies therefore correspond to 0.2 to 6.25 mg/mL of vitreous. As such, the concentration chosen in the present study was to be within 0.01 to 1 mg/mL.

Human ARPE19 and SVG cells, within 18 to 22 passages from the time of purchase, were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12, containing 3 mM L-glutamine supplemented with 10% fetal bovine serum and antibiotic mixtures of 100 U/mL penicillin G and 100 µg/mL streptomycin sulfate. Cell suspensions of both cell types with a cell volume of 5000 cells/mL were seeded onto 24-well tissue culture plates. After overnight incubation at 37°C and in an environment containing 95% O₂ and 5% CO₂, the seeded cells were washed gently with phosphate-buffered saline to remove cell debris before fresh culture medium was reintroduced.

Preparation of Triamcinolone

Triamcinolone acetonide (TA, 9α-fluoro-16α-hydroxyprednisolone; Kenacort-A, Bristol-Myers-Squibb, New York, NY) was serially diluted to appropriate concentrations directly from the original vials. The concentrations used were derived from the known concentrations that have been used in experimental and clinical settings. Table 1 shows the dosage, equivalent vitreous concentrations, and route of administration used in these studies. As the present study was in an in vitro setting and the stock of TA was diluted in culture medium directly, lower concentrations were used. Triamcinolone was supplied in particle suspension form in benzyl alcohol. To ensure even dispersion of the stock TA (40 mg/mL) particles, the content of the entire vial was added to 39 mL of culture medium to provide a stock concentration of 1 mg/mL. This mixture was then vortexed and, before the suspended TA particles settled, was serially diluted immediately to obtain 0.1 and 0.01 mg/mL of TA-containing culture medium. Diluted TA was well mixed before adding into the ARPE19 and SVG cells. This concentration range of TA in the present study translates to approximately 30 to 3000 µM.

Cytotoxic Effect of Triamcinolone

TA (0.01–1 mg/mL) at the corresponding concentrations or the highest concentration of vehicle (benzyl alcohol, 0.025%) were added to the ARPE19 and SVG cells (day 0). After 24 hours (day 1), these cells were washed and the determination of the amount of cell proliferation was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were washed with phosphate-buffered saline (PBS), and MTT at 0.5 mg/mL in serum-free medium was added to the culture and incubated for 3 hours. Formazan extraction was performed with isopropanol and the quantity determined colorimetrically by using a spectrophotometer (NanoDrop, Rockland, DE) at λ = 570 nm, with the correction of interference at 690 nm in triplicate and four individual samples per group. Effects of TA on ARPE19 and SVG cell viability and proliferation were determined on days 3 and 5 after the initial TA exposure. The highest concentration of benzyl alcohol (0.025%) was used to test its effect alone on ARPE19 and SVG over this 5-day period. The relative difference between the concentrations of TA, and its application to these two cell types on different days was further compared using the median lethal dose (LD₅₀).

Gene Expression Study

Samples of ARPE19 and SVG cells after exposure to different TA concentrations were collected at 0, 10, 20, 30, 40, 50, 60, and 90 minutes for isolation of RNA. For total RNA extraction, the cells were first harvested by lysis buffer (RNeasy Lysis Buffer [RNL]; Qiagen GmbH, Hilden, Germany), which contained 1% β-mercaptoethanol, and homogenized by passing through a spin column (QIASHredder; Qiagen GmbH). The total RNA samples were isolated by using a kit according to the protocol of the manufacturer (RNeasy Mini Kit; Qiagen GmbH).

The RNA samples were quantified with the spectrophotometer (NanoDrop), and 500 ng of the total RNA was used for reverse-transcription with 3 µg/µL random primer p[dN]6 (Roche Diagnostics GmbH, Mannheim, Germany) and a reverse transcriptase kit with RNase inhibitor (Superscript II RNase H⁻ Reverse Transcriptase kit and RNase OUT RNase inhibitor; Invitrogen, Carlsbad, CA).

Different amounts of cDNA corresponding to 12.5, 25.0, 37.5, and 50.0 ng RNA were amplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for 30 cycles to determine the optimized amount of cDNA used in PCR. The cDNA corresponding to 12.5 ng RNA, at which the amplification of the signal was linear, was selected and amplified with the following primer pairs: *GAPDH* forward, 5'-gaa ggt gaa ggt cgg agt-3', and reverse, 5'-gaa gat ggt gat ggg att tc-3'; *caspase-3* forward, 5'-tat tct tgg cga aat tca aag gat-3', and reverse, 5'-aaa gta gcg tca aag gaa aag gac-3'; *c-fos* forward, 5'-aga cag acc aac tag aag atg a-3', and reverse, 5'-agc tct gtg gcc atg gcc ccc-3'; *c-jun* forward, 5'-gtg acg gac tgt tct atg act g-3', and reverse, 5'-ggg ggt cgg cgt ggt gat g-3'; *c-myc* forward, 5'-tcc agc ttg tac ctg cag gat ctg a-3', and reverse, 5'-cct cca gca gaa ggt gat cca gac t-3'; and *p53* forward, 5'-ttg ccg tcc cca gaa atg gat ga-3', and reverse, 5'-tct ggg aag gga cag aag atg ac-3'.

These PCR products were separated by 2% agarose gel electrophoresis and stained with 0.5 µg/mL ethidium bromide, and the band signals were exposed to UV before they were scanned and quantified with a gel image analyser (GelDoc Quantity One; Bio-Rad, Hercules, CA). Band intensities were quantified and normalized against that of GAPDH. Each set of the experiments was repeated in triplicate for statistical analysis.

