Retinoic Acid in Silicone and Silicone–Fluorosilicone Copolymer Oils in a Rabbit Model of Proliferative Vitreoretinopathy

Masaaki Nakagawa, Miguel F. Refojo, Jesus F. Marin, Motoaki Doi, and Felipe I. Tolentino

Purpose. The authors evaluated the effect of retinoic acid (RA) in silicone oil (SiO) and in silicone–fluorosilicone (SiFO) copolymer oil in a new rabbit model of proliferative vitreoretinopathy (PVR).

Methods. To create the PVR model, three groups of rabbits were administered vitreous injections of approximately 100,000 homologous fibroblasts, 75,000 platelet-rich plasma (PRP), and fibroblasts + PRP, respectively. These rabbits were followed up ophthalmoscopically and histopathologically for as long as 2 months. Five additional groups of rabbits underwent gas-compression vitrectomy in one eye. Four days later, group 1 was administered intraocular RA in SiFO (9 \( \mu g/ml \)) with approximately 150,000 fibroblasts and 70,000 PRP. Group 3 was administered the same amount of fibroblasts and PRP as group 1 with RA in SiO (9 \( \mu g/ml \)). Groups 2, 4, and 5 were administered the same amount of fibroblasts and PRP as groups 1 and 3 with 1 ml of SiFO, SiO, or balanced salt solution only, respectively. To evaluate RA toxicity, RA was injected in SiO (15 and 20 \( \mu g/ml \)) and RA in SiFO (10 \( \mu g/ml \)).

Results. All eyes that were administered fibroblasts or PRP developed vitreous membranes, but those with PRP alone did not develop proliferative changes or retinal detachment; fibroblasts alone produced proliferative changes and retinal detachment after 2 to 3 weeks; fibroblasts + PRP produced similar changes within 3 days of injection. Retinoic acid (15 \( \mu g/ml \)) in SiO and RA (10 \( \mu g/ml \)) in SiFO was well tolerated. Retinal atrophic changes were found in eyes with 20 \( \mu g/ml \) RA in SiO. The retinal detachment rate was lower \( (P < 0.05) \) in the eyes that were administered fibroblasts + PRP and RA than in the controls. Significant differences were found in the degrees of PVR among the groups.

Conclusions. RA could be useful in PVR treated with SiO or for eyes treated intraoperatively with heavier-than-water SiFO when it is used as a short-term retinal tamponade. Invest Ophthalmol Vis Sci. 1995;36:2388–2395.
TABLE 1. Properties of Silicone Oil and Silicone–Fluorosilicone Copolymer Oil

<table>
<thead>
<tr>
<th>Property</th>
<th>SiO</th>
<th>SiFO</th>
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<tr>
<td>Viscosity (centistokes, 25°C)</td>
<td>5,000</td>
<td>180</td>
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<tr>
<td>Density (g/cm³)</td>
<td>0.97</td>
<td>1.16</td>
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<tr>
<td>Refractive index (nD, 25°C)</td>
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<td>1.39</td>
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<tr>
<td>Surface tension (dyne/cm², 37°C)</td>
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<td>21</td>
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<tr>
<td>Interfacial tension against water (dyne/cm², 37°C)</td>
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SiO = silicone oil; SiFO = silicone–fluorosilicone copolymer oil.

Various lipophilic drugs that dissolve in SiO inhibit cell proliferation and suppress experimental PVR. Additionally, retinoic acid (RA) inhibits retinal pigment epithelium (RPE) cell migration and appears 100-fold more potent than any other retinoid. The anti-proliferative effect of RA in SiO and SiFO was observed in a fibroblast rabbit model of PVR showed dose-dependent prevention of retinal detachment.

In the current study, we describe a new animal model of PVR produced by intravitreous injection of homologous subconjunctival fibroblasts and platelets. In addition, we evaluated all-trans RA dissolved in SiO and silicone–fluorosilicone copolymer oil (SiFO) for retinal toxicity and its role in preventing PVR.

