Evaluation of the Toxicity of Triamcinolone Acetonide and Dexamethasone Sodium Phosphate on Human Lens Epithelial Cells (HLE B-3)

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Abstract

Purpose: The purpose of this study was to compare the in vitro effects of triamcinolone acetonide (TA) and dexamethasone sodium phosphate (DEX) on human lens epithelial cells (HLE B-3).

Methods: HLE B-3 cells were exposed for 24 h to commercially available TA (c-TA) and dimethylsulfoxide-solubilized TA (s-TA). The cells were treated with 1,000 (clinical dose), 750, 500, 200, and 100 μg/mL concentrations of c-TA, s-TA, and supernatant for 24 h. The cells were also treated with DEX at 2, 1, 0.5, 0.2, 0.1 (clinical dose), and 0.05 mg/mL. Cell viability, caspase-3/7 activity, and DNA fragmentation analyses were performed.

Results: The mean cell viabilities of HLE B-3 after exposure to c-TA at 1,000, 750, 500, 200, and 100 μg/mL were significantly reduced compared with control untreated cells. The s-TA also significantly reduced cell viability at 1,000, 750, 500, 200, and 100 μg/mL compared with dimethylsulfoxide control. The supernatant did not reduce cell viability. Caspase-3/7 activity significantly increased after treatment with c-TA and s-TA. DNA laddering revealed bands at 200 bp intervals with both c-TA at ≥100 μg/mL and s-TA at ≥500 μg/mL. The cell viabilities of HLE B-3 after 24 h exposure to DEX were significantly reduced at 2 and 1 mg/mL but not at lower concentrations tested. Caspase-3/7 activities in HLE B-3 cells were not increased significantly after treatment with DEX at any dose tested. DNA laddering did not reveal any band at any dose tested.

Conclusion: This study showed that TA at its clinical dose (1,000 μg/mL) in both commercial preparation and solubilized forms decrease HLE B-3 cell viability through an apoptotic pathway. DEX at its clinical dose (0.1 mg/mL) does not decrease cell viability or cause any increase of caspase-3/7 activity. This study suggests that for long-term sustained-release devices, DEX may be less damaging to human lens cells than TA.

Introduction

The recent focus on the angiostatic and antipermeability properties of steroids has encouraged clinicians to greatly extend the use of intravitreal corticosteroids in a variety of ocular conditions. Triamcinolone acetonide (TA) is an insoluble crystalline steroid that acts as a depot when injected intravitreally. For its prolonged duration of action, TA is broadly used in treatment of a variety of ocular diseases. Intravitreal TA (IVTA) is extensively used in treatment of macular edema due to diabetic retinopathy,1,2 venous occlusive disease,3 ocular inflammation,4,5 and choroidal neovascularization.6 The anti-inflammatory potency of TA is similar to that of methylprednisolone but significantly less compared with that of dexamethasone.7 The biological half-life of parenterally administered TA is 18–36 h,8 whereas the mean elimination half-life in nonvitrectomized eyes is 18.6 days, with measurable concentrations of TA for ~3 months after intravitreal injection in nonvitrectomized eyes.8

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Dexamethasone sodium phosphate (DEX) is also clinically used to reduce intraocular inflammation; but, because it is only available in a soluble form, its duration of action is much less than that of TA.\(^9\) DEX has a half-life of \(~3\) h in the rabbit eye, leading to clearance from the vitreous after \(~3\) days.\(^9\) For its short duration of action, intravitreal DEX has enjoyed a limited role in the management of chronic and/or refractory posterior segment diseases. However, advances in new sustained-release devices containing DEX have brought recent excitement in the use of DEX for management of a variety of chronic/refractory ocular conditions such as macular edema.\(^10\)

Studies have shown that although the steroids are injected into the vitreous, there is diffusion into the anterior chamber, and as a result, elevated concentrations of the steroids can be found in that region.\(^8,11,12\) Under these circumstances, the lens epithelial cells would be exposed to high concentrations of steroids, which may have a negative effect because the terminal differentiation of the epithelial cells into lens fibers is essential for development and growth of the lens.\(^13-16\) Therefore, steroid-induced damage to these parental lens cells could disrupt the normal biological processes and contribute to cataract formation.

