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Effect of Photodynamic Therapy and Anti-VEGF Therapy in the Treatment of Corneal Neovascularization : (FTIR) Study

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ABSTRACT

The aim of the present study was to compare the effect of photodynamic therapy (PDT) by using argon laser and bevacizumab (Avastin) on corneal neovascularization (CNV) in rabbits. Thirty-Nine New Zealand male rabbits aged 2.5 months and weighing 2.5 - 3kg. All rabbits' eyes were examined by slit lamp biomicroscope before induction of corneal neovascularization. Three rabbits (N=6 eyes) were used as control, and 36 rabbits (N=72 eyes) were generally anesthetized using intramuscular Xylaject (0.2 ml/kg) and ketamine hydrochloride (0.6 ml/ kg). Benoxinate eye drops (0.4%) was used for local anesthesia. Corneal neovascularization (CNV) was induced by a three-interrupted 7.0 silk sutures at midstromal depth approximately 1 mm from the limbus. After four days' sutures were removed and slit lamp examinations were performed to determine the area of corneal neovascularization. Twelve rabbits with corneal NV were left without any treatment. photodynamic therapy group (N=12 rabbits) were generally anesthetized and rose bengal ($C_{20}H_4Cl_4I_4O_5$) with a dose of 50 mg/Kg was administered by slow intravenous injection in the marginal ear vein. Argon laser (532 nm) was applied for 5 minutes with a power of 150 mW/cm2. Bevacizumab group was the last group (N=12 rabbits) were the rabbits were subjected to intravitreal injection of 25 mg / eyes. For a period of 4 weeks, three rabbits were selected and sacrificed weekly (N = 6 eyes each) from several groups.

Keywords: Corneal neovascularization, Photodynamic therapy, Rose bengal, Argon laser, Bevacizumab, Fourier transform IR.

I. INTRODUCTION

Eye is one of the most important organs in the body of the organism and through which to see the various objects. Cornea is a component of the eye as the first receiver of light falling on the eye through which objects can be seen. The cornea collects light at one point on the surface of the retina by helping of the crystal lens of the eye. The cornea has also the unique feature (except for cartilage) of being normally avascular, but under pathologic conditions vessels invade the cornea from the limbal vascular plexus. A wide variety of insults including infection, inflammation, ischemia, degeneration, trauma, and loss of the limbal stem cell barrier can cause corneal neovascularization (NV) [1]. Although corneal NV can occasionally serve a beneficial role in the clearing of infections, wound healing, and in arresting stromal melts [2], its disadvantages are numerous. Corneal NV often leads to tissue scarring, edema, lipid deposition, and persistent inflammation that may significantly

alter visual acuity [3]. The corneal NV may not only reduce visual acuity but also it results in the loss of the immune privilege of the cornea, thereby worsening the prognosis of subsequent penetrating keratoplasty (PK) [4]. Vascular endothelial growth factor (VEGF) is thought to be a key mediator in the corneal neovascularization process [5]. The notable role of VEGF in the pathophysiology of CNV has been demonstrated in experimental models of CNV [6].

Recently, subconjunctival of bevacizumab (Avastin) has also been considered as a new treatment modality for corneal NV [7,8]. While there is substantial evidence for the intravitreous administration of bevacizumab in the treatment of choroidal NV, data regarding the safety and efficacy of topical bevacizumab in the treatment of CNV are, as yet, preliminary. Topical bevacizumab was demonstrated to inhibit CNV after chemical injury in an experimental animal model [9].

Vascular endothelial growth factor (VEGF) is thought to be a key mediator in the process of neovascularization [10]. The prominent role of VEGF in the pathophysiology of corneal NV has been demonstrated in experimental models of corneal NV [11]. It has been shown that VEGF is up-regulated in inflamed and vascularized corneas in humans and in animal models [12]. It has also been shown that inhibition of angiogenesis by neutralization of VEGF can promote corneal graft survival in animal models [13]. VEGF inhibitors such as pegaptanib sodium (Macugen; [OSI] Eye tech/Pfizer, Inc, New York, NY), ranibizumab (Lucentis; Genentech Inc., San Francisco, CA) and bevacizumab (Avastin; Genentech Inc., San Francisco, CA) are currently used for the treatment of neovascular age-related macular degeneration (AMD) [14]. The first two agents have been approved by the FDA for use in neovascular AMD; the third drug which is a full-length humanized antibody against VEGF, has been approved for use in oncology but is also widely used off-label to treat choroidal neovascularization [15], central retinal vein occlusion [16], proliferative diabetic retinopathy [17], and iris neovascularization [18] with encouraging results. Bevacizumab has now been widely adopted and is arguably part of the standard of care for the treatment of neovascular AMD for many patients [14,15].