Expression of Results and Statistics

The results were expressed as units of absorbance of MTT at 570 nm ± SD. Because each group originated from a single pool of cells, the changes in absorbance reflect the changes in the total numbers of viable cells of the same population over time and in relation to concentrations of TA or vehicle used. The relative difference between the control (drug free and vehicle) and TA-treated groups were analyzed with ANOVA. To show the nature and the extent of cytotoxicity, TA concentrations were plotted against the total percentage of viability of ARPE19 and SVG cells. The percentage of viability in the presence of TA was calculated by dividing the absorbance reading of cells under

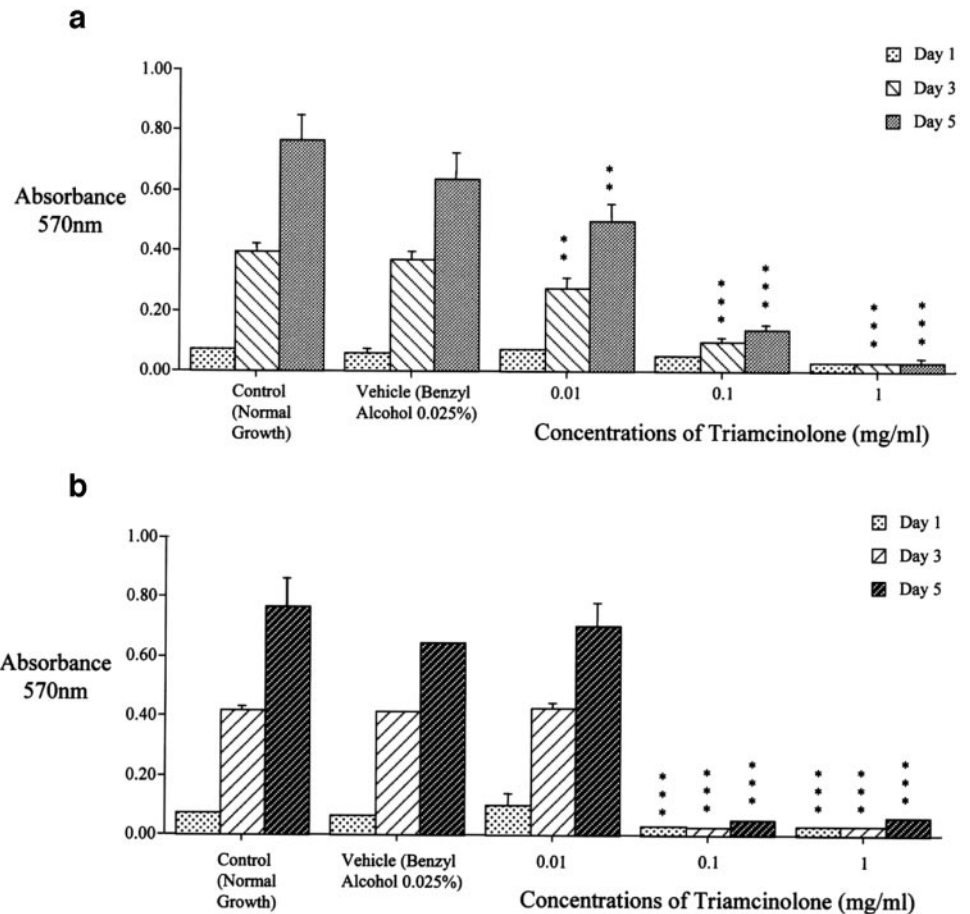


FIGURE 1. The proliferation of ARPE19 (a) and SVG (b) cells in the presence of various concentrations of TA and over 5 days of treatment period. Significant reductions (mean \pm SD) in the number of ARPE19 cells were observed across all concentrations of TA except for the results obtained on day 1. The cytotoxic effect of TA on SVG cells was less severe than on ARPE19 cells at 0.01 mg/mL but became significantly reduced in the presence of 0.1 and 1 mg/mL TA. The highest concentration of benzyl alcohol had no effect. $**P < 0.01$, $***P < 0.001$, control drug-free growth versus TA-treated cultures, ANOVA; $n = 4$ in all cases.

different TA concentrations by the absorbance reading of cells under normal growth (assumed 100% viability on day 0) without TA or its vehicle. Comparisons between the effect of TA on ARPE19 and SVG were expressed as $LD_{50} \pm SD$ —that is, the concentration of TA that is required to kill 50% of a population of cells over a given period of exposure. For the gene-expression study, band intensities of different primers are expressed as mean \pm SD. Significant differences were determined between control and TA-treated cells at the respective time points of sample collections. In all the experiments, $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Cytotoxicity of TA in ARPE 19 and SVG Cells

The present experiment showed that TA caused a significant reduction in the cell numbers of ARPE19 across the whole range of concentrations (0.01–1 mg/mL) as long as cells had been exposed to TA for more than 1 day (Fig. 1a). Although an insignificant difference was found with SVG cells being exposed to 0.01 mg/mL TA over 5 days, these cells were substantially more susceptible to TA toxicity than the ARPE19 cells when exposed to 0.1 and 1 mg/mL TA. Significant reductions in the number of SVG cells were observed as early as day 1 at 0.1 and 1 mg/mL, which was not the case in ARPE19 cells (Fig. 1). In general, the level of the remaining viable SVG cells appeared to be less than that of the APRE19 cells at 0.1 and 1 mg/mL (Fig. 1b). The morphologic characteristics of ARPE19 and SVG cells in the absence and presence of 0.1 mg/mL TA are shown in Figure 2. To illustrate better the different responses of these two cell types, log-linear plots of TA concentrations against the percentage of viability (mean \pm SD) were used. The results show that the toxicity of TA on ARPE19 cells was

