The Distribution, Release Kinetics, and Biocompatibility of Triamcinolone Injected and Dispersed in Silicone Oil

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PURPOSE. Triamcinolone acetonide (TA) has been proposed as an adjuvant to pars plana vitrectomy with silicone oil for the surgical treatment of proliferative vitreoretinopathy and proliferative diabetic retinopathy. However, to date no data about the distribution and pharmacokinetics of lipophilic TA injected into silicone oil have been reported.

METHODS. An artificial vitreous space chamber was filled with silicone oil. TA was either injected or dispersed into silicone oil. TA release using a continuous flow model was measured spectrophotometrically. To determine the antiproliferative or cytotoxic effect of the released TA, monolayer cultures of retinal pigment epithelial cells (ARPE19) and retinal ganglion cells (RGC5) were used. Bromodeoxyuridine incorporation, MTT assay, and scanning electron microscopy were performed.

RESULTS. Injected TA sank slowly through the silicone oil and started to sediment below the silicone oil bubble shortly after injection. After the simulated intravitreal injection, no TA could be retrieved from the silicone oil bubble. In contrast, when a suspension of silicone oil and TA was prepared before injection, stable noncytotoxic amounts of TA (25 μg/mL) could be retrieved for up to 90 days. After mere injection (without previous suspension in silicone oil), the sedimented TA crystals showed a pronounced cytotoxic effect.

CONCLUSIONS. Intravitreally injected TA does not mix with silicone oil. TA crystals that sediment at the lower border of a silicone oil bubble may be harmful to retinal cells. A suspension of TA in silicone oil may exhibit safer extended release over several days. (Invest Ophthalmol Vis Sci. 2009;50:2337–2343) DOI:10.1167/iovs.08-2471

Silicone oil is the most commonly used internal tamponade for the repair of complicated retinal detachment caused by proliferative diabetic retinopathy (PDR), proliferative vitreoretinopathy (PVR), or other proliferative vitreoretinal disease.1,2 Modern silicone oils are biologically inert and provide short- to long-term tamponade of the retina. Tamponade force arises from the difference in density among vitreous humor, aqueous humor, and silicone oil bubble. Perisilicone proliferation of fibroglial membranes with subsequent repeat detachment, however, may compromise surgical success and remains a great concern.3 This unwanted proliferation of intraocular cells is often accompanied and stimulated by intraocular inflammation.

Various antiproliferative and anti-inflammatory drugs have been tried, including the perioperative use of daunorubicin,4 5-fluorouracil, and heparin,5 in irrigating solutions during pars plana vitrectomy with limited success. All those drugs (including the newer agents against the vascular endothelial growth factor such as bevacizumab and ranibizumab) have in common that they are hydrophilic and insoluble in silicone oil. The same is true for hydrophilic steroid formulations such as dexamethasone.

Corticosteroids have long been known to reduce intraocular inflammation and, depending on their concentration, to suppress cell proliferation. Triamcinolone acetonide (TA) is a synthetic lipophilic corticosteroid with low solubility in aqueous humor that has been used as a depot drug for decades. Because of its advantageous pharmacokinetic profile with rapid bioavailability and sustained release characteristics, Machemer et al.6,7 suggested the intravitreal use of crystalline TA to provide extended therapeutic drug levels within the vitreous cavity. Since then, intravitreal administration of crystalline TA suspension has become increasingly popular for the treatment of various intraocular disorders such as chronic uveitis,8 diabetic macular edema,9,10 age-related macular degeneration,11,12 and PVR.7

Because TA, like silicone oil, is lipophilic, it has been suggested as an adjuvant to pars plana vitrectomy with silicone oil for the surgical treatment of PVR and PDR. Several (uncontrolled) case series encouraging the combination of silicone oil tamponade with intravitreal TA have been published.13–17

However, no data about the distribution, the pharmacokinetics, and the biocompatibility of TA injected into silicone oil have been reported. Knowledge of the distribution and the biological effects of TA crystals in the vitreous space after injection into silicone oil is crucial because, as previously shown, clumped TA particles in intimate contact with the surface of retinal cells may be cytotoxic. Moreover, it must be determined how much TA actually is released from silicone oil to appreciate the potential of silicone oil as a “slow-release system” for TA. Finally, the biological effects of the released TA should be assessed.

