Squalamine Improves Retinal Neovascularization

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PURPOSE. Modalities for inhibiting neovascularization may be one avenue to the development of effective therapies for retinopathy. The effect of squalamine, an antiangiogenic amino sterol, on oxygen-induced retinopathy (OIR) was assessed in a mouse model.

METHODS. OIR was induced in C57BL6 mice by a 5-day exposure to 75% oxygen from postnatal day (P)7 through P12. Squalamine (25 mg/kg, subcutaneous)-treated animals received either daily doses for five days from P12 to P16 or one dose just after removal from oxygen on P12. Each set of animals was killed at P17 to P21. Retinopathy was assessed with a retinopathy scoring system evaluation of retinal wholemounts and by quantification of neovascular nuclei on retinal sections.

RESULTS. Animals receiving 5 days of squalamine after a 5-day exposure to oxygen had total retinopathy scores (expressed as median score with 25th and 75th quartiles in parentheses) of 4(3, 5) versus oxygen-only–reared animals with scores of 8(7, 9; P < 0.001). Animals reared in room air and animals exposed to squalamine only had similar retinopathy scores: 1(1, 2) and 1(0, 2). Oxygen-reared animals receiving single-dose squalamine also showed improvement, with a median retinopathy score of 4(4, 6.75) versus oxygen-only–reared animals with median retinopathy score of 9(7, 10; P < 0.001). There was a decreased number of neovascular nuclei extending beyond the inner limiting membrane on retinal sections in animals treated with 5 days (P < 0.01) and 1 day (P < 0.001) of squalamine.

CONCLUSIONS. Squalamine significantly improved retinopathy and may be a novel agent for effective treatment of ocular neovascularization. (Invest Ophthalmol Vis Sci. 2000;41:1507–1512)

Squalamine is a broad-spectrum aminosterol antibiotic originally isolated from the dogfish shark Squallus acantbias.1 It has recently been reported to inhibit tumor-induced angiogenesis and tumor growth.2,3 Squalamine also improved tissue oxygenation in rats bearing the 13762 mammary carcinoma.3 On a cellular level, squalamine inhibits growth factor–mediated endothelial cell proliferation and migration, including that stimulated by vascular endothelial cell growth factor (VEGF) at concentrations that are not toxic to endothelial cells in vitro.2

Vasoproliferative retinopathy is a leading cause of blindness and occurs primarily because of overexpression of VEGF.4,5 Steroids have been previously described to inhibit retinal neovascularization in the mouse,6 inhibit preretal neovascularization in a pig model,7 prevent hyperoxia–induced neovascularization in the rabbit,8 and inhibit subretinal neovascularization in a primate model.9 The goal of this study was to determine whether squalamine, a newly described antiangiogenic steroid with no mineralocorticoid or glucocorticoid function, could inhibit retinal neovascularization in a mouse model of oxygen-induced retinopathy (OIR).

METHODS

Mouse Model

C57BL6 mice were obtained from Taconic Laboratories (Germantown, NY). Infant mice were placed in an infant incubator (Ohmeda, Columbia, MD) with 75% oxygen at P7 through P12 with their nursing mothers as previously described.6,10–12 The oxygen was delivered at 75% ± 2%, measured with an oxygen analyzer (Hudson Ventronics, Temecula, CA), and checked at least twice daily during the oxygen exposure. Individual litters were either room air or oxygen reared. Within most litters, animals were divided into no treatment, treatment with squalamine, and/or vehicle treatment. On P12, the animals were returned to room air to induce relative retinal hypoxia. Animals were treated with vehicle or squalamine injections that were initially given for 5 days from P12 to P16. Subsequent experiments were performed using a single dose of squalamine on P12. Vehicle-treated animals were administered diluent (sterile water) for either 5 days or 1 day in parallel to the squalamine–treated animals. Animals were killed by lethal pentobarbital injection from P17 to P21, because retinal neovascularization is at its maximum and is consistent at that time.10 The protocol was reviewed and approved by the New York University Medical Center Institutional Animal Care and Use Committee for the Division of Laboratory Animal Resources and the Georgetown University Animal Care and Use Committee.
and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Squalamine Administration**

Squalamine was administered subcutaneously in the nape of the neck at 25 mg/kg · d in a single daily injection. This dose was selected because prior effective doses of 20 to 40 mg/kg · d of squalamine were used in oncologic studies in rats. Initial pilot experiments were performed on several litters, by using a 5-day regimen of squalamine after oxygen exposure from P12 through P16. Five-day squalamine experiments were then performed using room air- or oxygen-reared animals with intralitter assignments, as described. All animals were used in the data analysis. Subsequent experiments were performed using a single dose of squalamine at P12.