Adverse events typically associated with corticosteroid therapy include cataract formation and increased intraocular pressure.\(^17,18,19\) The purpose of this study was to compare the \textit{in vitro} effects of 2 steroids that are used clinically, TA and DEX, to determine their cytotoxic effects upon a human lens epithelial cell line (HLE B-3).

**Methods**

**Cell culture**

HLE B-3 cells (provided by Usha P. Andley) were grown in tissue culture in minimum essential medium containing Eagle’s salts, L-glutamine, gentamicin (50 \(\mu\)g/mL), and 20% fetal bovine serum. The cells were plated in 6- and 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) for cell viability (5.5 \(\times\) 10^5 cells per well) and caspase-3/7 (1.5 \(\times\) 10^5 cells per well) assays, respectively. Passages 15–20 were used for the experiments. As there are variations in enzyme activity with passage and time, experiments were performed in triplicates and all the experiments were repeated 3 times.

**Exposure to drug**

Commercially available TA (c-TA; Kenalog\(^\text{®}\); BMS, Princeton, NJ) was centrifuged at 5,000 rpm for 1 min and the supernatant was removed. To generate the solubilized TA (s-TA), the pellet was resuspended in equivalent amounts of dimethylsulfoxide (DMSO) to achieve the same concentration of DMSO found in the commercial suspension. The cells were treated for 24 h with 1,000 (clinical dose), 750, 500, 200, and 100 \(\mu\)g/mL concentrations of c-TA, s-TA, and the supernatant. Other cells were treated for 24 h with DEX (APP, Schaumburg, IL) at 2,000, 1,000, 500, 200, 100 (clinical dose), and 50 \(\mu\)g/mL concentrations.

**Cell viability assay**

Cell viability assay was performed as previously described.\(^20\) Briefly, cells harvested from the 6-well plates by treatment with 0.2% trypsin–EDTA were then incubated at 37°C for 5 min. The cells were centrifuged at 1,000 rpm for 1 min and then resuspended in 1 mL of culture medium. Automated cell viability analysis was performed (ViCell analyzer; Beckman Coulter, Inc., Fullerton, CA). The analyzer performed an automated trypan blue dye-exclusion assay, generating percentages of viable cells.

**Caspase-3/7 assay**

Caspase-3/7 activities were detected with carboxyfluorescein apoptosis detection kits (FLICA; Immunochemistry Technologies LLC, Bloomington, MN). At the designated time period, the wells were rinsed briefly with fresh culture media, replaced with 300 \(\mu\)L/well of 1 \(\times\) FLICA solution in culture media, and incubated at 37°C for 1 h under 5% CO\(_2\). Cells were washed with phosphate-buffered saline. The following controls were included: untreated HLE B-3 cells without FLICA were used as a background control; untreated HLE B-3 cells with FLICA for comparison of caspase activity of treated cells; wells without cells with buffer alone; tissue culture plate wells without cells with culture media; DMSO + DEX to exclude cross-reaction of FLICA with DMSO + culture media; and HLE B-3 cells with DMSO and FLICA to account for any cross-fluorescence between untreated cells and DMSO.

Quantitative calculations of caspase activities were performed with a fluorescence image scanning unit instrument (FMBIO III; Hitachi, Yokohama, Japan). The caspase activity was measured as the average signal intensity of the fluorescence of the pixels in a designated spot, that is, mean signal intensity.

**DNA fragmentation assay**

HLE B-3 cells (5 \(\times\) 10^5) were plated overnight in 100-mm dishes and then incubated for another 24 h with c-TA, s-TA, and DEX in serum-free medium. DNA was extracted (QIAamp DNA Micro kit; Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Samples were separated by electrophoresis on 3% agarose gels and stained with 5% ethidium bromide. A 100-bp marker was used and images were captured with a fluorescence image scanning instrument (FMBIO III; Hitachi).