METHODS

Preparation of Triamcinolone Acetonide

Triamcinolone (Volon-A; Bristol-Myers-Squibb, Munich, Germany) containing 40 mg TA was used diluted or undiluted from the original vials.

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Supported by the European Community and by Marie Curie Research Grant WLG5-CT-2001–60034.

Submitted for publication June 21, 2008; revised September 8, October 19, November 15, and December 2, 2008; accepted March 2, 2009.

Disclosure: M.S. Spitzer, None; R.T. Kaczmarek, None; E. Yoruek, None; K. Petermeier, None; D. Wong, None; H. Heimann, None; G.B. Jaissle, None; K.U. Bartz-Schmidt, None; P. Szurman, None

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To yield preservative-free TA, purification was performed before dilution according to a standard protocol for human use (University Pharmacy, Tübingen, Germany). The content of the vial was transferred to a sterile syringe (1 mL) and centrifuged at 2000 rpm for 20 minutes (Multifuge 35-R; Heraeus, Osterode, Germany), and the supernatant was decanted. After removal of the supernatant (0.9 mL), the TA crystals were resuspended in 0.9 mL balanced salt solution (BSS; Alcon, Ltd., Ft. Worth, TX). Centrifugation and removal of the supernatant were repeated. TA crystals were then resuspended in a final volume of 0.2 mL PBS. Invariably, final TA concentrations were assured spectrophotometrically at 239 nm (EAR-100ATX, Labindustries, Berkeley, CA).

Model Eye Chamber

A cylindrical polymethylmethacrylate chamber with an internal diameter of 20 mm, a length of 20 mm, and a volume of approximately 6.3 mL was used. Ports in the top and bottom of the chamber allowed filling and sampling of silicone oil and TA. The model allows a clear view of fluids and substances. Before filling with silicone oil, the chamber was filled with 0.5% bovine albumin in phosphate-buffered saline (PBS) and allowed to equilibrate for 10 minutes to render the surface hydrophilic. The chamber was filled with silicone oil (AcriSil-ol 1000/AcriSil-ol 3000; Zeiss-Acritec, Oberkochen, Germany). TA (purified and nonpurified) was injected with a 27-gauge needle through the port in the bottom of the model eye chamber.

TA concentration was assayed spectrophotometrically at 239 nm (Ultrospec 1000; Pharmacia Biotech, Pharmacia, CA). Samples were taken from the top, the sides, and the lower border of the silicone oil bubble.

Suspension of Triamcinolone in Silicone Oil

TA (30, 3, and 1 mg) was injected in 4 mL silicone oil (AcriSil-ol 5000; Zeiss-Acritec) and vortexed at 2500 rpm for 20 minutes and then transferred to an automated rotating shaker and mixed at 300 rpm for 3 days until a TA/silicone oil suspension with evenly distributed TA crystals was obtained. BSS (1 mL) was added to this suspension. Every 24 hours, the BSS was exchanged and the TA concentration was measured.

Continuous Flow Model

To simulate physiological conditions with constant aqueous humor production of approximately 2.5 to 3 μL/min, a model of continuous fluid flow was used. A suspension of TA and silicone oil was obtained, as described, and placed in a plastic tube with 0.5 mL BSS at the bottom and a temperature of 37°C. An infusion line connected to an automated pump infusing BSS at a flow rate of 0.2 mL/h (3.3 μL/min) was introduced into the silicone oil bubble. A second infusion line, with the tip at the bottom of the tube, was introduced to allow fluid to leave the system for TA release measurements without removal of parts of the silicone oil bubble (Fig. 1). Every 24 hours the TA concentration released into the receiver fluid (BSS) was measured.