**Retinal Perfusion**

Fluorescein-conjugated dextran perfusion of the retinal vessels was performed as previously described using high-molecular-weight fluorescein conjugated dextran in 4% paraformaldehyde in phosphate-buffered saline (PBS). Briefly, animals were given a lethal dose of pentobarbital sodium (120 mg/kg) and when deep anesthesia was obtained, a median sternotomy was performed. The left ventricle was identified, and 1 ml of a 50-mg/ml solution of fluorescein-conjugated dextran was injected. Eyes were enucleated and placed in 4% paraformaldehyde for 3 to 24 hours. The retinas were dissected using light microscopy and flat mounted. In the 5-day squalamine treatment experiments the following numbers of animals were used: the room air (control) group, n = 19 from four litters (16 killed at P17 and 3 killed at P19); room air + squalamine group, n = 17 from 4 litters (9 killed at P17 and 8 killed at P19); oxygen group, n = 20 from 4 litters (17 killed at P17, 2 at P18, and 2 at P19); and oxygen + squalamine group, n = 25 from 8 litters (25 killed at P17). For the single-dose squalamine experiments the numbers of animals were as follows: the room air (control) group, n = 13 from 5 litters (1 killed at P17, 2 at P18, 4 at P19, and 6 at P20); room air + squalamine group, n = 19 from 6 litters (3 killed at P17, 2 at P18, 8 at P19, 2 at P20, and 4 at P21); oxygen group, n = 21 from 6 litters (13 killed at P18, 6 at P19, and 2 at P21); and oxygen + squalamine group, n = 22 from 4 litters (2 killed at P18, 17 at P19, and 3 at P21).

Retinae were scored in a masked fashion using a previously validated retinopathy scoring system as shown in Table 1. The minimum score according to this method is 0, and the maximum score is 17. Maximal vasoproliferation in this mouse model has previously been reported to occur from P17 to P21. In addition, our laboratory has previously reported concordance of scores in mice killed from P17 to P20.

**Quantification of Extraretinal Neovascularization**

After perfusion of animals with 4% paraformaldehyde, eyes were removed, placed in optimal cutting temperature embedding compound, and frozen at −70°C. Serial sections (7-10 μm) were cut through the cornea parallel to the optic disc using a cryostat. Tissue sections were stained with periodic acid–Schiff stain and hematoxylin. For the 5-day treatment with squalamine, there were the following numbers of animals: room air (control) group, n = 7 from 5 litters (6 killed at P17 and 1 at P18); room air + squalamine group, n = 6 from 2 litters (all 6 killed at P17); oxygen group, n = 8 from 4 litters (4 killed at P17, 2 at P18, and 2 at P19); and oxygen + squalamine, n = 5 from 2 litters (all five killed at P17). In the single-dose squalamine experiments, the animal numbers were as follows: the room air (control) group, n = 10 from 6 litters (3 killed at P17, 1 at P19, and 6 at P20); room air + squalamine group, n = 8 from 4 litters (4 killed at P17, 2 at P18, and 2 at P19); oxygen group, n = 15 from 11 litters (1 killed at P17, 8 at P18, 5 at P19, and 1 at P20); and oxygen + squalamine group, n = 9 from 6 litters (1 killed at P18, 7 at P19, and 1 at P21). Multiple sections were scored in a masked fashion by counting the number of nuclei extending beyond the inner limiting membrane into the vitreous, as previously described and used in our laboratory. A minimum of eight sections at least 50 μm apart over a maximum distance of 450 μm were counted and averaged for each eye. The average number for each eye was pooled and averaged across replicates for each treatment condition, and these averages were used in the statistical data analysis.

**Animal and Organ Weights**

Animals and individual organs were weighed on a standard laboratory balance. In addition, a log of animal death was kept throughout the course of the experiments.

**Statistical Analyses**

Analysis of variance using the Kruskal-Wallis test was performed to test for differences between the treatment groups.
The Mann–Whitney test was used to compare the total retinopathy scores and retinopathy subscores between individual groups. Student’s t-tests assuming unequal variance were performed to compare the number of nuclei in the retinal sections, weights, and organ-to-body weight ratios. Significance was defined as \( P < 0.05 \).