a linear event, regardless of the durations of exposure (Fig. 3a). The effect of TA on SVG cells was not as straightforward. There was little change in the viability of SVG cells at the lowest concentration (maintained 85%–100% viability at 0.01 mg/mL across all 5 days), followed by a rapid decline and then a near total destruction without much recovery (Fig. 3b). Furthermore, percentages of the viability of SVG cells were similar on both days 3 and 5 suggesting that the maximum effect of TA on these cells had been reached. The log-linear plots clearly show that the cytotoxicity of TA on SVG cells was also more severe than on ARPE19 cells at 0.1 mg/mL and 1 mg/mL on days 3 and 5. This was illustrated by the percentage (\pm SD) of viability of ARPE19 cells (concentration in parentheses, significant difference between SVG and ARPE19 cells) on day 3, 24.96 ± 3.66 (0.1); 7.44 ± 1.64 (1.0) and day 5, 18.26 ± 2.31 (0.1); 3.85 ± 1.66 (1.0), compared with SVG cells on day 3, 5.96 ± 0.56 (0.1, $P < 0.001$); 7.57 ± 0.99 (1.0), and day 5, 6.98 ± 0.79 (0.1, $P < 0.001$); 8.21 ± 0.93 (1.0, $P < 0.01$). The vehicle alone at the highest concentration did not affect ARPE19 and SVG cells. Albeit insignificant, there was a slight reduction in the number of cells both types by day 5 with the vehicle treatment (Fig. 1). Nevertheless, the cytotoxicity observed in the present study was primarily attributable to the presence of TA.

To compare the relative efficacy of TA in these two cell types, the LD_{50} s of TA were calculated using log-linear plot data. Table 2 shows the relationship between LD_{50} concentrations of TA on ARPE19 and SVG cells over 5 days. The LD_{50} values of TA suggest that SVG cells were approximately 11 times more sensitive than ARPE19 cells to the presence of TA, after exposure for 1 day, as indicated by the significantly lower LD_{50} s obtained. However, the cellular response to TA at days 3 and 5 became more comparable between the two cell types. In

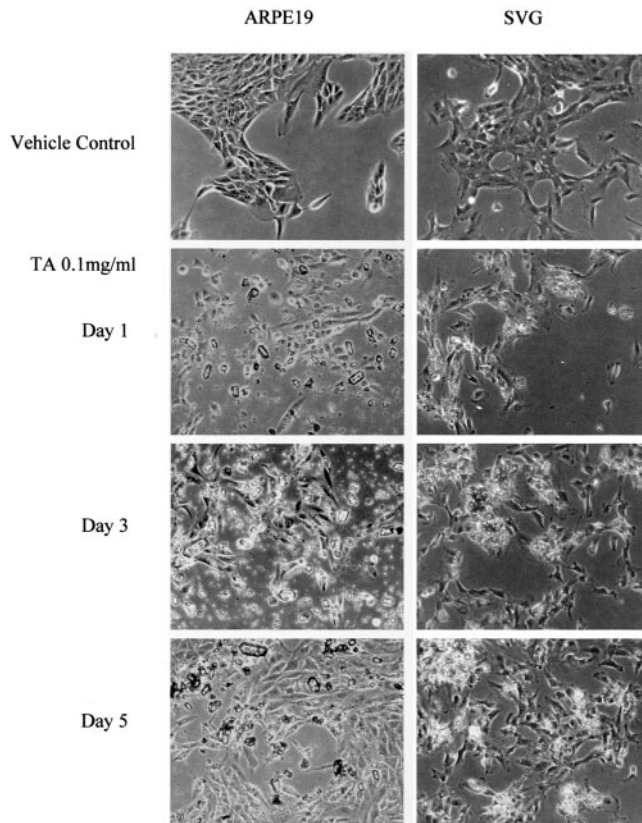


FIGURE 2. Phase-contrast micrographs showing ARPE19 and SVG cells in the presence of triamcinolone (TA) across the 5 days of treatment. TA crystals appeared as dark pigments on the phase-contrast images. Only cells treated with 0.1 mg/mL TA are shown as an example. Because TA is in particle suspension form, it tends to settle on top of cells in clumps. It can be seen that there were significantly fewer cells across the corresponding days in the presence of 0.1 mg/mL TA.

fact, the responsiveness was reversed, with ARPE19 cells slightly but significantly more sensitive than SVG cells to the presence of TA.

Gene Expression Study

Whereas the MTT assay was used to quantitate cell necrosis, the apoptotic response of both cell types was investigated with gene expression study. Of all the RNA expression levels investigated, only *c-fos*, *c-jun*, and *caspase-3* were significantly elevated in ARPE19 cells that had been treated with TA (Figs. 4a–c, respectively). The changes in expression peaked between 20 to 30 minutes and were concentration dependent. The difference among these samples was that *c-fos* and *caspase-3* expressions were significantly elevated only after being treated with 0.1 mg/mL TA, whereas *c-jun* expression was significantly elevated across all concentrations. The other RNA protein expressions, *c-myc* and *p53*, were not significantly elevated (Figs. 4d, 4e, respectively) under the present TA concentrations used and sample collection time points. In contrast, only *c-jun* and *caspase-3* expressions were significantly elevated in SVG samples under identical treatments with maximum levels also observed between 20 and 30 minutes in a concentration-related manner (Figs. 5b, 5c). The increase in *c-jun* expression was not so clear. A significant increase was found only in *c-jun* expression with 0.1 mg/mL TA treatment at 20 minutes and 1 mg/mL at 30 minutes. In addition, similar to the MTT results obtained with SVG cells, no difference in *caspase-3* protein expression was observed at the lowest concentration of TA but reached the same maximum at 0.1 and 1

