

Effects of triamcinolone acetonide on human trabecular meshwork cells *in vitro*

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Aim: To study the effects of triamcinolone acetonide (TA) on cultured human trabecular meshwork (HTM) cells. **Materials and Methods:** HTM cells were cultured and treated with 125, 250, 500 and 1000 µg/mL concentration of TA for 24 h. The cells were treated with both crystalline TA (TA-C) (commercial preparation) and solubilized TA (TA-S). Cell viability was measured by a trypan blue dye exclusion test. The activity of caspase-3/7 was measured by a fluorescence caspase kit and DNA laddering was evaluated by electrophoresis on 3% agarose gel. Levels of lactate dehydrogenase (LDH) were assessed with LDH cytotoxicity assay kit-II. **Results:** Mean cell viabilities of HTM cells after 24 h exposure to TA-C 125, 250, 500, and 1000 µg/mL were 75.4 ± 2.45% ($P < 0.0001$), 49.43 ± 1.85% ($P < 0.0001$), 17.07 ± 2.39% ($P < 0.0001$), and 3.7 ± 0.9% ($P < 0.0001$), respectively, compared with the untreated HTM cells 92.49 ± 1.21%. The mean cell viabilities with 125, 250, 500, and 1000 µg/mL of TA-S were 94.47 ± 1.60% ($P > 0.05$), 90.13 ± 0.40% ($P < 0.01$), 85.57 ± 0.47% ($P < 0.001$), and 71.67 ± 3.30% ($P < 0.0001$), respectively, compared to DMSO-equivalent cultures. Untreated HTM control had a cell viability of 96.57 ± 1.98%. DMSO-treated controls of 125, 250, 500, and 1000 µg/mL had a cell viability of 94.73 ± 0.57%, 96.97 ± 1.08%, 93.97 ± 1.85%, and 97.27 ± 1.15%, respectively. There was no increase of caspase-3/7 activity in cultures treated with either TA-C or TA-S. DNA laddering showed no bands in the TA-C or TA-S treated cultures. There were significantly higher LDH release rates at all concentrations of TA-C compared to TA-S. **Conclusions:** Results show that the effect of TA-C and TA-S on HTM cells is due to cell death by necrosis at all concentrations except 125 µg/mL of TA-S. Elevated levels of LDH confirmed necrotic cell death. Our study also infers the relative safety of TA-S over TA-C.

Key words: Triamcinolone acetonide, human trabecular meshwork cells, *in vitro*

Triamcinolone acetonide (TA) is extensively used in intravitreal injections to treat macular diseases such as macular edema due to diabetic retinopathy,^[1] venous occlusive diseases,^[1,2] ocular inflammation,^[3,4] and also in the cases of choroidal neovascularization from AMD.^[5] With recent clinical and experimental studies confirming the pivotal role of intravitreal triamcinolone acetonide (IVTA) in the successful management of routine and refractory cases of inflammatory, neovascular and macular edematous conditions, the use of IVTA has increased in the past decade.^[6] The indications have widened to treat other anterior ocular diseases such as neovascularization of the iris. With its expanded use, the complications related to the injection of TA are being reported more frequently.

Complications of IVTA, include increased intraocular pressure (IOP),^[7,8] cataract formation,^[9] retinal detachment, vitreous hemorrhage, and pseudoendophthalmitis.^[10,11] Bioanalytical and pharmacokinetic studies have shown TA in the aqueous following IVTA.^[12] Alternative review shows that the most common complications reported are the rise

in IOP causing secondary chronic open angle glaucoma that requires medications or antiglaucoma surgeries,^[13] progression of cataracts,^[14] and accumulation of triamcinolone crystals, resulting in pseudohypopyon. The passage of emulsified TA into the anterior chamber occurs commonly in many clinical scenarios. It can be seen in patients with zonular dehiscence and in those with rupture of the posterior capsule. Presumably, the TA crystals are carried into the anterior chamber by currents generated by saccadic eye movements in these conditions.

Moshfeghi *et al.* reported a rate of 0.8% of pseudohypopyon after IVTA.^[11] Although pseudohypopyon does not cause any ocular morbidity, persistence of even a trace amount of TA may lead to prolonged ocular hypertension occasionally seen in some patients. TA crystals induce cytotoxic and stress response in trabecular meshwork (TM) cells.^[15] Although several mechanisms have been put forth to explain the elevated IOP with IVTA, it is still not clear as to the role of TA on the human trabecular meshwork (HTM) cells.

A dose of 25 mg of IVTA led to ocular hypertension in about 50% of treated eyes that lasted for approximately 6 months.^[11] However, in another study using 4 mg of IVTA, there was no effect on IOP at 7 days after injection.^[16] Although, the intravitreal TA lasts for 3-6 months, persistence of even a trace amount of the drug can lead to a rise in the IOP. HTM cells constantly bathe in the aqueous and an altered aqueous humor outflow resistance plays an important role in the regulation of IOP. Previous works in our laboratory have shown the toxicity of TA on the retinal pigment epithelial (ARPE-19), neurosensory retinal (R28), and human lens epithelial cell lines.^[19,20] TA might affect the trabecular meshwork structural framework by increasing the protein expression and inhibiting

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the proteases or may have a direct effect on the trabecular meshwork cells. This might disrupt the aqueous outflow and prove glaucomagenic.¹⁷

The aim of this study was to investigate the effects of TA on the HTM cell line. We tested the effects of two forms of TA on the trabecular cells, one being the commercially available crystalline TA suspension (TA-C) and other being the TA without the supernatant, but solubilized in dimethyl sulfoxide (DMSO). Solubilized TA (TA-S) is a solution without any particulate matter. This is in contrast with the TA-C that has a suspension of varying sized particles/crystals that may cause a mechanical effect on the cells. Comparing the TA-S and TA-C formulations, will help distinguish the possible toxicity of the TA crystals on the cells.