**Statistical analysis**

Data were subjected to statistical analysis by ANOVA (Prism, ver. 3.0; GraphPad Software, Inc., San Diego, CA). Newman–Keuls multiple-comparison test was done to compare the data within each experiment. \(P<0.05\) was considered statistically significant. Error bars in the graphs represent SEM, with experiments performed in triplicate.

**Results**

**Cell viability studies**

\textit{c-TA preparation.} The mean cell viabilities of HLE B-3 after exposure to c-TA at 1,000, 750, 500, 200, and 100 \(\mu\)g/mL were 26.3 \pm 5.8 \((P<0.001)\), 34.0 \pm 3.2 \((P<0.001)\), 43.6 \pm 3.3 \((P<0.001)\), 54.3 \pm 11.7 \((P<0.001)\), and 71.6 \pm 4.4 \((P<0.01)\), respectively, compared with control untreated cells (89.7 \pm 0.5; Fig. 1A).

\textit{s-TA preparation.} HLE B-3 cells exposed to s-TA also had a significantly reduced mean cell viability of 20.1 \pm 2.5 \((P<0.001)\), 30.4 \pm 6.6 \((P<0.001)\), and 36.2 \pm 2.4 \((P<0.001)\) at
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Cell viability was 89.3% at 500 μg/mL dimethylsulfoxide. HLE, human lens epithelial; s-TA, solubilized TA; DMSO, dexamethasone sodium phosphate; DEX, dexamethasone sodium phosphate.

**FIG. 1.** (A) c-TA causes dose-related cell viability loss of HLE B-3 cells, starting at a concentration of 100 μg/mL (*P < 0.001). (B) s-TA treatment causes dose-related decrease in cell viability of HLE B-3 cells, starting at a concentration of 500 μg/mL (*P < 0.001). (C) DEX causes dose-related cell viability loss of HLE B-3 cells, beginning at a concentration of 500 μg/mL (*P < 0.001). c-TA, commercially available triamcinolone acetonide; DEX, dexamethasone sodium phosphate; HLE, human lens epithelial; s-TA, solubilized TA; DMSO, dimethylsulfoxide.

TA supernatant

The supernatant alone did not reduce the viability of HLE B-3 cells at any concentration. The mean cell viabilities were 76.6 ± 7.2% (P > 0.05), and 85.3 ± 3.4% (P > 0.05), respectively, compared with equivalent DMSO control cultures. Values of DMSO controls at 1,000, 750, 500, 200, and 100 μg/mL were 65.5 ± 13.0, 69.0 ± 7.4, 81.2 ± 4.0, 86.9 ± 4.7, and 87.6 ± 0.0, respectively. Untreated control cultures were 14.8 ± 0.8% (P < 0.001), 3,232.7 (P < 0.001), 914.9 (P < 0.001), 10,886.5 (P < 0.001), respectively, compared with equivalent DMSO controls at 1,000, 750, 500, 200, and 100 μg/mL.

Caspase-3/7 activity

c-TA preparation. The mean caspase-3/7 activity of HLE B-3 after 24 h exposure to c-TA at 1,000, 750, 500, 200, and 100 μg/mL was 13,407.0 ± 1,079.7 (P < 0.001), 13,848.0 ± 1,496.3 (P < 0.001), 11,660.0 ± 590.3 (P < 0.001), 11,701.3 ± 611.7 (P < 0.001), and 10,814.6 ± 947.3 (P < 0.001), respectively, compared with control untreated cells (2,600.0 ± 510.9) (Fig. 1A).

s-TA preparation. Caspase-3/7 activity in HLE B-3 significantly increased after treatment with s-TA for 24 h. The cells treated with s-TA 1,000, 750, 500, 200, and 100 μg/mL showed mean fluorescence of 10,160.0 ± 498.2 (P < 0.001), 10,886.5 ± 1,429.0 (P < 0.001), 8,308.5 ± 485.7 (P < 0.001), 7,632.0 ± 914.9 (P < 0.001) and 2,932.5 ± 830.8 (P > 0.05), respectively. Values for untreated cells and DMSO-equivalent cultures for 1,000, 750, 500, 200, and 100 μg/mL were 3,479.0 ± 281.4 and 3,232.7 ± 24.5, 3,057.7 ± 222.9, 3,325.2 ± 883.6, 3,867.5 ± 116.6, and 3,215.0 ± 487.9, respectively (Fig. 1B).