MATERIALS AND METHODS

All-trans RA (all-trans vitamin A acid, 98%) was obtained from Eastman Kodak (Rochester, NY). Crude SiO and SiFO were obtained from Hüls America Inc. (Bristol, PA). Because the commercially available compounds have a relatively large amount of low-molecular-weight components, SiO and SiFO were pretreated using a previously reported technique for purification, with a resultant viscosity of 5000 centistokes and density of 0.97 g/cm³ in the case of SiO and 180 centistokes and 1.16 g/cm³ for SiFO (Table 1).

Rabbit Model of Proliferative Vitreoretinopathy

Methods. Homologous subconjunctival fibroblast suspension was prepared by harvesting the subconjunctival tissue of several 5- x 5-mm rabbit conjunctival flaps. The tissues were shredded and cultured in 35-mm petri dishes containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, gentamicin (50 g/ml), and amphotericin B (5 µg/ml) as nutrient media. The cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and air. Confluent cultures were trypsinized, centrifuged, and resuspended in culture flasks containing nutrient media. On the day of injection, confluent cultures were again trypsinized, centrifuged, resuspended in DMEM, and counted using the microhemocytometer. Two fibroblast batches obtained after two passages were used in the experiments, one at a concentration of approximately 1 million cells per milliliter and the other at approximately double this concentration. To inject approximately the same number of cells in all rabbit eyes, the volume of cell suspension injected was either 0.1 ml or 0.05 ml, respectively.

Platelet-rich plasma for injection was prepared by collecting marginal venous blood from the rabbit ears in glass centrifuge tubes containing 3.8% sodium citrate solution (1 part/9 parts whole blood). The fresh citrated blood then was centrifuged at 50g for 10 minutes at 23°C using the Beck centrifuge model TJ-6 (Beckman Instrument, Palo Alto, CA). The upper third of the resultant supernatant was used to obtain a platelet count using an automated Coulter counter Model B (Coulter Electronics, Hialeah, FL). Obtained densities were 500,000 to 1.0 million platelets per milliliter of plasma.

We randomly assigned 15 pigmented rabbits weighing 2.5 to 3.5 kg into three groups of five rabbits each. Rabbits in group A were administered an intravitreous injection of 0.1 ml homologous subconjunctival fibroblasts (108,000 cells) suspended in DMEM. Rabbits in group B were administered an intravitreous injection of 0.1 ml of PRP (76,000 platelets in rabbit 1 and 72,000 platelets in rabbits 2 to 5). Rabbits in group C were administered an intravitreous injection of 0.1 ml PRP (68,000 platelets) and 0.05 ml homologous subconjunctival fibroblasts (100,000 cells) in DMEM, except rabbit 5, which was administered 75,000 platelets in 0.1 ml plasma and 108,000 fibroblasts in 0.1 ml DMEM from the same batch used in all rabbits of group A.

Surgical Procedure. All surgeries were performed under aseptic conditions and pursuant to the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Only the right eye of each experimental rabbit was used. These animals were anesthetized using 0.7 ml/kg of body weight of three parts intramuscular ketamine hydrochloride (100 mg/ml) (Aveco, Fort Dodge, IA), one part acepromazine maleate (10 mg/ml) (Aveco), and three parts xylazine (20 mg/ml) (Phoenix Pharmaceuticals, St. Joseph, MO). The pupils were dilated with 0.5% tropicamide (Bausch & Lomb Pharmaceutical Division, Tampa, FL), and 10% phenylephrine hydrochloride (Winthrop Pharmaceuticals, New York, NY). A superior rectus bridge suture was placed and a peritomy was performed, after which a partial-thickness scleral incision was made 4 mm posterior to the limbus. The incision bed was diathermized and secured by a single preplaced 7-0 vicryl suture. The suspensions for injections then were introduced through the incision bed into the middle of the vitreous cavity using a tuberculin syringe and a 27-gauge needle. Simultaneous viewing through the operating microscope and a plano-
41 Eyes of 41 Pigmented Rabbits