Materials and Methods

HTM cells were obtained from Dr. Vincent Raymond, MD, PhD, at the Laboratoire de Genetique et Geonmique Oculaires, CREMO (Quebec, QC). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Low glucose; Invitrogen-Gibco, Carlsbad, CA), 1000 mg/L D-glucose, L-glutamine, 25 mM HEPES buffer, and 110 mg L-sodium pyruvate, 25 µg/mL, 2.5 µg/mL, amphotericin B, 10% fetal bovine serum.

The cells were plated in 6 and 24-well plates (Fisher Scientific, Park Lane, Pittsburgh, PA) for cell viability (6×10^5 cells/well) and caspase assays (1.2×10^5 cells/well), respectively, and were incubated at 37°C until confluent. The cells were then exposed to TA-C or TA-S at four different concentrations. As controls for the TA-S cultures, some cells were treated with dimethyl sulfoxide (DMSO) at all equivalent concentrations. Before the treatment, the cells were incubated for 2 h in 2% fetal bovine serum containing medium, to make them relatively nonproliferating. This simulates the HTM cells, which remain in a nonproliferating phase.

HTM cells were treated for 24 h with TA-C (Kenalog, Bristol-Meyers Squibb, Princeton, NJ) in the concentration of 125, 250, 500, and 1000 µg/mL. For the TA-S solution, the TA-C was centrifuged at 5000 rpm for 1 min and the supernatant containing the vehicle was discarded. The pellet of TA was resuspended in an equivalent amount of DMSO to achieve the same concentrations of TA as in TA-C. The new TA solution was resuspended in an equivalent amount of culture medium to achieve the desired concentrations of solubilized Triamcinolone (TA-S) (125, 250, 500, and 1000 mg/mL) for 24 h. HTM cells were also treated with equivalent amounts of DMSO as control. We maintained untreated controls in all our experiments.

Cell viability assay was performed as described by Luthra *et al.* and Narayanan *et al.*¹⁸⁻²⁰ Cell passages used were between 10 and 15. Briefly, cells were harvested from the 35-mm dishes by treatment with 0.2% trypsin-EDTA at 37°C for 5 min. The cells were centrifuged at 1000 rpm for 1 min. The supernatant was removed and the cell pellet resuspended in 1 mL of culture medium. Automated cell viability analysis was performed using ViCell analyzer (Beckman Coulter Inc., Fullerton, CA). The analyzer performs an automated trypan blue dye-exclusion assay and gives the percentage viability of cells. All concentrations were performed in triplicates and the experiments repeated three times.

Caspase-3/7 is a key effector in the pathway of apoptosis, and its activation heralds the cell commitment to disassembly. Therefore, we analyzed the caspase-3/7 activity to study the molecular pathway of cell death. Sequential activation of caspase plays a central role in the execution phase of cell apoptosis.

The caspase-3/7 activity was detected using caspase detection kits (Carboxyfluorescein FLICA Apoptosis Detection kits; Immunochemistry Technologies, LLC, Bloomington, MN). The FLICA reagent has an optimal excitation range from 488 to 492 nm and an emission range from 515 to 535 nm. Apoptosis was quantified as the level of fluorescence emitted from FLICA probes bound to caspase. Nonapoptotic cells appeared unstained, whereas cells undergoing apoptosis fluoresced brightly.

At the designated time period, the wells were rinsed briefly with fresh culture media, replaced with 300 µL/well of $1 \times$ FLICA solution in culture media, and incubated at 37°C for 1 h. Cells were washed with phosphate buffered saline. The following controls were included: Untreated HTM cells without FLICA to exclude auto fluorescence from cells; untreated HTM cells with FLICA for the comparison of the caspase activity of treated cells; tissue culture plate wells without cells with buffer alone to represent the background levels; tissue culture plate wells without cells with culture media with DMSO to exclude cross reaction of FLICA with DMSO with culture media; HTM 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-3/7 activity was measured as average signal intensity of the fluorescence of the pixels in a designated spot-Mean Signal Intensity (msi). In addition, cultures were observed through an inverted fluorescent microscope (Leica, Solms, Germany) with a band pass filter (excitation 490 nm, emission 520 nm) to view the green fluorescence of caspase positive cells.

Apoptotic cell death is characterized by the cleavage of DNA into oligonucleosomal multimers of approximately 200 basepair fragments which can be visualized by an oligosomal ladder by 3% agarose gel electrophoresis. DNA fragmentation is an early sign of cellular apoptosis. Hence, we did DNA fragmentation assay, a sensitive assay to look for any apoptotic cell death, which could be noncaspase mediated.