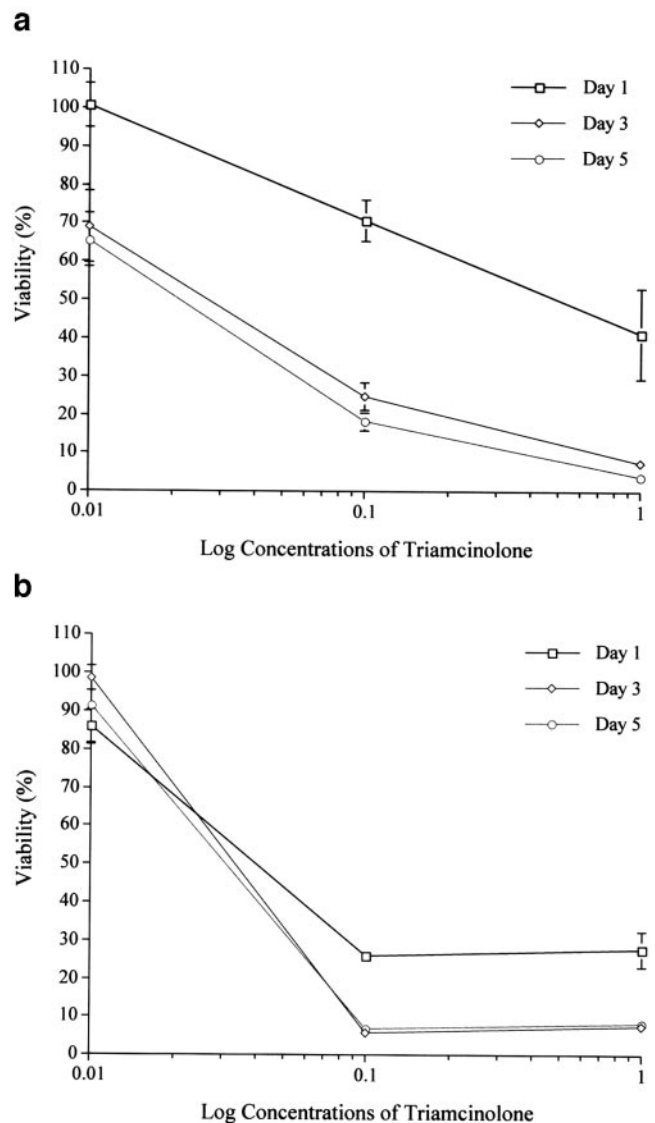


FIGURE 3. Log-linear LD₅₀ plots of concentrations of TA against the viability of ARPE19 (a) and SVG (b) cells after 1, 3, and 5 days. The percentage of viability in the presence of TA was calculated by dividing the absorbance reading of cells under different concentrations of TA by the absorbance reading of cells under normal growth (assumed 100% viability on day 0) in the absence of TA or vehicle.

mg/mL TA treatments (Fig. 5c). No difference in the expression of *c-fos*, *c-myc*, or *p53* was observed (Figs. 5a, 5d, 5e). These data imply that the presence of TA caused apoptotic cell

TABLE 2. The LD₅₀s of TA on ARPE19 and SVG Cells over 5 Days

Durations of Exposure	LD ₅₀ (μg/mL)	
	ARPE19	SVG
Day 1	740 ± 57	64 ± 2**
Day 3	53 ± 3	57 ± 1*
Day 5	40 ± 1	53 ± 2**

The LD₅₀ values of TA suggest that SVG was about 11 times more sensitive than ARPE19 to the presence of TA if being exposed for one day as indicated by the significantly lower LD₅₀ values obtained. However, the cellular response to TA at Day 3 and Day 5 became more comparable between the two cell types.

* $P < 0.05$, ** $P < 0.001$ ARPE 19 versus SVG (ANOVA).