Gas Compression Vitrectomy

1) SiFO + RA (n=14)  2) SiFO (n=11)  3) SiO + RA (n=6)  4) SiO (n=6)  5) BSS (n=4)

Fibroblasts + Platelets in the Vitreous

Slit-lamp, Funduscopy, IOP, and Histopathology

FIGURE 1. Outline of the experiments performed to compare the effect of 9 µg/ml of retinoic acid (RA) in silicone oil (SiO) or in silicone–fluorosilicone–copolymer oil (SiFO) in two groups of eyes of the fibroblasts + platelet-rich plasma rabbit model of proliferative vitreoretinopathy. The control groups were eyes of the model injected with the respective oils or with balanced salt solution but without RA.

Concave vitrectomy lens assured better control. The incision was closed with the preplaced suture as the syringe and needle were withdrawn. Postoperative indirect ophthalmoscopy confirmed adequate intraocular perfusion. An anterior chamber paracentesis was performed to normalize intraocular pressure.

Postoperative medications consisted of topical solutions of atropine 1% (Akorn, Abita Springs, LA), bacitracin–neomycin–polymyxin (Fougera, Melville, NY), and dexamethasone sodium phosphate (Merck Sharp & Dohme, West Point, PA). The rabbits were followed up daily for the first week, and weekly thereafter using the biomicroscope and indirect ophthalmoscope for as long as 2 months. Clinical observations were recorded carefully in a sketch and categorized according to the classification of Fastenberg et al. Photographs were taken when it was feasible. The animals were killed at 1- to 2-week intervals to observe and document the intraocular reactions of the different groups at various time intervals, ranging from 10 to 57 days. Eucleated eyes were embedded in paraffin, stained with hematoxylin and eosin, periodic acid Schiff, and Masson trichromic, and examined by light microscopy.

Retinoic Acid Effect in the Fibroblast + Platelet-rich Plasma Rabbit Model of Proliferative Vitreoretinopathy

Only the fibroblast + PRP model was used for this part of the experiment because it provided the most consistent and severe PVR, as determined in the Methods section.

Materials. Saturated solutions of RA in SiO or SiFO were obtained by occasionally shaking RA in excess oil in capped glass containers wrapped in aluminum foil at room temperature for 1 week or longer, followed by filtration using a 0.22-mm Millipore (Bedford, MA) filter. The RA concentration was measured by high-performance liquid chromatography using a Waters (Millipore, Milford, MA) HPLC 600 system equipped with a Waters 990 photodiode detector and a 250-mm × 4.6-mm Adsorsphere C18-5U column (Alltech, Deerfield, IL). The mobile phase consisted of 70% acetonitrile and 30% water containing 5% vol/vol acetic acid and 0.02% vol/vol triethylamine (flow

<table>
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<th>Rabbit Number</th>
<th>Enucleation (Days After Injection)</th>
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* Presence of localized tear noted at day 2, probably surgically induced.
PRP = platelet-rich plasma.

TABLE 2. Clinical and Histologic Staging of Fundus Changes in the Three Experimental Groups
Effect of Retinoic Acid in Silicone Oils in PVR

Immediately before surgery. Before the vitreous injection, the rabbit eyes were dilated with 10% phenylephrine hydrochloride and 1% cyclopentolate (Akorn). Gas compression vitrectomy with perfluoropropane (C₅F₁₈, 0.4 ml; PCR Inc., Gainesville, FL) was performed as described previously.³⁶ Four days later, the rabbits were reanesthetized, and a gas–oil exchange (1.2 ml) was performed in all animals. The exchange was performed under an operating microscope with the pu-

rate, 2 ml/min; wavelength, 350 nm). Before intraocular injection, the RA concentration in the SiO or the SiFO was adjusted to the concentration used in the experiments.