HTM cells (1×10^6 cells/dish) were plated overnight in 60 mm dishes and then incubated for another 24 h with 125 and 250 µg/mL of TA-C and 250 and 500 µg/mL of TA-S and DMSO-equivalent cultures. These concentrations were chosen because the cell death at these concentrations was not complete. Still there were good numbers of viable cells or dying cells that expressed quantifiable caspase-3/7 activity. 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. Images were captured with a fluorescence image scanning instrument (FMBIOIII; Hitachi, Yokohama, Japan).

LDH cytotoxicity assay is a common technique for measuring cell death. It quantifies the release of lactate dehydrogenase (LDH), a stable enzyme found in the cytoplasm, into the supernatant upon rupture of the plasma membrane. It is a colorimetric assay and measures the absorbance at a particular wavelength. The absorbance correlates positively with the number of compromised cell membranes as in necrosis.

LDH release rate was detected using LDH cytotoxicity assay kit-II (Biovision, Mountain View, CA). 2×10^4 of HTM cells were plated in 96-well plate. After 24 h of exposure to TA-C and TA-S, the plate was shaken well to ensure LDH was evenly distributed in the culture medium. The cells were centrifuged at 6000 rpm for 10 min to precipitate the cells. 50 μ L/well of supernatant was transferred into an optically clear 96-well plate. The cells were then treated with 100 μ L/well LDH reaction mix. LDH reaction mix was prepared by mixing the WST substrate mix with LDH assay buffer. After 30 min of incubation at room temperature, the LDH activity was quantified by multiwell spectrophotometer (Perkin Elmer, Wellesley, MA) at OD 450 nm filter. The plate was

read at multiple time points until the consistent reading was observed.

Normative data were subjected to statistical analysis by ANOVA (Prism, ver. 3.0; GraphPad Software Inc., San Diego, CA). Multiple-comparison test was conducted with posthoc Tukey test. Error bars in the graphs represent SEM (Standard Error of Mean) with experiments performed in triplicate. $P < 0.05$ was considered statistically significant.

Results

HTM cells showed a progressive concentration dependent decrease in cell viability after exposure to both TA-C and TA-S [Figs. 1 and 2] [Table 1a].

Cell viability of untreated HTM cell cultures was similar to DMSO equivalent cultures of TA-S at all the concentrations. There was significant loss of cell viability at all the concentration of TA-S concentrations except 125 μ g/mL compared to untreated HTM cultures. TA-C showed higher loss of cell viability compared to TA-S at all the concentrations tested [Table 1b].

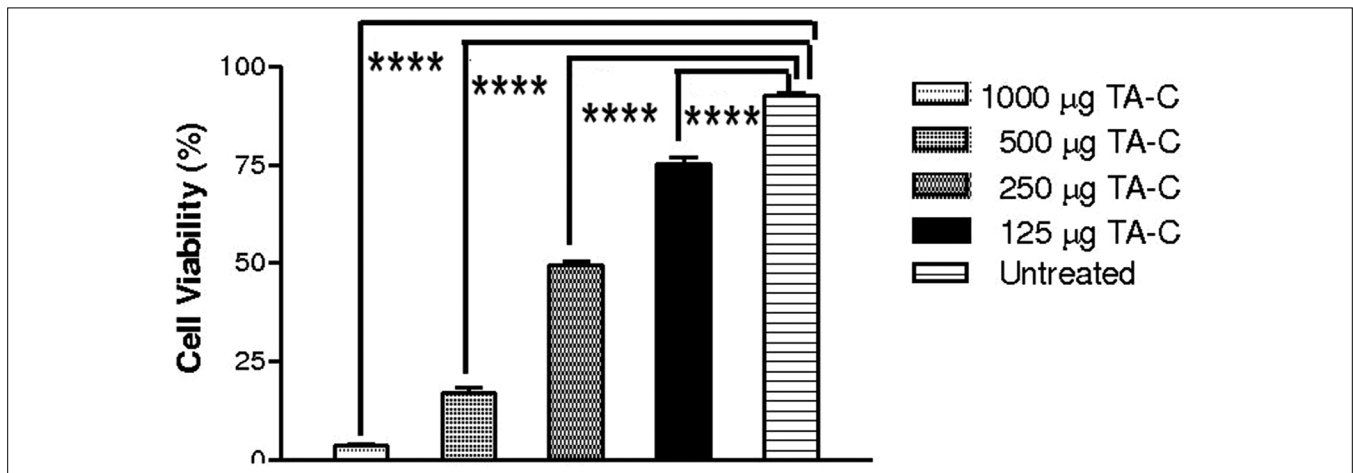


Figure 1: HTM cells showed a dose-dependent decrease in cell viability after treatment at all concentrations of TA-C for 24 h compared with untreated control cultures. Statistically significant ($P < 0.0001$)

