Antiproliferative Effect of Retinoic Acid in Intravitreous Silicone Oil in an Animal Model of Proliferative Vitreoretinopathy

J. Javier Araiz,*† Miguel F. Refojo,*† Milagros H. Arroyo,*† Fee-Lai Leong,* Daniel M. Albert,† and Felipe I. Tolentino*†

Purpose. To evaluate, in vitro, the solubility and stability of all-trans RA in silicone oil (SiO) and, in vivo, the stability and the antiproliferative effect of all-trans RA in SiO on an experimental model of PVR.

Methods. The solubility and stability of RA in SiO, in vitro and in vivo, were evaluated by HPLC. Rabbits underwent unilateral gas-compression vitrectomy and gas-SiO exchange. Rabbits received 10 μg (n = 17), 5 μg (n = 11), and 2 μg (n = 9) of all-trans RA in SiO, and SiO only (n = 12). All rabbits received an intravitreous injection of 150,000 fibroblasts.

Results. RA is stable in SiO in vitro, but some isomerization from all-trans to 13-cis was observed under light exposure. In vivo, after 1 week, trace amounts of RA in SiO were observed in the controls and in the experimental animals, suggesting a steady state between the release of RA from the SiO and from the retina to the SiO. The rate of fractional retinal detachment was significantly lower in the animals that received 10 and 5 μg of RA than in the controls (P < 0.05). No statistical differences were found between the eyes treated with 10 and 5 μg of RA. Eyes that received 2 μg of RA showed no difference from the control group.


Proliferative vitreoretinopathy (PVR) is the most common cause of failure following rhegmatogenous retinal detachment surgery.1–5 This entity is frequently associated with giant retinal tears, posterior segment trauma, and excessive cryotherapy. It is believed to be a consequence of a biological process of wound repair and involves proliferation of retinal pigment epithelial (RPE) cells, glial cells, and fibroblasts. Despite meticulous surgical membrane removal and the use of silicone oil (SiO) as a long-term tamponade, failure occurs in a large number of cases due to difficulty with complete removal and continuous growth of the membranes. To address this problem, a number of experiments have been reported using different antiproliferative agents alone or in combination with vitreoretinal surgery.6–16 Most of these agents have narrow safety margins and could produce severe toxic reactions; also, most are water-soluble and, therefore, cannot be used with SiO. For these reasons, we searched for an agent soluble in SiO that might act as a long-term tamponade as well as a drug delivery system.

Retinoids are known to have an antiproliferative effect on epithelial, mesenchymal, and neoplastic
cells. More specifically, retinoic acid (RA) is being used topically and systemically to treat several skin disorders and neoplastic diseases. In the eye, RA is used to treat xerophthalmia and experimental corneal wounds. The antiproliferative effect of retinol in SiO has been investigated in our laboratory in an animal model of PVR with intravitreal injected fibroblasts, and showed a dose-dependent prevention of retinal detachments. In vitro, RA has shown the most potent inhibitory effect on RPE cell proliferation among all retinoids.

In this study, we evaluated the ability of all-trans RA dissolved in SiO to inhibit membrane proliferation in an animal model of PVR based on the intravitreal injection of homologous subconjunctival fibroblasts (REF).

**MATERIALS AND METHODS**

**In Vitro Studies**

**Solution of Retinoic Acid in SiO.** All-trans RA (all-trans Vitamin A acid, 98%) was obtained from Eastman Kodak (Rochester, NY; lot 909587A). Commercial grade 1000 cS SiO (polymethylsiloxane) was obtained from Huls Petrarch Systems (Bristol, PA). The low-molecular-weight components in the SiO (LMWC) were removed as previously described, and the viscosity of the fractionated SiO was adjusted to 5000 cS. Saturated solutions of RA in SiO (about 20 μg/ml) were prepared and stored in dark bottles at room temperature and shaken occasionally. After at least 2 weeks, the mixture was filtered through a 0.45-μm filter (Millipore, Bedford, MA). To determine the amount of RA dissolved in the SiO, the RA was extracted from SiO, three times, with acetone (1:1 v/v). The RA concentration was then measured by high-pressure liquid chromatography (HPLC), using a Water’s HPLC 600 system equipped with a Waters Adsorbosphere C18-5U column (Alltech, Deerfield, IL). The mobile phase consisted of 70% acetonitrile and 30% water containing 5% V/V acetic acid and 0.02% V/V triethylamine (flow rate 2 ml/min, wavelength 350 nm).