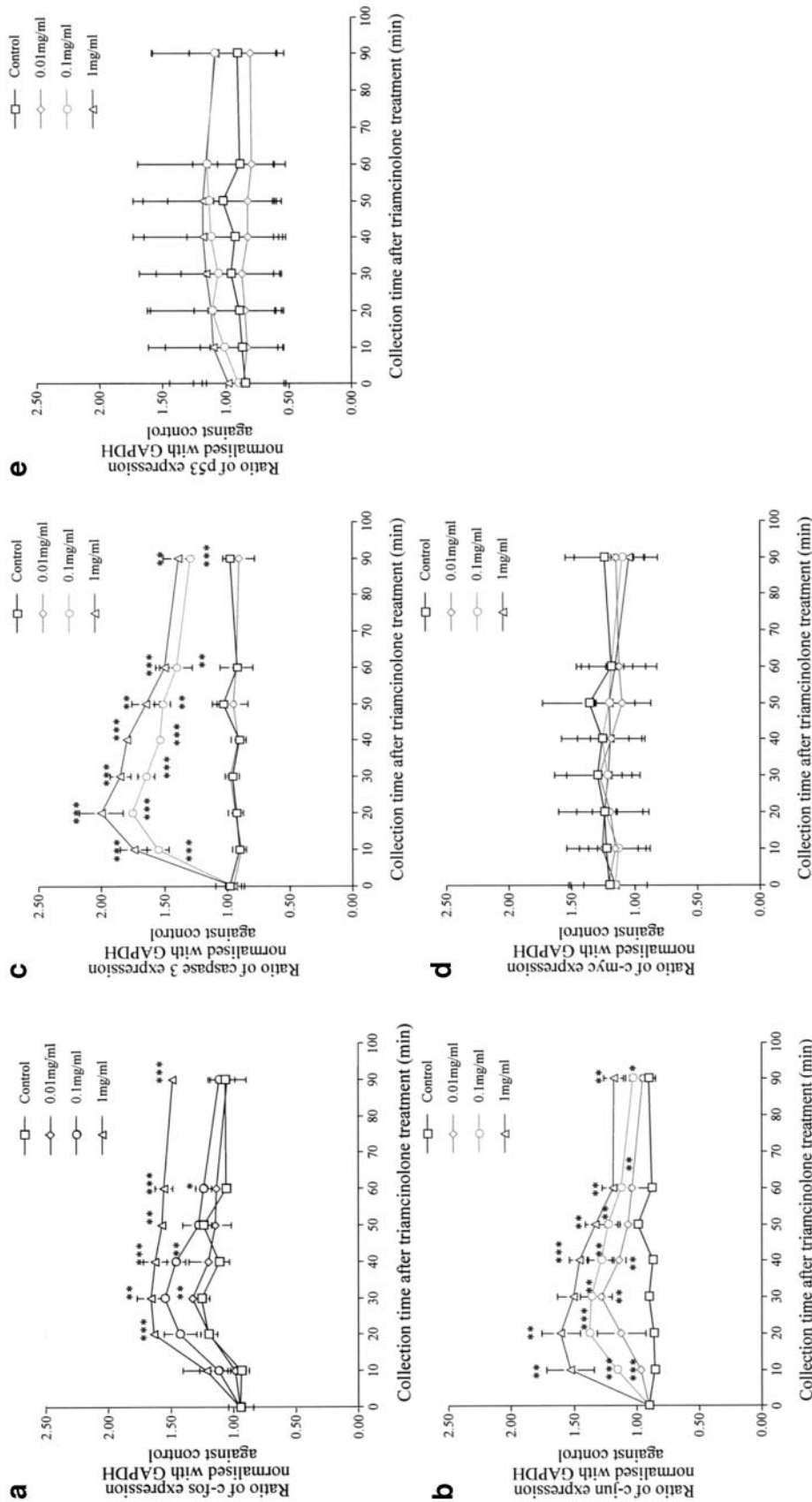


FIGURE 4. Quantification of PCR signal intensities of c-fos (a), c-jun (b), caspase-3 (c), c-myc (d), and p53 (e) in ARPE19 cells treated with triamcinolone (0.01–1 mg/mL). Samples were collected at 0, 20, 30, 40, 50, 60, and 90 minutes. Only c-fos, c-jun and caspase-3 showed significant alteration in expression ($P < 0.05$) in triamcinolone-treated ARPE19 cells, and the maximum alterations all occurred at approximately 20 to 30 minutes in all cases. All experiments were performed in triplicate, and the ratios of expressions normalized with GAPDH against the control were compared by ANOVA. $**P < 0.05$; $**P < 0.01$; $***P < 0.001$, $n = 3$ in all cases.