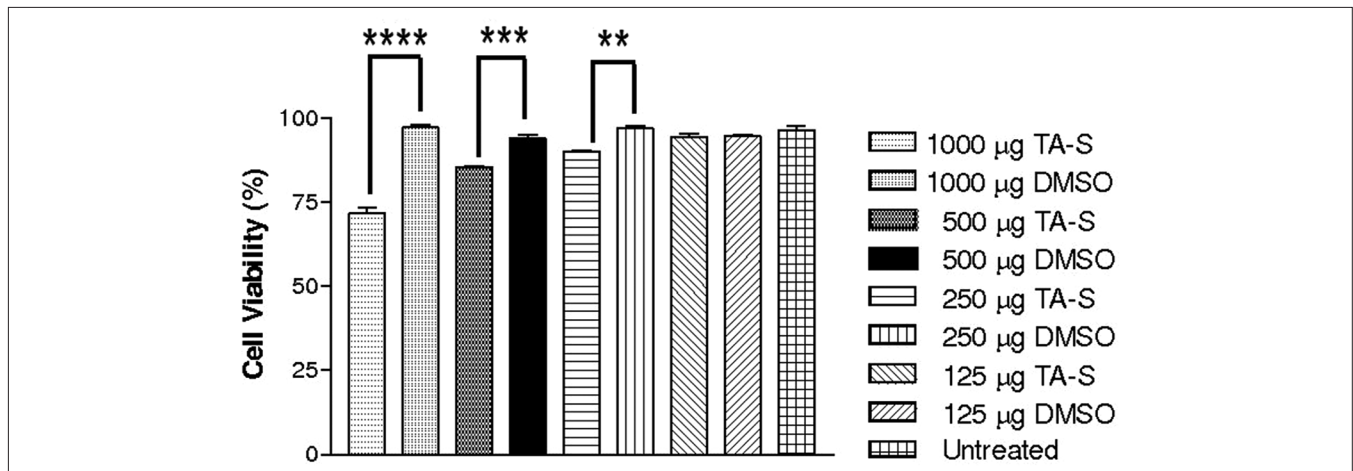


Figure 2: HTM cells showed a dose-dependent decrease in cell viability at all concentrations of TA-S except 125 μ g/mL when treated for 24 h compared to respective DMSO-controls. **Statistically significant ($P < 0.01$) ***Statistically significant ($P < 0.001$) ****Statistically significant ($P < 0.0001$)

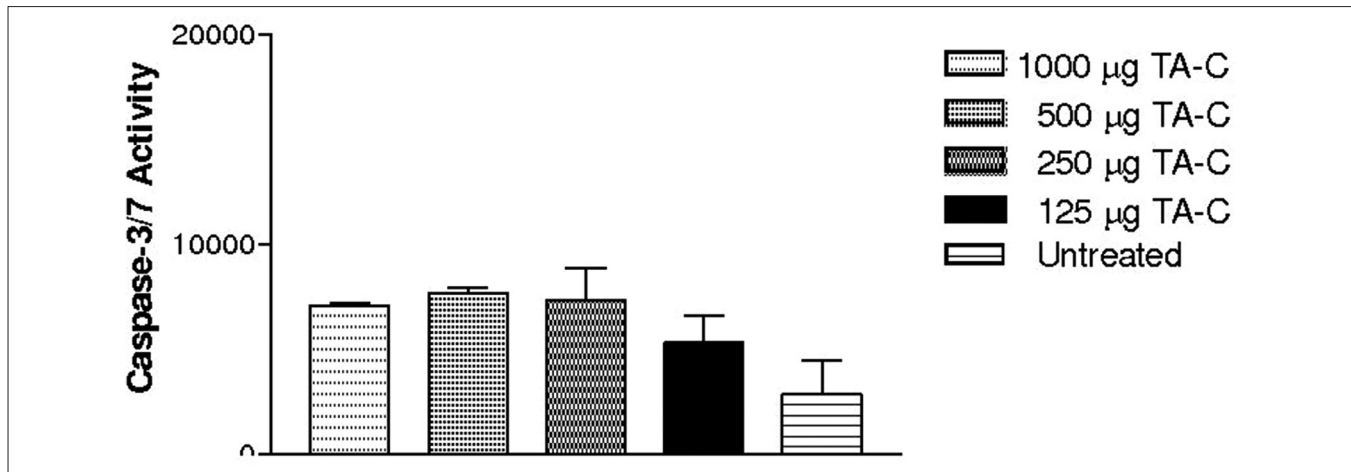


Figure 3: HTM cells did not show statistically significant caspase-3/7 activity at any concentration of TA-C

Table 1a: The mean cell viabilities of human trabecular meshwork cells after 24 hours exposure with triamcinolone acetamide treatment

	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL	Untreated control (%)
TA-C (%)	75.4±2.45	49.43±1.85	17.07±2.39	3.7±0.9	92.49±1.21
TA-S (%)	94.47±1.60	90.13±0.40	85.57±0.47	71.67±3.30	96.57±1.98
DMSO (TA-S equivalent) control	94.73±0.57%	96.97±1.08	93.97±1.85	97.27±1.15	

TA-C: Crystalline triamcinolone, TA-S: Solubilized triamcinolone, DMSO: Dimethyl sulfoxide