Cell Culture

The ARPE19 cell line was purchased from American Type Culture Collection (Manassas, VA). The rat ganglion cell line (RGC5) was kindly provided by Neeraj Agarwal (University of North Texas Health Science Center, Fort Worth, TX).

ARPE19 and RGC5 cells were maintained in Dulbecco’s modified Eagle’s medium containing 3 mM l-glutamine, 1% glucose, 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate. To differentiate the biological effect of direct TA sedimentation, the cells were incubated either with or without direct contact with the sampled fluids and particles. This was achieved by use of a culture plate membrane filter system based on the principle of a Boyden chamber. The area of the cell culture dishes covered by TA crystals was assessed microscopically, and the percentage of the culture surface covered was documented. Samples were added directly on top of the cell layer or on top of the protective filter inserts, as previously described.

Stationary Toxicity Assay

The cytotoxicity assay was designed to assess the level of direct toxicity in a confluent, growth-arrested cell layer. A quantity of 5 × 10⁴ cells was grown to confluence in a 24-well tissue culture and incubated for 24 hours in a serum-free medium to ensure a static, nonproliferating environment. Under these serum-free conditions, the cells were more vulnerable to cytotoxic stress. To determine the biological effect of direct TA apposition after release from the silicone oil, the cells were incubated with or without direct contact with crystalline particles. The released TA was added directly on top of the cell layer (adherent epidermal epithelial cells) or on top of a protective Boyden chamber filter insert (nonadherent TA) that prevented direct cell contact. The activity of mitochondrial dehydrogenase was measured based on the cleavage of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT =M5655; Sigma, Deisenhofen, Germany) according to the manufacturer’s instructions. MTT cell assay measures cell mitochondrial activity rate and, when metabolic events lead to apoptosis or necrosis, reduction in cell viability.

Cell Viability/Cytotoxicity Assay

To further assess the potential cytotoxicity of the released TA on the two cell lines under nonstarvation conditions, cell viability was assessed with a viability/cytotoxicity kit (LIVE/DEAD Viability/Cytotoxicity Kit #1 [L-7013]; Molecular Probes, Eugene, OR). During drug exposure, the same experimental setting with or without a Boyden chamber, as mentioned, was used. Staining was performed according to the manufacturer’s instructions. Adequate negative controls (cells without TA) and positive controls (cells treated with Triton X-100 [Serva, Heidelberg, Germany] 0.5% in PBS) were run with each set of experiments. Cell viability was analyzed by fluorescence microscopy after 48 hours of incubation. Green and red cells were counted per...
eight fields at 200-fold magnification. The percentage of cells with green fluorescence (interpreted as viable cells) was then calculated.

Bromodeoxyuridine (BrdU) ELISA

The unwanted proliferation of RPE cells is one of the hallmarks of PVR. Consequently, we assessed the effect of dissolved or sediments TA on RPE cell proliferation. Cellular proliferative activity of RPE cells was directly monitored by quantification of BrdU incorporation into genomic DNA during cell growth. DNA synthesis was assessed by a colorimetric cell proliferation ELISA assay (Calbiochem, La Jolla, CA) according to the manufacturer’s instructions. Briefly, cells from non-confluent cultures were seeded onto 24-well tissue culture plates and incubated under conditions identical to those described. During drug exposure, cellular DNA was simultaneously labeled for 24 hours with BrdU-labeling reagent containing 10 μg/mL BrdU. After fixation, the cells were incubated with 1 μg/mL monoclonal anti-BrdU antibody for 60 minutes at room temperature. After the cells were washed and peroxidase-conjugated goat anti-mouse IgG was added, the cells were incubated for 30 minutes. After the addition of substrate solution, the reaction was stopped with H₂SO₄, and absorbance was analyzed within 30 minutes with the use of an ELISA plate reader (SLT Spectra 400; ATX, Salzburg, Austria) at dual wavelengths of 450 and 540 nm.