**Surgical Procedure.** All rabbits were anesthetized intramuscularly with 12.5 mg chlorpromazine hydrochloride (Rugby, Rockville Center, NY) and 100 mg/kg ketamine hydrochloride. Only the right eyes of the rabbits were used. Topical anesthesia with 0.5% proparacaine hydrochloride was instilled in the eye imme-

**FIGURE 2.** Micrographs of the inferior retina of one eye after 4 weeks with 10 μg/ml retinoic acid in silicone–fluorosilicone–copolymer oil. (A) Light micrograph shows no specific changes (hematoxylin and eosin; original magnification, ×50). (B) In the electron micrograph, some vacuoles are seen in the pigment epithelial cells. Bars = 1 μm.

**FIGURE 3.** Four weeks after the injection of 20 μg/ml retinoic acid in silicone oil. (A) Cellular disorganization is observed in the superior retina by light microscopy (hematoxylin and eosin; original magnification ×50). (B) Electron micrograph of same eye shows thinning of the photoreceptor layer and disappearance of the lamellar structure in the outer segments. Bars = 1 μm.
Groups

<table>
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<tr>
<th>Stage</th>
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FIGURE 4. Rates of retinal detachment (greater than or equal to those at stage 3) at 1 month (by ophthalmoscopy) in the fibroblast + platelet-rich plasma rabbit model of proliferative vitreoretinopathy: group 1, 29%; group 2, 82%; group 3, 33%; group 4, 100%; and group 5, 100%. The incidence of tractional retinal detachment in groups 1 and 3 treated with retinoic acid was significantly lower ($P < 0.05$) than in control groups 2 and 4.

RESULTS

Proliferative Vitreoretinopathy Rabbit Model

In all experimental eyes, the vitreous appeared diffusely hazy during the first 24 hours; thereafter, the hazy material progressively condensed to gray, whitish membranes that produced various degrees of vitreous opacification, particularly in groups B and C. A mild anterior chamber reaction was present and resolved within 2 to 4 days. The results are summarized in Table 2.

Retinal Tolerance of Retinoic Acid in SiO and SiFO.

After the surgical techniques described above, we injected RA in SiO at concentrations of 15 $\mu$g/ml ($n = 6$) and 20 $\mu$g/ml ($n = 6$), and we injected RA in SiFO at concentrations of 10 $\mu$g/ml ($n = 6$). Half the eyes were enucleated and processed for histopathology at 2 weeks and the other half at 4 weeks.

Retinoic Acid in the Proliferative Vitreoretinopathy Rabbit Model. Forty-one pigmented rabbits of both sexes (2.2 to 4.6 kg) were used in these experiments. The surgical procedure was the same as described above, except that at the gas–oil exchange, group 1 ($n = 14$) was administered 1.2 ml SiFO with RA (9 $\mu$g/ml); group 2 ($n = 11$) was administered SiFO only as a control for group 1; group 3 ($n = 6$) was administered 1.2 ml SiO with RA (9 $\mu$g/ml); group 4 ($n = 6$) was administered SiO only as a control for group 3; and group 5 ($n = 4$) was administered only balanced salt solution (BSS) (Akorn Inc., Metairie, LA). Immediately after the gas–oil exchange, all the injected eyes were administered approximately 150,000 fibroblasts (0.1 ml suspension) and approximately 70,000 platelets (0.1 ml plasma) (Fig. 1). After surgery, the fundus was examined immediately and was checked for hemorrhages or surgically induced detachments. Slit lamp biomicroscopy and indirect ophthalmoscopy were repeated and recorded at 1, 3, 7, and 14 days and 1 month after injection. All examinations were masked as to the treatment received by each rabbit, and were performed concurrently by two observers (MN, MD). The findings were recorded in fundus drawings. The resultant PVR was graded from stages 1 to 5 according to the classification of Fastenberg et al..

All enucleated eyes were embedded in paraffin, and sections were stained with hematoxylin and eosin for light microscopy or embedded in epoxy resin, sectioned, and examined by electron microscopy.