Table 1b: Results of post hoc tukey test for cell viability

Tukey's multiple comparisons test	Mean diff.	95% CI of diff.	Significance
1000 µg TA-C vs. untreated	-88.79	-93.80 to -83.78	$P < 0.0001$
500 µg TA-C vs. untreated	-75.42	-80.43 to -70.41	$P < 0.0001$
250 µg TA-C vs. untreated	-43.06	-48.07 to -38.05	$P < 0.0001$
125 µg TA-C vs. untreated	-17.09	-22.10 to -12.08	$P < 0.0001$
1000 µg TA-S vs. 1000 µg DMSO	-25.60	-30.27 to -20.93	$P < 0.0001$
500 µg TA-S vs. 500 µg DMSO	-8.400	-13.07 to -3.729	$P < 0.001$
250 µg TA-S vs. 250 µg DMSO	-6.833	-11.50 to -2.162	$P < 0.01$
125 µg TA-S vs. 125 µg DMSO	-0.2667	-4.938 to 4.404	$P > 0.05$

TA-C: Crystalline triamcinolone, TA-S: Solubilized triamcinolone, DMSO: Dimethyl sulfoxide, CI: Confidence interval

HTM cells showed no statistically significant increase in the caspase-3/7 activity at any concentration of TA-C treated cultures for 24 h [Fig. 3] [Tables 2a and b].

HTM cells showed no statistically significant increase in the caspase-3/7 activity at any concentration of TA-S compared to equivalent DMSO-treated cultures at 24 h [Fig. 4] [Tables 2a and b].

Caspase-3/7 activity of untreated HTM cell cultures were

similar to DMSO cultures at all the concentrations along with all the TAS concentrations tested [Table 2b].

To verify the findings of our caspase-3/7 assay, we performed DNA fragmentation assay with TA-C at concentration of 125 and 250 µg/mL. No 200 basepair (bp) banding pattern was noted. DNA fragmentation with TA-S at concentration of 250 and 500 µg/mL and DMSO-equivalent controls also did not show any 200 bp banding. This supports our caspase-3/7 findings of nonapoptotic cell death with TA-C and TA-S [Fig. 5a and b].

LDH release rates of untreated HTM cell cultures were similar to DMSO equivalent cultures of TA-S at all the concentrations except 1000 µg/mL ($P < 0.05$). There was significant release rate at 250, 500, and 1000 µg/mL of TA-S concentrations whereas 125 µg/mL release rate was not significant compared to untreated HTM cultures. TA-C showed higher LDH release compared to TA-S at all the concentrations tested [Figs. 6 and 7] [Tables 3a and b].

Discussion

In this study, we analyzed the effects of TA on HTM cells and noted loss of cell viability at all concentrations 125, 250, 500, and 1000 µg/mL of the commercially available TA solution, TA-C. However, when the TA crystals were solubilized in DMSO (TA-S), the loss of cell viability was less at the higher concentrations and absent at 125 µg/mL. On direct comparison of TA-S and TA-C, TA-S was found to be less toxic to HTM cells at all the concentrations compared to TA-C on cell viability and LDH release assay. This cytotoxicity of TA could be one of the contributing factors for ocular hypertension following IVTA.

Our cytotoxicity studies of TA with HTM cells corroborate the findings by Wang *et al.*^[15] As in our case, Wang *et al.* found

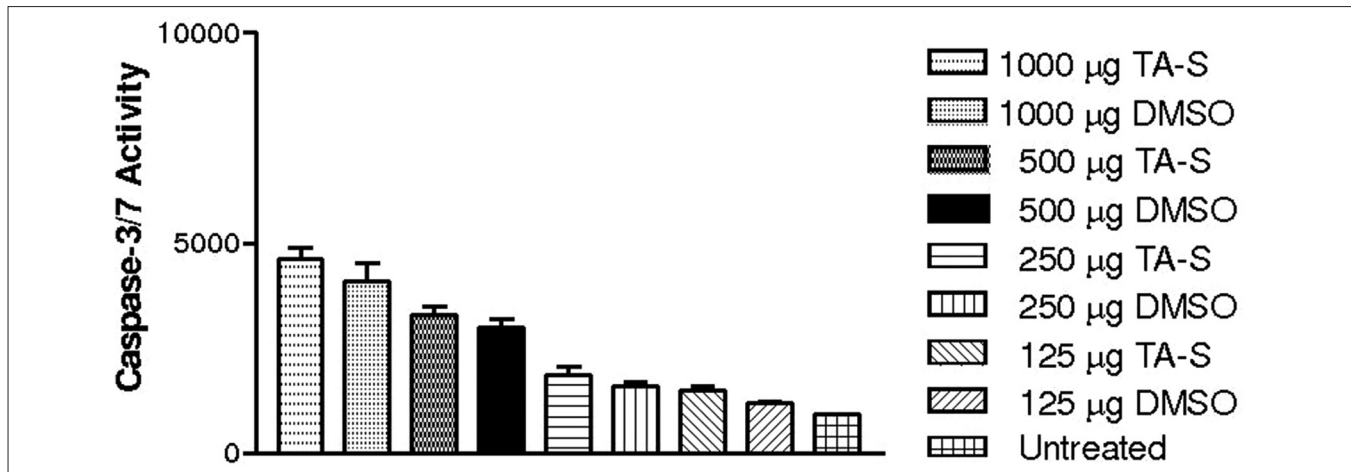


Figure 4: HTM cells did not show statistically significant caspase-3/7 activity at any concentration of TA-S and DMSO-equivalent cultures