Statistical Analysis

Results were given as units of mean absorbance ± SD relative to control for the MTT and BrdU assays. For the cell viability/cytotoxicity assay, results were reported as percentage of viable cells ± SD relative to control. At least 10 samples per group were measured in triplicate. The relative difference between the control and the TA-exposed groups was analyzed with ANOVA. In all experiments, P < 0.05 was considered statistically significant. All analyses were performed with statistical software (SPSS version 12.0; SPSS, Inc., Chicago, IL).

Scanning Electron Microscopy

After fixation the RPE cells were buffer rinsed, dehydrated in a graded ethanol series, and critical point dried through liquid CO₂ (CPD 930; Balzers Hochvakuum GmbH, Wiesbaden, Germany). Sheets with RPE cells were mounted on aluminum stubs, sputter coated with 30 nm gold (Sputter Coater S150B; Edwards High Vacuum, West Sussex, UK), and examined under a scanning electron microscope (DSM 950; Carl Zeiss AG, Oberkochen, Germany).

RESULTS

Distribution Kinetics of Triamcinolone after Injection as a Bolus into Silicone Oil

The injected TA sank slowly through the silicone oil and started to sediment below the silicone oil bubble 5 minutes after injection (Fig. 2). After the simulated intravitreal injection, no TA could be retrieved from the silicone oil bubble. Samples taken from above the oil bubble or the side wall of the eye chamber also contained no TA. From the small liquid interface between the silicone oil bubble and the crystalline sediment, TA in a concentration of 35.8 μg/mL (82 μM) could be retrieved.

Identical results were obtained after gentle shaking of the artificial eye chamber for 5 to 10 days to simulate the influence of body and eye movements on the distribution of the TA. Distribution dynamics for purified and unpurified TA were, in principle, identical except that unpurified TA traversed the silicone oil bubble slightly more quickly than the purified TA (unpurified TA, 3 minutes, 12 seconds; purified TA, 3 minutes, 50 seconds).

Cytotoxic Effects of Triamcinolone Sediments

A strong decrease of viable cells was seen when sedimented crystalline particles were present on top of the cells and were not separated by the protecting filter (Filter). In a confluent cell layer, cell viability significantly decreased within 24 hours in a dose-dependent manner. The more TA crystals were released from the silicone oil bubble and the denser the sediments on the cell layer, the higher the decrease in the number of viable RPE and RGC5 cells. In the MTT assay, when 10% of the cell culture dishes were visibly covered by TA crystals, cell viability decreased to 74.4% ± 4.4% (P < 0.01) in the RPE cell culture compared with control and to 48.2% ± 5.1% (P < 0.01) in the RGC5 culture. In the presence of more than 50% of epiretinal TA deposits, cell viability decreased to 20.3% ± 4.9% (P < 0.001) compared with control and to 13.9% ± 1.4% (P < 0.001) in RGC cells. Silicone oil or dissolved TA (either from the aqueous interface or from below the Boyden chamber) did not exhibit a cytotoxic effect (Fig. 3). The cytotoxicity of sedimented TA crystals after injection into silicone oil could be observed in a stationary and in a proliferating cell culture (Figs. 3, 4). To assess the viability of RGC5 and RPE cells under nonstarvation conditions in the presence or absence of TA sediment (again using Boyden chambers), intracellular esterase activity (cell viability/cytotoxicity assay) was used to identify the living cells. Percentages of cells with esterase activity (viable cells, green fluorescence) were as fol-
Antiproliferative Effect of Dissolved Triamcinolone

Dissolved TA from the small liquid interface between the silicone oil bubble and the sedimented TA and the TA that was retrieved from below the Boyden chamber (or from the aqueous phase between the sediment and the silicone oil bubble) had a concentration of 35.8 g/mL (82 M). This concentration significantly reduced cell proliferation relative to control (by 41.7% ± 7.1%; \( P < 0.05 \)) without causing cellular damage. A similar antiproliferative effect was observed for the TA released from the TA/silicone oil suspensions as long as no TA crystals were present (reduction of cellular proliferation by up to 38.6% ± 9.4%; \( P < 0.05 \); compare Fig. 6).
months. Repeat studies (at least three times for every concentration) showed consistent results with small variability.