**RA Stability in SiO.** After the mixture of RA in SiO was filtered, we divided the solution into four bottles (10 ml each). Two bottles were kept in an incubator at 37°C with a transparent door, and the other two were kept at room temperature (21°C). One bottle from each pair was protected from light with aluminum foil.

**RA concentration was measured on the day the solution was filtered, and at days 7, 14, 28, 42, and 56 under the abovementioned conditions of temperature and illumination.**

**In Vivo Experiments**

The in vivo experiments were performed in accordance with the ARVO Resolution on the Use of Animals in Research. Adult pigmented rabbits of both sexes (average weight 3 kg; range 2.6–3.8 kg) were used in this study.

**Surgical Technique.** The rabbits (n = 49) were anesthetized intramuscularly with 0.5 ml chloropromazine hydrochloride (25 mg/ml solution) (Rugby, Rockville Centre, NY) and ketamine hydrochloride (100 mg/ml per kg of body weight) (Aveco, Fort Dodge, IA). Proparacaine hydrochloride 0.5% in ophthalmic solution (Squibb, Princeton, NJ) was administered for topical anesthesia. Mydriasis was obtained by instilling 1% cyclopentolate hydrochloride (Akorn, Abita Springs, LA) and 10% Phenylephrine Hydrochloride (Winthrop Pharmacr., New York, NY) 30 min before the operations.

All rabbits underwent unilateral gas compression vitrectomy (C₂F₆, 0.4 ml) as previously described. Four days later, the rabbits were reanesthetized and a gas/SiO exchange (1 ml) was performed in all of them. Briefly, a 6-0 Vycril mattress suture was placed 4 mm posterior to the limbus. As SiO was introduced through a 20-gauge needle, the displaced gas escaped out of the eye around the needle. At the end of the procedure, 70–80% of the vitreous cavity was filled with SiO. In group 1 (n = 17), the concentration of RA in SiO was 10 μg/ml. In group 2 (n = 11) and group 3 (n = 9), the concentration of RA was 5 μg/ml and 2 μg/ml of SiO, respectively. In the control group (n = 12), SiO only was introduced into the vitreous cavity.

Fibroblasts for culture were obtained from the subconjunctival space of pigmented rabbits. Immediately after the extraction, the tissue was carefully minced into 1–2 mm sections and placed in petri dishes with Dulbecco’s modified Eagle medium (DMEM, Sigma, St Louis, MO) containing 15% fetal bovine serum (FBS, Gibco, Grand Island, NY), 200 nM of L-Glutamine, 100 international units/ml of penicillin, and 100 μg/ml of streptomycin. The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed twice weekly. When cells become confluent, fibroblasts were enzymatically detached with 0.5% trypsin and 0.53 mM NaEDTA (Gibco, Grand Island, NY) and subcultured. Immediately after the gas/SiO exchange, 150,000 of the harvested homologous fibroblasts suspended in 0.1 ml of culture medium were injected through a 30-gauge needle into the vitreous cavity 4 mm posterior to the limbus. Dexamethasone sodium phosphate (MSD, West Point, PA) and bacitracin-neomycin-polymyxin (Fougera, Melville, NY) in ophthalmic ointment were applied to the eye immediately after surgery.
Fibroblast viability was confirmed in all of the batches of cultured cells used by injecting control animals with the cells at the same time that the experimental animals were operated on. Viability of the left-over cells was also sporadically determined by the Tryphan Blue Exclusion test and found to be greater than 95%.

Postoperative Follow-up. Slit lamp biomicroscopy and funduscopic examination using indirect ophthalmoscopy were performed before surgery and at postoperative days 1, 4, and 7 and each week thereafter. Two rabbits from group 1 were excluded from the study because of severe panophthalmitis occurring immediately after the gas/SiO exchange. All remaining animals (n = 47) were followed up for a period of 4 weeks. Of these animals, 32 were followed up for a period of 8 weeks to conform to the recommendations of Ophir. Two examiners were always present to obtain concurrent observations.

The size of the SiO bubble was estimated and recorded and the extent of membrane formation and retinal traction was graded according to Fastenberg classification.

Results were quantified by determining the prevalence of tractional retinal detachment (TRD) (Fastenberg 3) within a given period of time, and by assigning one point to each Fastenberg grade of periretinal proliferation. A Chi-square test and an Anova, respectively, were used to analyze the results.