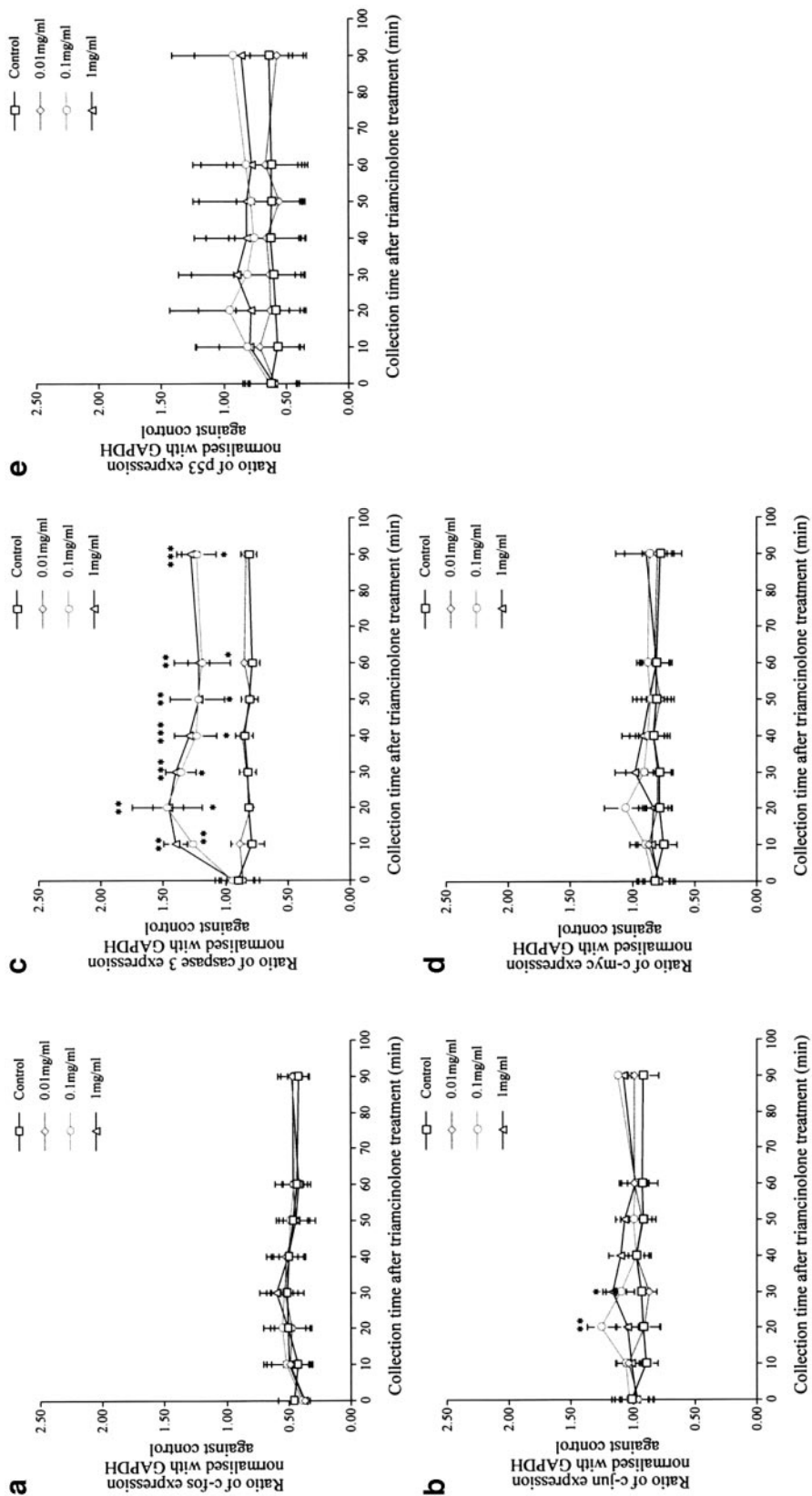


FIGURE 5. Quantification of PCR signal intensities of c-fos (a), c-jun (b), caspase-3 (c), c-myc (d), and p53 (e) in ARPE19 (Fig. 4) and SVG (Fig. 5) cells treated with triamcinolone (0.01–1 mg/mL). Samples were collected at 0, 10, 20, 30, 40, 50, 60 and 90 minutes. Only c-jun and caspase-3 show significant alteration in expression ($P < 0.05$) with maximum alteration occurred at around 20–30 minutes in both cases. All experiments were performed in triplicate and the ratios of expressions normalized with GAPDH against the control were compared using ANOVA (** $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 3$ in all cases).

death in both APRE19 and SVG cells, as caspase-3 expression was elevated in a concentration-dependent fashion. However, that only c-jun and caspase-3 levels were elevated in SVG cells and the extent of expression was also less severe than in ARPE19 cells suggests that the response to TA was different between these cells under identical experimental conditions.

DISCUSSION

When an agent is administered to the body, its effect on different organs and cells and on different cells within the same organ could be very different. When an agent is suspected to possess a certain degree of toxicity, it is important to consider such an effect within the immediate site of administration. The key question was to evaluate how different cells would react to TA when they had been exposed to it for more than a few days and in different concentrations.

In the present study, we evaluated two distinct cell types, human retinal pigment epithelial and astroglial cells, which are cells of similar origin (ectoderm). Although as early as 1981 McCuen et al.¹⁸ used rabbits to demonstrate the lack of toxicity of a single intravitreal injection of 1 mg triamcinolone, their finding should not be taken for granted. It is clear that although TA has a pharmacological profile that could be beneficial to ocular inflammations, its potential cytotoxicity has to be considered carefully, because its action may be quite different within the entire ocular structure, which contains a variety of cell types. As such, in the present study, we used two different cell types to compare and contrast the likely differences in response to the presence of TA under different concentrations and durations of exposure. The retinal pigment epithelium is in close proximity to the vitreous space, separated from it only by the neuroretina. The ARPE19 cell line was used, because it has morphologic and functional properties similar to retinal pigment epithelial cells *in vivo* and is capable of differentiating and proliferating *in vitro*.²⁰⁻²¹ In this study, ARPE19 cells served merely as a tool to demonstrate the effect of TA on cells in the outer region of the retina and SVG to represent cells from the neuroretina near the inner limiting membrane, for the sole purpose of comparison. The astroglial cells help to elucidate TA's effects on neuronal-type cells, as there are ganglion cells and nerve fiber layers within the retina. Furthermore, if TA were to be injected, the inner layers would be the first exposed to the maximum concentration and the retinal pigmented cells would be the last, because TA is diffused from the vitreous space toward the posterior part of the eye.

The present study, in which we used the changes in MTT absorbance to indicate the degree of necrosis and the gene expression data to reflect the extent of apoptosis of APRE19 and SVG cells in the presence of TA, has illustrated several relevant points. Under the present experimental conditions, it appeared that TA was cytotoxic to both cell types, but it has higher efficacy on SVG cells than on ARPE19 cells. The response of ARPE19 cells in the presence of TA was linear and changes in viability of these cells were related to the concentrations and durations of exposure. Although the SVG cells were more resistant to TA at the lowest concentration (0.01 mg/mL) than were ARPE19 cells, they were more susceptible than ARPE19 cells to the cytotoxic action of TA at 0.1 and 1 mg/mL. On the contrary, the SVG cells were more vulnerable than ARPE19 cells to the effect of TA by approximately 11-fold even after 1 day of exposure. However, this difference was reduced by days 3 and 5, with the ARPE19 cells becoming significantly more sensitive than the SVG cells over time.

Similarly, the difference in the response of these cells to TA was demonstrated in the gene expression study. The RNA expression data suggest that APRE19 cells may tolerate the cytotoxic effect of TA better than SVG cells, at least within the time points studied. The elevated caspase-3 expression in both