**RESULTS**

**Five-Day Regimen of Squalamine**

Retinopathy scores are expressed as median with 25th and 75th quartiles in parentheses. Animals that received 5 days of squalamine from P12 through P16 after exposure to a hyperoxic environment had a lower retinopathy score than their counterparts that were exposed to oxygen only: \( 4(3, 5) \) and \( 8(7, 9) \), respectively (Fig. 1A). Squalamine-only–treated animals reared in room air had a retinopathy score similar to that of the untreated control group: \( 1(0, 1) \) and \( 1(1, 2) \), respectively. Vehicle-treated animals (sterile water diluent) had retinopathy scores of \( 1(0, 1) \) in the room air–reared group \( (n = 6 \text{ from three litters}) \) and \( 8(7, 9) \) in the oxygen-reared group \( (n = 2 \text{ from two litters}) \), which were similar to the scores of untreated animals in both groups. Within each of the groups, day of sacrifice did not affect the scores. Retinal neovascularization was notably improved after the squalamine treatment in the specific categories of blood vessel tuft formation, extraretinal neovascularization (ERNV), and blood vessel tortuosity (Table 2). Squalamine-treated eyes had less clock hours or circumferential disease with regard to all parameters that were scored. Control animals had slightly positive retinopathy scores, because mice are born with an immature retinal vasculature that is sometimes not fully mature by P17 to P21. Vessel remodeling occurs into adulthood\(^1\) and may also help explain the small positive values noted in the retinopathy scores for room-air-reared mice.

In confirmation of the retinopathy score observations, squalamine-treated, oxygen-reared animals had less ERNV as measured by neovascular nuclei \( (12.3 \pm 4.7) \) counts on retinal sections compared with oxygen-reared animals \( (56.0 \pm 24.4) \) as shown in Figure 1B. Control animals showed few neovascular nuclei, as has been previously described in this model.\(^6\)\(^{-12}\) This observation in room-air-reared animals may again be explained as vessel remodeling into adulthood\(^1\).\(^4\) There were no observed histologic differences (other than ERNV) between oxygen-exposed and squalamine-treated retinal sections when compared with control sections.

![Figure 1](https://example.com/figure1.png)

**TABLE 2.** Subscores of Categories of Retinopathy in Animals in 5-Day Squalamine Experiments

<table>
<thead>
<tr>
<th>Category</th>
<th>Control ((n = 19))</th>
<th>Control + Squalamine ((n = 17))</th>
<th>Oxygen ((n = 20))</th>
<th>Oxygen + Squalamine ((n = 25))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessel growth</td>
<td>1 (0, 1)</td>
<td>1 (0, 1)</td>
<td>1 (1, 2)</td>
<td>1 (1, 1)</td>
</tr>
<tr>
<td>Blood vessel tufts</td>
<td>0.5 (0, 1)</td>
<td>0 (0, 0)</td>
<td>2 (1, 2)</td>
<td>0 (0, 1)*</td>
</tr>
<tr>
<td>Extraretinal neovascularization</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>1 (1, 2)</td>
<td>0 (0, 0)*</td>
</tr>
<tr>
<td>Central vasoconstriction</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>2 (1, 2)</td>
<td>2 (2, 2)</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>1 (1, 1)</td>
<td>0 (0, 1)</td>
</tr>
<tr>
<td>Blood vessel tortuosity</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>1 (1, 2)</td>
<td>0 (0, 0)*</td>
</tr>
</tbody>
</table>

Values are expressed as median (25th, 75th quartile).

*\( P < 0.05 \) when compared with oxygen alone by Mann–Whitney test for the oxygen + squalamine group.
Single Dose of Squalamine

Subsequent experiments were performed to test the hypothesis that a single dose of squalamine on P12, the day the animals are removed from a hyperoxic environment, would be sufficient to alter the development of retinal neovascularization. Results again showed an improvement in retinopathy as measured by total retinopathy scores (Fig. 2A) and subscores in categories of blood vessel tuft formation, ERNV, and blood vessel tortuosity (Table 3). Vehicle-treated animals had median retinopathy scores of 0(0, 1) in the room air group (n = 9 from 4 litters) and 10(9, 10) in the oxygen-reared group (n = 8 from 2 litters), which were similar to the untreated animals in both groups. Further, a decrease in the number of neovascular nuclei was found in oxygen + single-dose squalamine–treated animals (16.3 ± 6.8) compared with oxygen-only–reared animals (46.2 ± 24.1) as shown in Figure 2B.