The rabbits were killed by intravenous administration of sodium pentobarbital (100 mg/kg) (Anthony Products Co., Arcadia, CA) 4 or 8 weeks after the injection of SiO. All operated eyes were enucleated and fixed in formalin 10%. The eyes were cut sagitally at a plane close to the optic nerve head. All eyes were examined grossly and selected eyes were embedded and stained with hematoxilin-eosin and PAS and examined by light microscopy. The eyes were evaluated for preliminary RA retinal toxicity and for the presence and severity of periretinal membranes.

RA Release From Intravitreous SiO
To evaluate the release of RA from SiO in vivo, we performed the surgical procedure described above in an additional 14 rabbits. We divided the animals into three groups according to the medium used for gas/SiO exchange (group a [n = 6], 10 µg/ml of RA in SiO with injection of 150,000 fibroblasts; group b [n = 5], 10 µg/ml of RA in SiO without injection of fibroblasts; and group c [n = 3], SiO only). Animals from each group were killed 1 week (four rabbits from group a, four from group b, and two from group c) and 2 weeks (two rabbits from group a, and one from each of groups b and c) after surgery and the SiO was removed from the eyes for RA analysis. The SiO was removed from the vitreous cavity and separated by centrifugation, dissolved in methylene chloride, dried with anhydrous sodium sulfate, and filtered, and the solvent was evaporated first with a stream of nitrogen and then under vacuum. RA was extracted with acetone from SiO and the sample analyzed by HPLC as described in the in vitro studies.

RA Retinal Toxicity Evaluation
To evaluate the potential retinal toxicity of RA, we performed the same surgical techniques described above. We used RA concentrations in SiO of 10 µg/ml (five rabbits) and 5 µg/ml (five rabbits); in the control group (five rabbits), gas was exchanged for SiO only. Fibroblasts suspension were not injected, to avoid any artifactual retinal or ocular alteration attributed to fibroblast proliferation.

The eyes were followed up by slit lamp and indirect ophthalmoscopic examination 1 day before surgery, postoperative days 1 and 4, and each week thereafter. Four weeks after the surgery, the rabbits were killed and the eyes enucleated. All eyes were studied grossly and selected cases were processed histologically.

RESULTS
In Vitro RA Stability in SiO
RA stability in SiO is shown in Figures 1A–C. After 8 weeks, the amounts of RA detectable by HPLC in SiO were 95.5% (in darkness, at 37°C), 94.7% (in darkness, at 21°C), 86.3% (under illumination, at 37°C), and 88.8% (in light, at 21°C) of the original amount. The amounts of RA degradation products were slightly higher under illumination than in dark. Under light exposure, isomerization was seen from all-trans RA to 13-cis RA. This phenomenon occurred mainly during the first week of light exposure (Fig. 1A,B). In the samples that were protected from light, all-trans RA was stable (Fig. 1A). A very small but increasing amount of RA degradation products were seen during the time we measured the RA stability in vitro.

Clinical and Histopathological Findings
Before gas/SiO exchange, a mild posterior subcapsular cataract was noted in all eyes because of the expanding perfluoropropane gas. The cataracts developed slowly and in only six cases was visualization of the fundus partially obscured for the period during which the rabbits were examined. Generally, a mild, self-limited inflammation of the conjunctiva and anterior segment was observed in the first 3–5 days after the exchange.

In groups 1 and 2 (10 µg and 5 µg of RA, respectively), 1 week after intravitreal fibroblasts inoculation, 1 of 15 eyes (6.6%) and 3 of 11 eyes (27.3%), respectively, developed TRD. At the same time, 4 of
Effect of Retinoic Acid in PVR

FIGURE 1. Stability of RA in SiO in vitro. (A) All-trans RA; (B) 13-cis RA; (C) RA (all-trans and 13-cis). Note the expected isomerization from all-trans RA to 13-cis RA in the samples exposed to light.

12 eyes (33.3%) from the control group developed similar changes.

Two weeks after fibroblasts inoculation, 2 of 15 eyes (13.3%; group 1) and 3 of 11 eyes (27.3%; group 2) developed TRD, while 8 of 12 eyes (66.6%) from the control group developed TRD.

Three weeks after intravitreal fibroblasts injection, 8 of 15 (53.2%) of the eyes in group 1 and 3 of 11 (27.3%) of the eyes in group 2 developed TRD. In the control group, 11 of 12 eyes (91.6%) showed TRD at the same time.