**DNA fragmentation assay**

c-TA preparation. Caspase-3/7 is the hallmark of apoptosis because it is the final common pathway of apoptosis. To verify apoptotic activity, DNA fragmentation analysis was performed, which showed DNA bands laddered in ~200 bp increments, consistent with apoptosis (Fig. 3A).

s-TA preparation. DNA fragmentation analysis showed bands in ~200 bp increments, consistent with apoptosis (Fig. 3B).

Dexamethasone sodium phosphate

DNA laddering did not reveal any band, corroborating the lack of apoptosis evidenced by the absence of caspase-3/7 activity (Fig. 3C).
Discussion

The use of intravitreal corticosteroids in the management of a variety of ocular conditions has recently gained widespread acceptance. Formation and/or progression of cataract are common complications of intravitreal steroids. In a study by Jonas et al., IVTA in an elderly population of patients led to clinically significant cataract formation and eventual cataract surgery in about 15%–20% of eyes within 1 year. A single IVTA injection was noted to induce posterior subcapsular cataract development, whereas multiple injections result in all-layer cataract progression. Although cataract is a reversible cause of vision loss, with the recent improvements in the cataract surgery techniques, it is now considered to be a relatively low-risk procedure, and it is generally associated with more long-term complications in patients with chronic posterior segment diseases. It is therefore of clinical significance to identify whether 1 steroid formulation is less cataractogenic than another.

Loss of cell viability resulting from triggered apoptosis or necrosis in HLE cells has been shown to be involved in the pathogenesis of cataract formation. Glucocorticoids induce apoptosis in hematological cells and are used as a chemotherapeutic agent for leukemias, lymphomas, and myeloma. Glucocorticoid-mediated apoptotic cell death could be the final result of the negative modulation of proinflammatory cytokines or glucocorticoid receptor alteration or may be a consequence of cell cycle arrest. Charakidas et al. demonstrated the presence of lens epithelial cell apoptosis and proliferation in human age-related cortical cataract. Although they found a relatively low rate of apoptotic death, which they felt was unlikely to cause significant lenticular opacity, it has been recognized that steroids can induce apoptosis, and it is possible that the higher rate of apoptotic death might lead to cataract formation. For example, Li et al. have shown that lens epithelial cell apoptosis occurs in calcimycin-induced cataract formation.

In our study, cell viability assays have demonstrated that both TA and DEX have cytotoxic effects on the HLE B-3 cells in a concentration-dependent fashion, although the effect was more pronounced in cells treated with TA. The usual clinical dosage of IVTA is 4 mg, and assuming that the vitreous volume is 4 mL, the intravitreal clinical concentration of TA would be 1,000 μg/mL (assuming equal dispersion of the drug throughout the vitreous cavity, although typically this does not happen in a nonvitrectomized eye as the drug tends to aggregate). Our study shows that this “clinically equivalent” dose and also doses as low as 1/5 of the clinical dose of both crystalline c-TA and s-TA decrease HLE cell viability. In contrast, the assigned clinical dosage of

FIG. 2. (A) c-TA treatment causes increased caspase-3/7 activity at all concentrations tested (*P<0.001). (B) Treatment with s-TA causes higher levels of caspase-3/7 activity at all concentrations tested (*P<0.001) except 100 μg/mL (P>0.05). (C) DEX did not show caspase-3/7 activity at any of the concentrations tested (P>0.05).
intravitreal dexamethasone is 0.1 mg/mL (100 μg/mL), which is 10 times below the ranges that caused loss of lens cell viability in the HLE B-3 cells. The observed greater toxic potential of TA compared with DEX has been recently shown by Yeung et al. in a cultured retinal pigment epithelium (RPE) cell line.\textsuperscript{34} Although many clinical studies have shown cataract development and progression after intravitreal use of TA,\textsuperscript{21,35,36} to the best of our knowledge, this is the first study comparing the effect of doses of TA and DEX used clinically on lens epithelial cells \textit{in vitro}.