The degree of suppression of neovascular nuclei in hyperoxia-exposed mice after treatment with a single dose of squalamine (64%) was comparable with that seen after 5 days of squalamine treatment (78%), suggesting that a long-lived biologic response to squalamine occurs. Representative retinal wholemounts are shown in Figure 3 from both sets of experiments. Although there is some variability within the room-air–reared and oxygen-reared groups (see error bars for quartiles in Figs. 1A and 2A and error bars for SDs in Figs. 1B and 2B), retinopathy developed in all animals exposed to 75% oxygen, and squalamine consistently improved the retinopathy. Squalamine did not affect normal vascularization or development of the retina as measured by the retinopathy scoring system or by histologic observation of retinal sections.

Effect of Squalamine on Growth

Systemic administration of squalamine to the mice did not have any gross adverse developmental effects when assessed by animal weight gain and individual organ weight-to-body weight ratios (Table 4). Normal growth of animals and individual organs suggests that squalamine does not alter normal vessel growth while inhibiting pathologic neovascularization during the period from P12 to P21. There were no deaths in the room-air-reared group with 5 days of squalamine treatment, the oxygen-reared group with 5 days of squalamine treatment, or the room-air-reared vehicle group. There was one death each in the room-air-reared group, the room air + single-dose squalamine group, and the oxygen-reared vehicle group. There were two deaths in the oxygen-reared group and oxygen-reared + single dose squalamine group.

Table 3. Subscores of Categories of Retinopathy in Animals in Single-Dose Squalamine Experiments

<table>
<thead>
<tr>
<th>Category</th>
<th>Control (n = 13)</th>
<th>Control + Squalamine (n = 19)</th>
<th>Oxygen (n = 21)</th>
<th>Oxygen + Squalamine (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessel growth</td>
<td>0 (0, 0)</td>
<td>0 (0, 1)</td>
<td>1 (0, 1)</td>
<td>0.5 (0, 1)</td>
</tr>
<tr>
<td>Blood vessel tufts</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>2 (2.3)</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td>Extraretinal neovascularization</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>2 (1.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Central vasoconstriction</td>
<td>0 (0, 0)</td>
<td>0 (0, 0.5)</td>
<td>2 (1.3)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>1 (1.1)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Blood vessel tortuosity</td>
<td>0 (0, 1)</td>
<td>0 (0, 0)</td>
<td>2 (1.2)</td>
<td>1 (1.1)</td>
</tr>
</tbody>
</table>

Values are expressed as median (25th, 75th quartile).

*P < 0.05 when compared to oxygen alone by Mann–Whitney test for the oxygen and squalamine group.
DISCUSSION

This is the first study that has examined inhibition of angiogenesis by squalamine in a noncancerous model—i.e., a mouse model of oxygen-induced retinal neovascularization. The measurements of retinal neovascularization collected in this study show that squalamine significantly reduces retinal neovascularization in a mouse model of oxygen-induced proliferative retinopathy, as measured by a previously validated retinopathy scoring system and by quantitation of extraretinal nuclei on retinal sections. Systemic squalamine given either during the 5 days from P12 through P16 or as a single dose on P12 significantly reduced retinal neovascularization.

FIGURE 3. Representative retinal wholemounts showing control (A), oxygen-treated (B), 5-day squalamine + oxygen-treated (C), and single-dose squalamine + oxygen-treated (D) animals. Note the smooth vascular pattern in the control group (A) compared with the loss of central vasculature and presence of multiple blood vessel tufts at the junction of the loss of central vasculature and the remainder of the retinal blood vessels in the oxygen-treated retina (B). Both squalamine-treated retinae (C, D) had some loss of central vasculature as did the retina in (B), but few blood vessel tufts, ERNV, and less tortuous vessels than the oxygen-only-reared retina. Magnification, ×4.5.

<table>
<thead>
<tr>
<th>Room Air</th>
<th>Room Air + 5-Day Squalamine</th>
<th>Room Air + Single-Dose Squalamine</th>
<th>Oxygen + 5-Day Squalamine</th>
<th>Oxygen + Single-Dose Squalamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 10)</td>
<td>(n = 12)</td>
<td>(n = 10)</td>
<td>(n = 6)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>Total body weight</td>
<td>8.96 ± 2.08</td>
<td>7.15 ± 1.40</td>
<td>8.92 ± 1.50</td>
<td>7.70 ± 1.63</td>
</tr>
<tr>
<td>Brain</td>
<td>0.045 ± 0.011</td>
<td>0.045 ± 0.005</td>
<td>0.041 ± 0.0034</td>
<td>0.046 ± 0.009</td>
</tr>
<tr>
<td>Heart</td>
<td>0.008 ± 0.001</td>
<td>0.009 ± 0.002</td>
<td>0.009 ± 0.002</td>
<td>0.007 ± 0.001</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.015 ± 0.002</td>
<td>0.015 ± 0.001</td>
<td>0.016 ± 0.004</td>
<td>0.015 ± 0.004</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.016 ± 0.002</td>
<td>0.017 ± 0.002</td>
<td>0.016 ± 0.001</td>
<td>0.016 ± 0.001</td>
</tr>
<tr>
<td>Liver</td>
<td>0.042 ± 0.008</td>
<td>0.038 ± 0.004</td>
<td>0.041 ± 0.007</td>
<td>0.044 ± 0.009</td>
</tr>
</tbody>
</table>