Table 2a: The mean caspase-3/7 activity of human trabecular meshwork cells after 24 hours exposure with triamcinolone acetonide treatment

	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL	Untreated control
TA-C	5312.09±2182.52	7347.55±2583.72	7671.25±501.31	7096.90±132.96	2864.11±2722.28
TA-S	1497.52±167.05	1879.04±288.49	3306.54±335.23	4629.40±471.62	923.97±2722.28%
DMSO (TA-S equivalent) control	1214.19±37.29	1594.76±175.86	2998.96±322.87	4075.93±772.83	

TA-C: Crystalline triamcinolone, TA-S: Solubilized triamcinolone, DMSO: Dimethyl sulfoxide

Table 2b: Results of post hoc tukey test for caspase-3/7 activity

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significance
1000 µg TA-C vs. untreated	4233	-1022 to 9487	<i>P</i> >0.05
500 µg TA-C vs. untreated	4807	-447.5 to 10062	<i>P</i> >0.05
250 µg TA-C vs. untreated	4483	-771.2 to 9738	<i>P</i> >0.05
125 µg TA-C vs. untreated	2448	-2807 to 7703	<i>P</i> >0.05
1000 µg TA-S vs. 1000 µg DMSO	553.5	-482.5 to 1589	<i>P</i> >0.05
500 µg TA-S vs. 500 µg DMSO	307.6	-728.4 to 1344	<i>P</i> >0.05
250 µg TA-S vs. 250 µg DMSO	284.3	-751.7 to 1320	<i>P</i> >0.05
125 µg TA-S vs. 125 µg DMSO	283.3	-752.7 to 1319	<i>P</i> >0.05

TA-C: Crystalline triamcinolone, TA-S: Solubilized triamcinolone, DMSO: Dimethyl sulfoxide, CI: Confidence interval

that after being treated with TA, the entire monolayer of cells were covered with TA-C crystals at 24 h. They also reported statistically significant loss in cell number upon treating with TA-C at concentration of 1 and 0.1 mg/mL. When the vehicle containing the preservatives was removed (similar to our TA-S solution), the cell-proliferation as measured by MTT assay was similar to control cultures thereby suggesting the drug without the preservatives to be much safer than the

commercial TA. In our study, we demonstrated that although there was significant loss of cell viability with TA-S at higher concentrations (250, 500, and 1000 µg/mL), still it was safer at these concentrations than TA-C in terms of cell viability.

The relative safety of TA-S over TA-C could be due to the fact that TA-S is free from the vehicle containing the preservative, benzyl alcohol, which is found to be cytotoxic by itself. The persistence of loss of cell viability due to TA-S could be related to the direct chemical toxicity of the steroid.

We then studied the molecular pathways involved in the cell death. The activation of caspase-3/7 represents a cell commitment to disassembly and is the hallmark of apoptosis. Caspase-3/7 assays showed no activity at any concentration of TA-C and TA-S, indicating noncaspase-mediated cell death. We then ran the DNA fragmentation assay with TA-C and TA-S, but did not find any banding pattern, which was consistent with lack of caspase-3/7 activity. This was in contrast to the apoptosis noted in a HTM cell line after TA treatment in a study by Wang *et al.*, with TA.^[15] This study examines the effect after 24 h and shows no significant caspase activity that is in agreement with the study by Wang *et al.*^[15]; however, they have found significant apoptosis at day 3 and 5.

The contradictory mechanisms of cell death to the same cytotoxic stimuli are also observed in several other studies using bovine HTM cells and dexamethasone. Sibayan *et al.* did not notice any apoptosis with dexamethasone at concentration of 0.01-100 µM in bovine trabecular meshwork cells.^[21] In contrast, Gu *et al.* noticed apoptosis with dexamethasone at concentration of 0.24-0.96 mmol/L in the same cell line.^[22] Such a phenomenon is also seen with TA and ARPE-19 cells. Chang *et al.* has shown necrosis to be the main mode of cell death in ARPE-19 cells.^[23] This is again in contrast to