Loss of cell viability can be attributed to both apoptosis and/or necrosis. Caspase-3/7 is a key effector in the apoptosis pathway, and its activation signals the full commitment to disassembly of the cell. It is known that caspase detects apoptotic and dying cells, but once the cell is dead it will not be caspase positive. Because of high numbers of dead cells at higher doses (1,000 μg/mL), we do not see dose-dependent increase at higher c-TA doses. Our study demonstrates that the loss of cell viability in TA-treated HLE B-3 cells is associated with activation of caspase-3/7 pathway, suggesting an involvement of an apoptotic mechanism, which was then further supported by DNA fragmentation analysis. In contrast, caspase-3/7 activities in HLE B-3 cells were not significantly increased after treatment with DEX at all concentrations tested. Other studies have shown that dexamethasone causes necrosis on corneal endothelial cells,\textsuperscript{37} but it does not cause apoptosis in retinal pigment epithelial cells.\textsuperscript{38} However, recently, Petersen et al. reported that DEX induces caspase-3 apoptosis in HLE cells. This could be due to difference in cell line used and different conditions of incubation. To mimic normal physiological behavior of HLE cells, we used 20% serum for incubation and treatment, whereas Petersen et al. used serum-free culture media during treatment.\textsuperscript{39} Our findings indicate that the cytotoxic effects of TA and DEX are mechanistically different. Further, lower solubility of TA leads to slower, and perhaps unequal, dispersion in vitreous and, therefore, variable concentration at different locations in vitreous cavity. We hypothesize that this could add to cataractogenic toxicity of TA if higher levels remain near the posterior pole of the lens.

We acknowledge that there are limitations to our study. First, concentrations used in this study cannot be directly extrapolated to clinical practice, as this study was performed \textit{in vitro}. In addition, the cells were exposed for only 24 h, which is not the case in clinical conditions, although by looking for subtle downstream changes such as mitochondrial membrane potential changes and caspase upregulation we are able to detect early effects that are likely to be predictive of later damage. Third, TA drug crystals came in direct contact with the cells, which happens in clinical conditions such as in vitrectomized eyes. However, to eliminate the crystal contact effect, the TA was solubilized and there was a decrease in cell viability and an increase in caspase-3/7 activity. In general, the \textit{in vitro} cell toxicity experiments have been predictive or corroborative of clinically observed toxicity with other agents such as vital dyes.\textsuperscript{20} Regardless, the results of this study suggest that doses of TA clinically used may be more cytotoxic to HLE cells than DEX and, hence, perhaps, more cataractogenic. Despite having more potency, DEX has also been shown to be less cytotoxic in other ocular cell lines than TA.\textsuperscript{34,40–44} The varying levels of cytotoxicity for DEX and TA may be due to differences in their chemical formulations. TA has been shown to be more toxic than DEX primarily because of its crystalline formulation. Although these crystals make TA an excellent choice for long-term drug delivery, on the other hand, these crystals have been shown as a cause of toxicity. Szurman et al. showed that filter-purified, nonadherent TA is nontoxic to ARPE-19 cells at concentrations of up to 1,000 μg/mL.\textsuperscript{44} However, filtering this compound may result in decreased efficacy of TA, making it undesirable to use clinically. To summarize, the decreased lens epithelial cell toxicity observed \textit{in vitro} with DEX compared with TA, combined with the greater potency of DEX over TA, suggests that DEX may be a better option for prolonged clinical use in the eye.

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**Author Disclosure Statement**

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