cell types was expected as demonstrated by the MTT data. However, the TA-induced apoptotic cell death appeared to be all-or-nothing with SVG (Figs. 4, 5), and this pattern was similar to the MTT assay data. This is shown by the observation that SVG cells have the same maximum level of caspase-3 expression at 0.1 and 1 mg/mL (Fig. 5c), but no difference from the control at 0.01 mg/mL of TA. Similarly, the pattern of MTT data in Figure 1b shows that there was no change in MTT absorbance at 0.01 mg/mL but that it reached a similar minimum absorbance at 0.1 and 1 mg/mL. This means the response of SVG cells to TA was critical in terms of both the concentration and the duration of treatment. Kong et al.²² have demonstrated that low concentrations of chemical-induced oxidative stress can elevate gene expressions of c-jun and c-fos, which protect the cells against toxic insult and enhance cell survival, whereas high concentrations can lead to apoptosis with the activation of the caspase-3 pathway. Similar events might have been observed here. Indeed, it is known that activation of caspase-3 is one of the downstream events from the initial stimulation of the c-Jun NH₂-terminal kinase (JKN) pathway (one of the mitogen-activated protein kinase [MAPK] cascades). The stressful stimulus can cause calcium overload, and this induces mitochondrial depolarization, swelling, and release of cytochrome *c*. There is thus subsequent activation of caspase 3.^{23,24} In fact, there is evidence that shows that the inhibition of caspase-3 can increase the survival of injured retinal ganglion cells.²⁵ The pharmacological inhibition of the overexpressed genes, inhibition of proteases, and the control of intracellular calcium concentrations can all prevent apoptosis.^{26,27} The activation of JKN also triggers an induction of c-jun and c-fos expression, which can lead to cell proliferation, differentiation, and cellular survival, as well as to apoptosis.²⁸⁻³⁰ In the case of ARPE19 cells, significant increases in c-fos and c-jun expression were found across all three TA concentrations. The lack of increase in caspase-3 at the lowest concentration of TA may simply be due to the protective effects of both c-fos and c-jun or may be because TA had not reached a critical enough concentration to induce an increase in caspase-3 expression. However, as the concentration of TA increased, so did the caspase-3 expression level, leading to significant cell death. In the case of SVG cells, no increase in c-fos expression was observed. Despite significant increases in c-jun expressions with 0.1 mg/mL TA treatment at 20 minutes and 1 mg/mL at 30 minutes (Fig. 5b), no increase in expression was found at other time points and with TA 0.01 mg/mL treatment. Combining the effects observed with TA-treated ARPE19 and SVG cells, the results certainly support the view that elevations of c-fos and c-jun have protective effects on cell survival.²² It seems that SVG cells were relatively resistant to TA at 0.01 mg/mL, as indicated by both MTT assay data and caspase-3 expression. However, the cells became apoptotic once the level reached and exceeded 0.1 mg/mL. It can therefore be concluded that the SVG cells' response to TA treatment was somewhat different from that of APRE19 cells, with the activation of the caspase-3 pathway much more readily than the c-fos and c-jun pathways and, as such, the SVG cells tended to be more vulnerable than the ARPE19 cells.

The reason for the absence of expression of the other apoptosis-associated genes *c-myc* and *p53* is unknown. On speculation, it may be that MAPK activation tends to trigger a higher rise in the caspase-3 and the activator proteins (c-jun and c-fos) in response to TA, more so than in *p53* and *c-myc*. The lack of change of *p53* and *c-myc* suggests that, TA's effect on these cells in the present conditions do not involve cell growth regulation and proliferation, as these two genes are well-known transcription factors that suppress cell growth.³¹⁻³²

A study has shown that the toxicity of six commercially available corticosteroid preparations is due to preservatives or

vehicles alone.³³ Despite slight reductions in the number of both cell types were observed at 0.025% benzyl alcohol on day 5, this result was not as significant. The toxicity of TA alone was by far more significant in causing cell necrosis at the equivalent concentration of 1 mg/mL.

The toxic effect of TA on one particular cell type may not be a reliable indicator of its toxic effects on other cells within the eye. It is important to bear in mind that TA is commonly administered by the intravitreal route (Table 1). It therefore tends to have direct physical contacts with different cell layers, with the retina being one of the key sites. Different cells within the retina may react to TA treatment very differently, or TA may trigger different intracellular proteins or genes under different concentrations of the same stimulus. It is clearly demonstrated in this study that the concentration or the duration of treatment both had different effects on the retinal pigment epithelium, as represented by the ARPE19 cells and neuroglia, as represented by SVG cells. When TA is to be given, a suitable dose and concentration must be chosen carefully that serves the purpose of anti-inflammation with minimal toxicity. It is also clear that TA could cause various degrees of cytotoxic response, depending on the duration of exposure and the initial concentration used and, as such, a critical balance between the two must be carefully predetermined. The present study does not provide an absolute concentration of TA that would satisfy these criteria. The concentrations chosen in this study were equal to or lower than the clinical equivalents (Table 1). Even so, determining the concentration that can be classified as safe necessitates further *in vivo* studies. Our laboratories are currently planning to investigate the level of stress and related toxic responses due to the administration of TA in animal models based on the *in vitro* data obtained in the present study.