FIGURE 5. Normal eye from group 1, 1 month after injection of silicone–fluorosilicone–copolymer oil, fibroblasts, platelets, and retinoic acid (9 $\mu$g/ml). Light micrograph shows no specific changes.
Enucleation at day 35 and histologic examination revealed a total retinal detachment with epiretinal and epipapillary membranes that pulled the papilla into the vitreous cavity. This eye was clinically and histologically classified as Fastenberg stage 5.

**Group B (Platelet-rich Plasma).** All eyes in this group showed progression of the initial vitreous haze to a grayish white membrane within the vitreous cavity, which condensed during week 1. By week 2, the membranes characterized a solid, grayish core surrounded by a thin, loose, fibrillar tissue. Clinically and histologically, the eyes were classified as Fastenberg stages 1 to 2. At the end of the observation period, the condensed membranes further thinned, but in one eye that continued to have a condensed membrane, focal traction was noted on the peripapillary retina. This eye was followed up until the second month, and focal traction did not progress. On enucleation, the PVR grade in this eye was clinically and histologically classified as Fastenberg stages 2 to 3.

**Group C (Fibroblasts and Platelet-rich Plasma).** All eyes in this group showed immediate membrane formation that became well organized within 24 hours. Dense fibrous membranes with peripheral extensions to multiple points in the retina produced traction detachments at these points, particularly in the medullary areas. These changes were observed as early as postoperative day 1 and in all eyes on postoperative day 3. The intravitreal membranes proliferated and contracted progressively, causing funnel-shaped retinal detachments with breaks. Clinically and histologically, all eyes were classified as Fastenberg stage 5 at the time of euthanasia.

**Retinal Tolerance of Retinoic Acid in SiO and SiFO**

Clinical and gross examinations of the eyes treated with 9, 10, and 15 μg/ml of RA did not show any evidence of retinal toxicity (Fig. 2). However, histopathologic retinal atrophic changes (thinning or disappearance of the photoreceptor layer and outer plexiform layer) were found in 2 of 5 eyes injected with 20 μg/ml RA in SiO (Fig. 3).

**Retinoic Acid Effect in the Fibroblast + Platelet-rich Plasma Proliferative Vitreoretinopathy Rabbit Model**

Before the gas–oil exchange, a mild posterior subcapsular cataract was noted in all eyes (probably the effect of C₃F₈ gas). Generally, mild, self-limited inflammation of the conjunctiva and anterior segment was observed in the first 3 to 5 days after the exchange. The following observations are based on examination 1 month after the experimental procedure (Fig. 4).

**Group 1.** Ten of 14 eyes (71%) did not have retinal detachments (Fig. 5). A traction retinal detachment was noted in four eyes (29%). These detachments ranged in Fastenberg’s classification from stages 3 to 4 (Fig. 5).

**Group 2.** A traction retinal detachment was observed in 9 of 11 eyes (82%), the severity of which varied from stages 3 to 4 (Fig. 6).

**Group 3.** A traction retinal detachment occurred in 2 of 6 eyes (33%) ranging from stages 3 to 5.

**Group 4.** Retinal detachments, the severity of which varied from stages 3 to 5, occurred in 6 of 6 eyes (100%) in this group as early as 1 week after surgery.

**Group 5.** Retinal detachments developed in all eyes, occurring 3 to 4 days after surgery. The group had total retinal detachments in all eyes that were classified as stage 5.

A comparison among SiFO-, SiO-, and BSS-injected eyes with retinal detachments revealed the following observations. SiFO-injected eyes tended to develop severe superior retinal detachments. SiO-injected eyes developed worse detachments in the inferior retina. BSS-injected eyes developed total retinal detachments with severe vitreous membrane formation.

Figure 4 demonstrates the frequency and severity of retinal detachment in the five groups of eyes at the end of the experiment. The incidence of retinal detachment resulting from PVR in RA-treated eyes (groups 1 and 3) was significantly lower (Mann-Whitney test, \( P < 0.05 \)) than in the controls (groups 2, 4, and 5).