Body weights are expressed in grams ± SD, and organ weights are expressed as organ weight-body weight ratio ± SD. No differences were seen in body weight or individual organ weights in any of the treatment groups.
cantly decreased retinopathy. There was less neovascularization when measured by blood vessel tuft formation, ERNV, and blood vessel tortuosity in the squalamine + oxygen-treated retinas when compared with the oxygen-only–treated retinas. Thus, squalamine is able to inhibit or decrease the neovascular response seen after exposure of the neonatal mice to 75% oxygen. In addition no deleterious systemic side effects of squalamine such as weight loss or impaired organ growth were found in this study. Squalamine did not affect normal vascular development as assessed by the retinopathy score or retinal histology. There may be a more favorable therapeutic index for squalamine in treating retinopathy than for an antiangiogenic agent that suppresses all vessel growth. Further experiments are required to define the basis for squalamine’s selectivity with respect to inhibition of abnormal blood vessel growth.

Both single and 5-day regimens of squalamine had similar effects. This may be explained by postulating a long half-life for squalamine—that is, the single dose lasts long enough to inhibit neovascularization in this model. The presence or persistence of squalamine in the neonatal eye was not measured in these studies. There also may be a critical time (i.e., day 12 and shortly thereafter) when retinal hypoxia triggers a maximal response and VEGF-stimulated proliferation is at its peak in the mouse model.

We speculate that squalamine broadly inhibits growth factor–stimulated endothelial cell growth, leading to inhibited neovascularization in the mouse retina. A previous report provided evidence that squalamine inhibited in vivo angiogenesis in a tumor model. In addition, squalamine has been reported to inhibit mitogen (including VEGF)–stimulated proliferation of endothelial cells. Although it was beyond the scope of this study to assess squalamine concentrations in the retina, squalamine may also improve retinal oxygenation as it did in a tumor model and thereby may suppress hypoxia-mediated signaling for VEGF or other growth factor production at a critical time in the development of retinopathy. Alternatively, if squalamine decreases VEGF production or downregulates VEGF receptor expression, then the metabolic demands of the tissue may be lower, and tissue oxygenation may be improved by a lower metabolic rate in the retinas of animals treated with squalamine. Specific target cells for squalamine in this model of OIR are not known.

Dexamethasone has been reported to inhibit retinal neovascularization in a primate model, mouse, and rabbit. Triamcinolone acetate has been shown to inhibit preretinal and optic nerve head neovascularization in the pig. The exact mechanism of corticosteroid-induced inhibition of retinopathy is unclear but may be related to several factors including decreasing inflammation and inhibiting angiogenic growth factors.

Concerns regarding the long-term effects of angiogenesis inhibitors are valid, particularly in the context of patients with multiple medical problems. For instance, inhibiting retinal neovascularization in retinopathy of prematurity or diabetic retinopathy may be important but may have effects on other systemic diseases (i.e., hernia surgery, bronchopulmonary dysplasia, decubitus ulcer). In this regard, it is meaningful that short-term treatment (i.e., a single dose) with squalamine in this animal model of oxygen-induced retinopathy led to a marked improvement of retinal neovascularization. Intermitent treatment of vasoproliferative retinopathy with squalamine may therefore be a preferred way to reduce possible side effects.

A short-term therapy that may prevent blindness could prove clinically sightsaving in ocular diseases such as diabetic retinopathy and retinopathy of prematurity. Squalamine is currently in phase II trials for patients with late-stage non–small-cell lung cancer. If squalamine is shown to be efficacious in preventing or inhibiting tumor growth or metastasis in human cancers, it may also be useful as a potential treatment to prevent human retinal neovascularization. Squalamine’s ability to block angiogenesis in a noncancerous model such as this mouse oxygen-induced retinopathy model is a significant finding, and it may ultimately become a new agent for use in the treatment of ocular neovascularization.

References