Four weeks after the injection of fibroblasts, 8 of 15 (53.2%) of the eyes in group 1 and 4 of 11 (36.3%) of the eyes in group 2 developed TRD, while 11 of 12 (91.6%) of the eyes of the control group developed TRD.

At 5–8 weeks after fibroblasts inoculation, there was no progression in the TRD of the observed eyes.

Two weeks after gas/SiO exchange, the incidence of TRD in the group treated with 10 µg of RA was significantly lower ($P < 0.05$) than in the control group. In group 2 (5 µg of RA), the incidence of TRD was significantly lower ($P < 0.05$) than in the control group after the third week after treatment. A summary of these data is shown in Table 1. In group 3 (2 µg of RA), 7 of 9 (77.8%) of the eyes developed TRD 1 week after the injection of fibroblasts. Thereafter, the number of eyes that developed TRD remained the same until the end of the experiments (8 weeks). No difference was found between this group and the untreated control group, except for the first week ($P < 0.05$), during which the incidence of TRD was higher among the eyes treated with 2 µg of RA. Differences between the group treated with 10 µg (group 1) and 5 µg (group 2) of RA were also not statistically significant.

The results of the comparison among the treated groups and the control group when the extent of peri-retinal proliferation was graded according to Fastenberg et al. are shown in Figure 2.

Histopathologic studies on the selected cases confirmed the clinical observations. Light microscopy of the representative control eyes revealed extensive multilayered fibroblast-rich epiretinal and vitreous tractional membranes (Fig. 3). The eyes treated with 10 µg or 5 µg of RA showed markedly thinner and less extensive membranes. Typically, the periretinal membranes in these eyes were less cellular and covered a limited portion of the retina with limited or no apparent traction (Fig. 4).

RA Release From Intravitreous SiO

In vivo, only traces of RA were detected by HPLC in the SiO extracted from the eyes 1 week after gas/SiO exchange. The detected amounts in the animals into which 10 µg of RA was injected (with or without fibroblasts) fell within a range of 0.07–0.2 µg (0.7–2% of the original amount). Similar amounts of RA were also detected in intravitreal pure SiO (without fibroblasts and RA) 1 week after injection.
TABLE 1. Progression of the Periretinal Proliferation in the Control and Treated Groups

<table>
<thead>
<tr>
<th>Experimental and Control Groups</th>
<th>Fastenberg Stages</th>
<th>Postoperative stages (weeks)</th>
<th>Retinal Detachments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Group 1 10 μg</td>
<td>F1</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 2 5 μg</td>
<td>F1</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 3 2 μg</td>
<td>F1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Control group</td>
<td>F1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>F5</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Two weeks after the gas/SiO exchange, the amount of RA detected in the intravitreal SiO was similar to the amounts found at 1 week.

RA Retinal Toxicity Evaluation

Clinical and gross examination of the eyes treated with 10 μg and 5 μg of RA did not show any evidence of toxicity in the retina.

Light microscopy studies showed no histologic changes in the retina suggesting RA toxicity (Fig. 5).

DISCUSSION

PVR is characterized by the formation of cellular membranes within the vitreous cavity and on both surfaces of the retina. In this process, glial and RPE cells are known to undergo a migration and cellular differentiation into fibroblast-like cells.

Modern vitrectomy techniques combined with the use of SiO as internal tamponade in severe and desperate cases of PVR has increased the surgical success rate. However, there is no evidence that SiO could prevent PVR. On the contrary, it has been reported that SiO alone might enhance membrane proliferation. The Silicone Study Group recently reported little difference between perfluoropropane gas and SiO, stating that the surgeon's preference is the only reason to select one over the other. In our opinion, the advantage that SiO offers over the use of C3F8 is that SiO can also be used as a reservoir to deliver antiproliferative drugs.

A number of studies have been performed using various antiproliferative agents to inhibit cellular proliferation and membrane contraction in PVR. Most investigators consider 1 month of follow-up after antiproliferative treatment to be an appropriate period of time to evaluate the results of the drug application in PVR, but because a routine follow-up of 2 months was suggested in a recent study, we expanded the initial protocol to 8 weeks of follow-up. In
FIGURE 3. Photomicrographs of eye from control animal (gas vitrectomy, gas/SiO exchange, and innoculation of fibroblasts without treatment with RA). (A) Low-power view showing PVR with morning glory detachment and adhesion of the inner limiting membrane of the detached retina (hematoxilin and eosin, ×1). (B) Higher-power view of the same eye showing adherent posterior retina. Note preretinal membrane (hematoxilin and eosin, ×4). (C) Peripheral retina showing cyclitic membrane causing traction (hematoxilin and eosin, ×10).

our experiments, none or minimal progression of the disease could be observed 4 weeks after treatment, confirming the above observations.