The high concentration of TA crystals in the 10-mg/mL suspension gave the preparation a cloudy appearance, and it was concluded that such a high concentration of TA dispersed in silicone oil might not be feasible for clinical use because it could obscure vision and fundus view. Moreover, it was observed after approximately 90 days that from the 10 mg/mL suspension, TA crystals started to sediment below the oil bubble. This sediment was highly cytotoxic in the MTT and cell viability/cytotoxicity assay (Figs. 3, 4).

Because the 1 mg/mL TA/silicone oil suspension showed only mild optical disturbance by crystalline particles, it was further tested in the continuous flow model. This model was set up to simulate physiological conditions with continuous fluid flow in the eye (of which aqueous humor production was the dominant factor). Under these conditions, TA in concentrations from 25 to 15 μg/mL was released for approximately 40 days, when the release stopped (Fig. 8). Released concentrations were high enough to inhibit the proliferation of RPE cells without causing cytotoxicity (Figs. 3, 5).

However, after 19 days, crystalline TA sediments were also released from the silicone oil bubble. These sediments, though they were observed in only small quantities, evoked a cytotoxic response when tested on RGC5 and RPE cells (up to 74.6% reduction of cell viability; $P < 0.001$; data not shown).

**DISCUSSION**

The use of intravitreal TA remains one of the leading therapeutic interventions for the treatment of various retinal vascular
diseases, such as diabetic macular edema and retinal vein occlusion. Several authors have suggested its use in silicone oil–filled eyes as adjunctive treatment of PVR and complicated PDR.13–17

The inhibition of inflammation and proliferation are both desirable in the postoperative period in silicone oil–filled eyes. Ideally, this beneficial effect of steroids would be effective over the entire retinal surface during the postoperative period. However, our study shows conclusively that when TA is injected into silicone oil–filled eyes, biologically active concentrations are present only below the oil bubble. Although the exposed retinal area may change with body movement, most of the retinal surface cannot be reached most of the time. Moreover, high local concentrations of TA crystals may be present on the retinal surface because of sedimentation. This sediment may interfere with vision when it is present in the macular area. The risk for macular TA deposits is especially high when the TA is injected at the end of vitrectomy with silicone oil tamponade because the patient may remain in a supine position for several minutes after the end of the procedure.

Silicone oil and TA are lipophilic; thus, one may assume that both substances may be soluble in each other or at least that both compounds should mix easily. It has been suggested that intravitreal injection of the slowly resorbable TA in silicone oil–filled eyes may serve as a slow-release system for anti-inflammatory and antiproliferative TA. However, our studies strongly suggest that after the injection of TA into silicone oil, no TA is retained in the oil bubble, possibly because silicone oil has a lower specific weight than TA preparations. Furthermore, TA has some hydrophilic properties and a solubility equilibrium of 82 μM at 37°C (equivalent to a maximally dissolved level of 36 μg/mL). Any commercially available TA preparation, whether purified or unpurified, contains some water that, together with its higher specific weight and amphiphilic character, renders TA insoluble in silicone oil. We investigated water-free TA powder as a possible alternative; however, the results were comparable to those with the commercially available TA preparations (data not shown).

Our findings that no TA was retained in the oil bubble seem to contradict a previous study that found TA to be present in silicone oil months after injection at the time of silicone oil removal.21 We assume that the TA that was recovered arose from remnants trapped below the oil bubble. Moreover, the concentrations reported were low (5–11 μg). We tested TA in such low concentrations and did not observe any antiproliferative effect in cell culture (data not shown). Silicone oil has a distinctive pattern of hydrophobicity dissimilar from that of TA and with unique surface and interfacial properties. Unlike TA, silicone is insoluble in dehydrated ethanol or methanol and, consequently, is immiscible with TA. Inhibitory effects of TA can be expected only in a small retinal area below the silicone oil bubble.