**DISCUSSION**

This study demonstrates a rapid, effective, and reliable method of producing PVR in rabbit eyes. Intravitreous implantation of a mixture of approximately 100,000 fibroblasts and plasma enriched with at least 70,000 platelets invariably produced stage 5 PVR after as little as 24 to 72 hours. In comparison, only 1 of 5 eyes in the fibroblast group and none in the PRP group developed stage 5 PVR. However, milder PVR (stages 1 to 3) developed in these two groups after 1 to 2 weeks, a finding that agreed with previous reports.

The high rate of PVR produced in our model can be explained by the abundance of growth factors in the PRP that enhances fibroblast proliferation. We think that the white, discrete, fibrillar opacities observed soon after injection, and most notably in groups B and C, are aggregated platelets. We think that platelets release growth factors and, together with fibronectin from plasma, stimulate the fibroblasts to proliferate, adhere to the vitreous fibers and retina, and begin the PVR process. Previous studies have shown that vitreous injection of PRP, with or without plasma proteins, results in membrane formation. However, these membranes were transient and eventually thinned to fine strands after the second week. Our experiments produced similar results in one of
the five rabbits that showed a focal nonprogressive peripapillary traction.

Antiproliferative agents\textsuperscript{20-22,24,25,27,28,41,42} have been used experimentally against PVR in fibroblast rabbit models similar to group A eyes in our rabbit model PVR experiment. However, the new method of producing PVR by the simultaneous injection of homologous fibroblasts and platelets in rabbit eyes provides a faster and more reliable PVR response. Therefore, our PVR animal model could be very useful to evaluate potential antiproliferative agents against PVR.

Vitrectomy combined with SiO as an internal retinal tamponade has increased the success rate of surgery to treat retinal detachment complicated with PVR. However, intravitreal SiO does not prevent PVR recurrence,\textsuperscript{15-17,30} and might enhance it.\textsuperscript{17} In our experiments, inferior retinal detachments were seen in SiO-injected eyes, superior retinal detachments developed in SiFO-injected eyes, and total retinal detachments developed in BSS-injected eyes, indicating that the retinal tamponade effects of SiFO and SiO depend on their respective densities. The lighter SiO with a lower density (0.93 g/cm\(^3\)) cushions the superior retina, and the heavier SiFO (1.16 g/cm\(^3\)) rests on the inferior retina. In each case, cellular membrane proliferation takes place in the space available between the respective oil and the inner retina.

Surgery to treat retinal detachment complicated with PVR fails in many cases because of disease recurrence. Antineoplastic and antiinflammatory drugs have been injected intravitreally to prevent PVR recurrence, but most drugs are not soluble in silicone oils. Retinoic acid, a naturally occurring oxidative product of vitamin A that is soluble in SiO and SiFO, affects cell differentiation and proliferation.\textsuperscript{43} Doyle et al\textsuperscript{33} reported a potent antiproliferative effect of several retinoids on RPE cells, and RA showed the strongest inhibitory effect. Araiz et al\textsuperscript{34} reported the antiproliferative effect of RA (5 and 10 \(\mu\)g/ml) dissolved in intravitreal SiO in a fibroblast rabbit model of PVR. These results are confirmed in our newer rabbit model of PVR. In addition, we confirmed the antiproliferative effect of RA (9 \(\mu\)g/ml) in SiFO. Histopathologically, retinal atrophic changes were found in 2 of 5 eyes injected with 20 \(\mu\)g/ml RA in SiO (Fig. 3). However, using the lower RA concentrations reported by Araiz et al\textsuperscript{34} and in the current study, no changes attributable to RA were observed in the rabbit retina. This indicates that RA at concentrations up to or below 10 \(\mu\)g/ml in SiO or in SiFO does not appear to cause retinal histologic toxicity and has an inhibitory effect on PVR.

Key Words
animal model, antiproliferative, proliferative vitreoretinopathy, retinoic acid, silicone–fluorosilicone–copolymer oil, silicone oil

Acknowledgments
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References
Effect of Retinoic Acid in Silicone Oils in PVR

2395