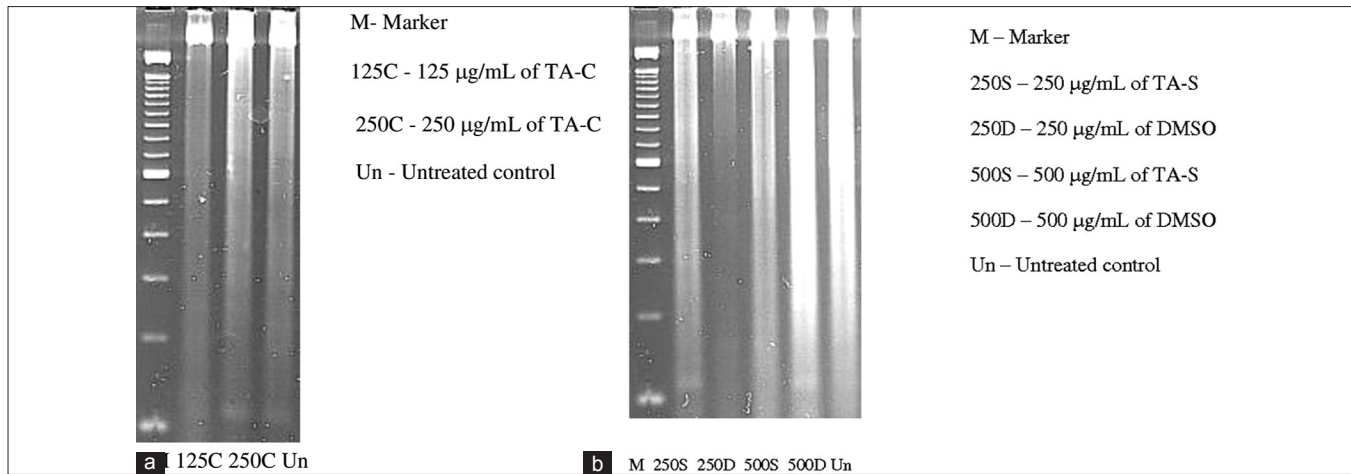


Figure 5: (a) DNA laddering showed no bands at 125 and 250 µg/mL concentration of TA-C cultures. (b) DNA laddering showed no bands at 500 and 250 µg/mL concentration of TA-S and DMSO-equivalents cultures

Table 3a: Lactate dehydrogenase release rate of human trabecular meshwork cells after 24 hours exposure with triamcinolone acetonide treatment

	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL	Untreated control
TA-C	27.85±1.20	29.50±0.71	21.90±1.13	27.55±0.78	8.0±1.41
TA-S	15.50±0.70	18.50±2.12	17.50±0.70	24.50±0.70	12.00±1.41
DMSO (TA-S equivalent) control	12.50±0.70	13.0±1.41	11.50±0.70	17.50±0.70	

TA-C: Crystalline triamcinolone, TA-S: Solubilized triamcinolone, DMSO: Dimethyl sulfoxide

Table 3b: Results of post hoc tukey test for LDH release rate

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significance
1000 µg TA-C vs. untreated	19.55	15.22 to 23.88	$P < 0.0001$
500 µg TA-C vs. untreated	13.90	9.568 to 18.23	$P < 0.001$
250 µg TA-C vs. untreated	21.50	17.17 to 25.83	$P < 0.0001$
125 µg TA-C vs. untreated	19.85	15.52 to 24.18	$P < 0.0001$
1000 µg TA-S vs. 1000 µg DMSO	7.000	2.528 to 11.47	$P < 0.01$
500 µg TA-S vs. 500 µg DMSO	6.000	1.528 to 10.47	$P < 0.01$
250 µg TA-S vs. 250 µg DMSO	5.500	1.028 to 9.972	$P < 0.05$
125 µg TA-S vs. 125 µg DMSO	3.000	-1.472 to 7.472	$P > 0.05$

TA-C: Crystalline triamcinolone, TA-S: Solubilized triamcinolone, DMSO: Dimethyl sulfoxide, LDH: Lactate dehydrogenase

the studies by Yeung *et al.* who reported the increasing caspase-3/7 activity and hence apoptosis in ARPE-19 cells.^[24] In disagreement with both these studies, Valamanesh *et al.* demonstrated that in rats the TA induced retinal cell death did not use a caspase-dependent or caspase-independent apoptotic pathways.^[25]

Kong *et al.* suggested that chemical-induced oxidative stress

can increase the expression of cytoprotective genes, which can safeguard the cells against apoptosis and enhance cell survival at some concentrations and not at others.^[26] Moreover, apoptotic caspase pathways can be cell-type specific and not stimuli specific.

Necrotic cell death is classically evaluated by the quantification of plasma membrane damage. Lactate dehydrogenase (LDH) is a stable enzyme, present in all cell types, and rapidly released into the cell culture medium upon damage of the plasma membrane. LDH cytotoxicity assay supported our earlier results with caspase-3/7 and DNA fragmentation assays. Statistically significant LDH release rate was observed at all concentrations with TA-C and at 250, 500, and 1000 µg/mL concentrations with TA-S. TA-C had significantly more LDH release at all the concentration compared to TA-S. This supports our hypothesis that the TA-induced HTM cell death was through the necrosis pathway.

The mechanism of raised IOP due to IVTA is not clear. The depletion of TM cells occurs with aging and also with continuous exposure to cytotoxic stimuli. The disruption of the trabecular meshwork beams affect the aqueous outflow and eventually could result in raised IOP.^[27,28] Other theories as that the raise in IOP could be because of activation of steroid receptors.^[29] or due to the deposition of glycosaminoglycans causing TM dysfunction.^[30] Singh *et al.* showed the rise in IOP as a result of HTM obstruction by the particulate matter of crystalline TA.^[31] We infer it could be due to the proposed cytotoxicity of TA on the HTM cells and subsequent TM dysfunction. There are some limitations to extrapolate our results directly to clinical practice.