Apart from that, intraretinal injection of TA in silicone oil–filled eyes results in areas of dense TA sediment in a circumscribed retinal area below the oil bubble, especially when high concentrations of TA are used. Because these TA deposits are trapped below the oil bubble, they may come into intimate contact with the cellular membranes of retinal cells. Sedimentation of these crystalline particles on the cell surface caused direct cytotoxicity with cellular membrane destruction. The experimental setting using a membrane filter system was designed to distinguish the direct action of crystalline particle sedimentation under the oil bubble from the pharmacologic action of dissolved TA or the possible effects of silicone oil.23 Our results confirm previous reports that only TA crystals with direct cell contact may evoke cytotoxicity.24–26 In most clinical situations, this effect can be neglected because it seems that the vitreous scaffold and the internal limiting membrane likely provide enough protection against this undesired side effect of TA.25 However, after membrane peeling or extensive retinal manipulation, such protective factors may be lacking. This may explain why one (retrospective) study comparing intravitreal TA in pars plana vitrectomy with silicone oil to standard vitrectomy with silicone oil for the treatment of complicated proliferative vitreoretinopathy did not find a clear benefit for the adjunctive treatment.26

A suspension of TA in silicone oil, though time consuming to produce, may be an alternative, especially when lower concentrations of TA that do not interfere with vision are used. However, such a suspension would have to be prepared shortly before intraocular instillation because, after the mixing process is stopped, the suspension slowly separates into two phases again over a period of several weeks (with silicone oil at the top and TA at the bottom; data not shown). Care must be taken, however, when applying the results of our continuous flow model for the release of TA from a silicone oil/TAsuspension to the clinical situation. Any attempt to model the human eye in terms of drug distribution and disposition is fraught with technical challenges and can be considered only a cursory representation of the physiological condition.27 Although we know the rate of aqueous production, we cannot know its exact circulation in the presence of a bubble of silicone oil. The shape and size of the aqueous environment may depend on the extent of vitrectomy and the degree of fill with silicone oil. Equally, we do not know the influence of disease processes (e.g., inflammation, retinal detachment, breakdown of the blood ocular barrier) on pharmacokinetics. Moreover, the intraocular geometry of eyes that undergo vitrectomy with silicone oil can vary considerably. For instance, in some eyes the lens is removed and in others the natural lens is displaced by an intraocular lens.

The release of TA crystals distributed evenly in a silicone oil bubble is likely not influenced by the convection of water through the oil because the silicone oil bubble is impermeable to water.

Sophisticated computer-based simulations may be better tools to predict the fate of TA dispersed into silicone oil.28 However, we did not want to try to quantify how much TA was delivered to the retina or was cleared by the aqueous humor. We only wanted to demonstrate that if a homogeneous suspension of TA in silicone oil is prepared before silicone oil

**FIGURE 8.** Release of TA from a 1 mg/mL TA in silicone oil suspension under continuous flow conditions (simulating permanent aqueous humor production). TA in concentrations from 25 to 15 μg/mL was released for up to 40 days when the release stopped. From day 19 onward, crystalline TA sediment also was observed below the silicone oil bubble.
instillation, the likelihood of the development of biologically critical epiretinal deposits of TA may be lower.

In conclusion, when TA is injected into silicone oil after vitrectomy, contact of TA crystals with retinal cells is enhanced. Thus, injections of high concentrations of TA into silicone oil-filled eyes may be harmful to the retina and should be avoided. In contrast, when a suspension of lower TA concentrations with silicone oil is prepared before intracocular filling, release of TA in antiproliferative and less cytotoxic concentrations can be achieved because the formation of epiretinal deposits is delayed and less pronounced than the injection of TA into silicone oil as a bolus. Such an approach may be beneficial as adjunctive treatment for the repair of complicated retinal detachment from PDR or PVR. The preparation of such a suspension is time consuming, but the preparation must be fresh because the TA and the silicone oil will segregate slowly into two phases over the weeks. Moreover, if the whitish TA crystals are spread evenly throughout the silicone oil bubble, they may interfere with vision and with the postoperative assessment of the fundus. Thus, the clinical use of TA/silicone oil suspension may not be feasible.

References