References

- Jonas JB, Sofker A. Intraocular injection of crystalline cortisone as adjunctive treatment of diabetic macular edema. *Am J Ophthalmol.* 2001;132:425-427.
- Martidis A, Duker JS, Greenberg P, et al. Intravitreal triamcinolone for refractory diabetic macular degeneration. *Ophthalmology.* 2002;109:920-927.
- Jonas JB, Kreissig I, Degenring R. Intraocular pressure after intravitreal injection of triamcinolone acetonide. *Br J Ophthalmol.* 2003;87:24-27.
- Jonas JB, Kressig I, Söfker A, Degenring RF. Intravitreal injection of triamcinolone for diffuse diabetic macular edema. *Arch Ophthalmol.* 2003;121:57-61.
- Danis RP, Ciulla TA, Pratt LM, Aniker W. Intravitreal triamcinolone acetonide in exudative age-related macular degeneration. *Retina.* 2000;20:244-250.
- Penfold PL, Wen L, Madigan MC, King NJC, Provis JM. Modulation of permeability and adhesion molecule expression by human choroidal endothelial cells. *Invest Ophthalmol Vis Sci.* 2002;43:3125-3130.
- Zamir E, Read RW, Smith RE, Wang RC, Rao NA. A prospective evaluation of subconjunctival injection of triamcinolone acetonide for resistant anterior scleritis. *Ophthalmology.* 2002;109:798-805.
- Yoshikawa K, Kotake S, Ichiishi A, Sasamoto Y, Kosaka S, Matsuda H. Posterior sub-Tenon injections of repository corticosteroids in uveitis patients with cystoid macular edema. *Jpn J Ophthalmol.* 1995;39:71-76.
- Ciulla TA, Criswell MH, Danis RP, Hill PE. Intravitreal triamcinolone acetonide inhibits choroidal neovascularization in a laser-treated rat model. *Arch Ophthalmol.* 2000;119:399-404.
- Jonas JB, Hayler JK, Sofker A, Panda-Jonas S. Regression of neovascular iris vessels by intravitreal injection of crystalline cortisone. *J Glaucoma.* 2001;10:284-287.
- Tano Y, Chanfler D, Machemer R. Treatment of intraocular proliferation with intravitreal injection of triamcinolone acetonide. *Am J Ophthalmol.* 1980;90:810-816.
- Sakamoto T, Miyazaka M, Hisatomi T, et al. Triamcinolone-assisted pars plana vitrectomy improves the surgical procedures and decreases the postoperative blood-ocular barrier breakdown. *Graefes Arch Clin Exp Ophthalmol.* 2002;240:423-429.
- Chandler DB, Hida T, Sheta S, Proia AD, Machemer R. Improvement in efficacy of corticosteroid therapy in an animal model of proliferative vitreoretinopathy by pretreatment. *Graefes Arch Clin Exp Ophthalmol.* 1987;225:259-265.
- Wingate RJ, Beaumont PE. Intravitreal triamcinolone and elevated intraocular pressure. *Aust NZ J Ophthalmol.* 1999;27:431-432.
- Modarres M, Parvaresh MM, Peymen GA. Accidental subretinal injection of triamcinolone acetonide. *Ophthalmol Surg Lasers.* 1998;29:935-938.
- McGhee CN, Dean S, Danesh-Meyer H. Locally administered ocular corticosteroids: benefits and risks. *Drug Safety.* 2002;25:33-55.
- Mackey TA, Oelkers C, Rix U, et al. Synthesis, pharmacokinetics, efficacy, and rat retinal toxicity of a novel mitomycin C-triamcinolone acetonide conjugate. *J Med Chem.* 2002;45:1122-1127.
- McCuen BW II, Bessler M, Tano Y, Chandler D, Machemer R. The lack of toxicity of intravitreally administered triamcinolone acetonide. *Am J Ophthalmol.* 1981;91:785-788.
- Kivilcim M, Peymen GA, El-Dessouky ES, Kazi AA, Cheema R. Retinal toxicity of triamcinolone acetonide in silicone-filled eyes. *Ophthalmol Surg Lasers.* 2000;31:474-478.
- Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res.* 1996;62:155-169.
- RE32369: Major EO. Immortal line of human foetal glial cells. U. S. Patent 4,707,448. November, 17, 1987.
- Kong AN, Yu R, Lei W, Mandelkar S, Tan TH, Ucker DS. Differential activation of MAPK and ICE/ced-3 protease in chemical-induced apoptosis: The role of oxidative stress in the regulation of mitogen-activated protein kinases (MAPKs) leading to gene expression and survival or activation of caspases leading to apoptosis. *Restor Neurol Neurosci.* 1998;12:63-70.
- He L, Poblenz AT, Medrano, CJ, Fox DA. Lead and calcium produce rod photoreceptor cell apoptosis by opening the mitochondrial permeability transition pore. *J Biol Chem.* 2000;275:12175-12184.
- He L, Perkins GA, Poblenz AT, Harris JB, Hung M, Ellisman MH, Fox DA. Bcl-xL overexpression blocks bax-mediated mitochondrial contact site formation and apoptosis in rod photoreceptors of lead-exposed mice. *Proc Natl Acad Sci USA.* 2003;100:1022-1027.
- Cellerino A, Bahr M, Isenmann S. Apoptosis in the developing visual system. *Cell Tissue Res.* 2000;301:53-69.
- Khokhar S, Chawla R. Apoptosis: an ocular perspective. *Natl Med J India.* 2002;5:148-153.
- Hajnoczky G, Davies E, Madesh M. Calcium signalling and apoptosis. *Biochem Biophys Res Commun.* 2003;304:445-454.
- Kim S, Iwao H. Stress and vascular responses: mitogen-activated protein kinases and activator protein-1 as promising therapeutic targets of vascular remodelling. *J Pharmacol Sci.* 2003;91:177-181.
- Cowan KJ, Storey KB. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stresses. *J Exp Biol.* 2003;206:1107-1115.
- Strniskova M, Barancik M, Ravingerova T. Mitogen-activated protein kinases and their role in regulation of cellular processes. *Gen Physiol Biophys.* 2002;21:231-255.
- Gartel AL, Shchors K. Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. *Exp Cell Res.* 2003;283:17-21.
- Oster SK, Ho, CS, Soucie EL, Penn LZ. The myc oncogene: marvelously complex. *Adv Cancer Res.* 2002;84:81-154.
- Hida T, Chandler D, Arena JE, Machemer R. Experimental and clinical observations of the intraocular toxicity of commercial corticosteroid preparations. *Am J Ophthalmol.* 1986;101:190-195.
- Jaffe GJ, Yang CH, Guo H, Denny JP, Lima C, Ashton P. Safety and pharmacokinetics of an intraocular fluocinolone acetonide sustained delivery device. *Invest Ophthalmol Vis Sci.* 2000;41:3569-3575.