Retinoic acid is a naturally occurring oxidative product of Vitamin A (retinol). In addition to the role that Vitamin A plays in the biosynthesis and regeneration of visual pigments, retinoids affect the differentiation and proliferation of many types of cells. Because of the liposolubility of the retinoids, they can be used with SiO as a vehicle for intravitreal delivery when SiO is used as the tamponade agent in PVR.

In vitro, Doyle et al reported a potent inhibitory effect of retinoids on RPE cell proliferation. Among all retinoids, RA showed the strongest inhibitory effect. In vivo, retinol showed a dose-dependent inhibition of fibroblast proliferation in an experimental rabbit model of PVR.

In the present study, the rate of TRD in the control group was similar to that reported in previous studies with similar animal models of PVR. However, the rates of TRD due to membrane formation in eyes treated with 10 μg and 5 μg of RA were significantly lower than in eyes from the control group. Although no significant differences were found between these two doses, the 5-μg/ml dose seemed to be somewhat more effective.

With doses of 2 μg/ml, no differences were found with the control group except in the first week after fibroblasts injection, in which doses of 2 μg/ml seemed to enhance cell proliferation.

At the doses that RA was employed in this study, no histological evidence of rabbit retinal toxicity was found. Electrophysiological studies were not done because SiO attenuates ERG signals, making ERG interpretation difficult.

Despite the difficulty of comparing the results of this and other studies using different antiproliferative drugs, because of the many variables in the methodol-
FIGURE 4. Photomicrographs of eyes from treated animals (gas vitrectomy, gas/SiO exchange, and inoculation of fibroblasts and treatment with 10 μg and 5 μg of RA dissolved in SiO). (A) Low-power view showing retina with artificial detachment (hematoxylin and eosin, X1). (B) and (C) Higher-power view of posterior retina. Note the absence (B) or the poor cellularity and minimal traction (C) of the preretinal membranes (hematoxylin and eosin, X10).

FIGURE 5. Photomicrograph of the eye (gas vitrectomy, gas/SiO exchange, and treatment with 10 μg of RA dissolved in SiO without fibroblasts inoculation) showing posterior attached retina with normal appearance (hematoxylin and eosin, X4).

FIGURE 6. Photomicrograph of the eye (gas vitrectomy, gas/SiO exchange, and treatment with 10 μg of RA dissolved in SiO with fibroblasts inoculation) showing posterior attached retina with normal appearance (hematoxylin and eosin, X4).

In the present study, traces of RA were detected in intravitreal SiO after 1 week, both in animals in which RA was administered in solution in SiO and animals in which only SiO was placed intravitreally. We interpret these results to mean a steady state between the RA released from the SiO and the RA released from the retina to the SiO. Clearance of RA from the SiO in the vitreous cavity within the first week suggests that RA influences the proliferation of cells at early stages. These findings are in agreement with the experimental study of Fisher et al., which concluded that a period of 2–5 days at the beginning of the disorder is critical for the initiation of the proliferative response. Also, Daniels et al. found that taxol inhibited PVR only
when administered within the first days after the development of retinal detachment.

The mechanisms by which RA inhibits cellular proliferation are not clear. Connor et al. found that eyes with intraocular fibrosis associated with PVR have more than three times the amount of TGF-β found in eyes with uncomplicated retinal detachments. However, Roberts et al. found that RA is capable of antagonizing the proliferation of cells stimulated by TGF-β. Furthermore, it has been recently reported that TGF-β production is markedly reduced by RA. Because there are data suggesting that cell proliferation and collagen production may be causally linked to TGF-β in many cell systems, it is possible that one of the mechanisms of RA modulation of collagen production relates to its effect on TGF-β accumulation. However, further studies are necessary to confirm this possible causal link.

Whatever the mechanisms are, RA given in a solitary administration in solution with intravitreal SiO produces a significant and lasting reduction in cellular proliferation in an experimental model of PVR. Because of its efficacy, RA may be very useful in humans as an adjunctive treatment in severe cases of PVR or reproliferation, particularly in those that require the use of SiO.

Key Words
antiproliferative, proliferative vitreoretinopathy, retinoic acid, silicone oil, vitamin A

References