- Shorter incubation: We incubated the HTM cells for 24 h

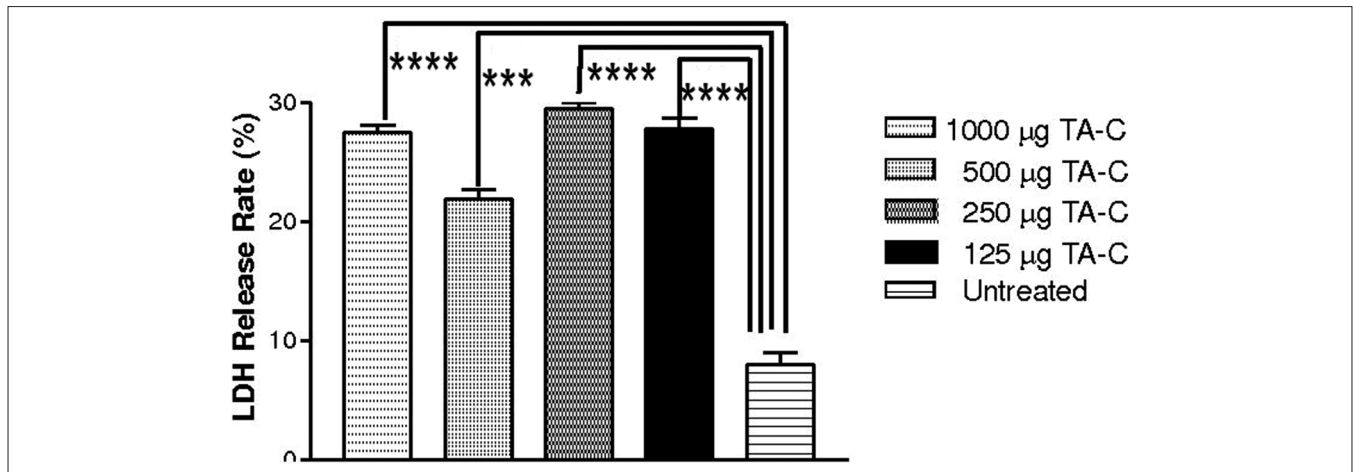


Figure 6: LDH release rate of HTM cells increased at all concentrations tested *** Statistically significant ($P < 0.001$), **** Statistically significant ($P < 0.0001$)

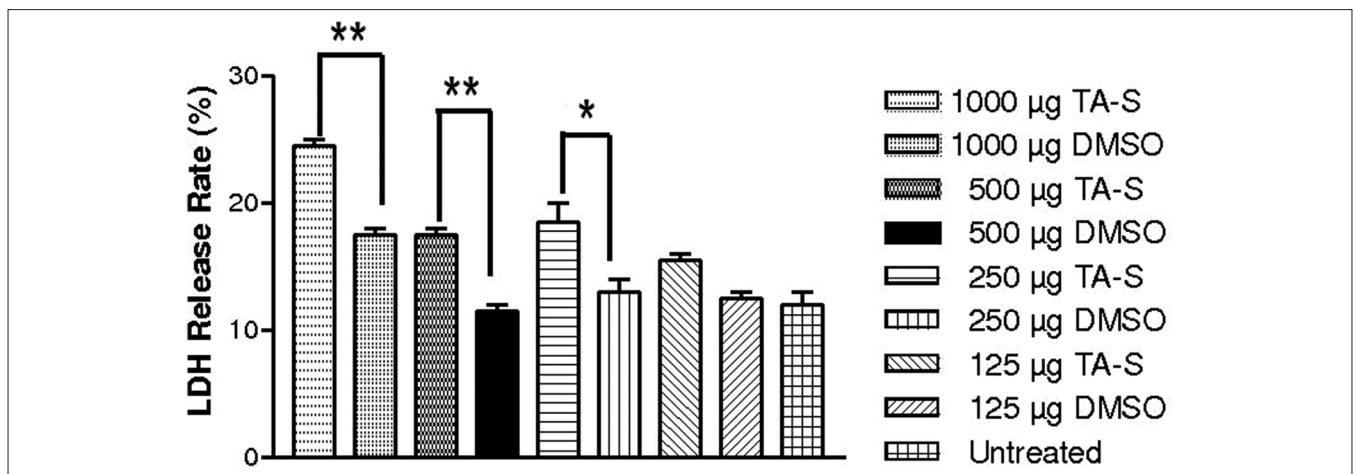


Figure 7: LDH release rate of HTM cells increased at concentration of all the concentrations except 125 µg/mL. *Statistically significant ($P < 0.05$) **Statistically significant ($P < 0.01$)

with TA, which would not explain the chronic exposure of TA crystals to the TM cells *in vivo*

- Varying concentrations: *In vivo*, the aqueous levels of TA following IVTA vary with different time points postinjection and also on the amount injected. This could not be reproduced in our study design
- HTM is a single cell type.

In spite of these variations, we still gained valuable information.

- We could understand the molecular mechanism of necrotic cell death in TM cells on exposure to TA
- HTM cells appeared to be very sensitive to TA compared to other cell types that did not decrease cell viability with the same levels of TA
- The differential toxicity of the TA in its two different forms was studied. TA-C was found to have greater cytotoxic effects than TA-S. The relative safety of TA-S over TA-C supports the rationale behind the current use of preservative free TA for intravitreal injections
- We could also get some clues to the question of raise in IOP following IVTA.

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