Bevacizumab

Bevacizumab, sold under the trade name Avastin, is medication used to treat a number of types of cancers and a specific eye disease. For cancer it is given by slow injection into a vein and used for colon cancer, lung cancer, glioblastoma, and renal-cell carcinoma. For age-related macular degeneration it is given by injection into the eye [19].

Common side effects when used for cancer include nose bleeds, headache, high blood pressure, and rash. Other severe side effects include gastrointestinal perforation, bleeding, allergic reactions, blood clots, and an increased risk of infection [20]. When used for eye disease side effects can include vision loss and retinal detachment. Bevacizumab is in the angiogenesis inhibitor and monoclonal antibody families of medication. It works by slowing the growth of new blood vessels [21]. Bevacizumab was approved for medical use in the United States in 2004. It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system [22]. It is listed for its use in treating eye disease [23].

Recently, off-label use of topical as well as subconjunctival bevacizumab has also been considered as a new treatment modality for corneal NV [24-28]. While there is substantial evidence for the intravitreous administration of bevacizumab in the treatment of choroidal NV, data regarding the safety and efficacy of topical bevacizumab in the treatment of corneal NV are, as yet, preliminary. Topical bevacizumab was demonstrated to inhibit corneal NV after chemical injury in an experimental rat model [25]. In humans, a small number of studies showed that topical bevacizumab can reduce corneal

NV in a few patients with significant corneal NV [26,27].

II. METHODS AND MATERIAL

New Zealand male rabbits (n = 39) aged 2.5 months and weighing 2–2.5 kg was selected from the animal house located at Research Institute of Ophthalmology, Giza, Egypt. The animals were maintained in a standard 12-h light/dark cycle with free access to water and balanced diet. All procedures were conducted according to the principles enunciated in the Guide for Care and Use of Laboratory Animals. They were subjected to experimental protocols approved by the local experimental ethics committee of ophthalmic and vision research.

The rabbits are divided into 4 groups as the following:

- **Control group** (n=3 rabbits)
- **Group with corneal neovascularization** (n=12 rabbits).
- **Group treated with photodynamic therapy** (n=12 rabbits) are generally anesthetized and rose bengal with a dose of 50 mg/kg is administered by slow intravenous injection in the marginal ear vein. After 15 minutes, rabbits' corneas are exposed to argon laser (Quantel-Medical, Vitra, France) at 1 cm distance from the cornea in a continuous mode of exposure at 532 nm for 5 minutes and a power of 150 mW/cm 2 (Ophthalmology department, national institute of laser enhanced science, Cairo University, Egypt).
- **Group treated with Avastin** (n=12 rabbits), the rabbits are subjected to subconjuctival injection of 25 mg /eye avastin (Genentech Inc., San Francisco, CA). For a follow up period of 1, 2, 3 and 4 weeks, 3 animals are selected weekly from each group, sacrificed, and the eyes are enucleated.
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Clinical examination

All rabbits' eyes were examined by slit lamp biomicroscope before induction of corneal neovascularization. The results indicate no signs of edema or intraocular inflammation in all eyes.

Corneal neovascularization injury

Three rabbits were used as control, and 36 rabbits (n=72 eyes) were generally anesthetized using intramuscular Xylaject (0.2 ml/kg) and ketamine hydrochloride (0.6 ml/ kg). Benoxinate eye drops (0.4%) was used for local anaesthesia. Corneal neovascularization was induced by a three-interrupted 7.0 silk sutures at midstromal depth approximately 1 mm from the limbus. Anti-inflammatory eye drops (Diclofenac) was used three times daily to treat pain and inflammation. After four days' sutures were removed and slit lamp examinations were performed to determine the area of corneal neovascularization.

FTIR spectroscopy analysis for lens protein

FTIR spectra of different cornea samples were recorded by means of a Thermo Nicolet iS5 FTIR spectrometer, USA, in the range 4,000:1,000 cm^{-1} at room temperature. The spectrometer is operated under a continuous dry nitrogen gas purge to remove interference from atmospheric carbon dioxide and water. The data was baseline corrected and smoothed by Savitzky Golay to eliminate the noise before Fourier transformation. One hundred scans were taken for each interferogram at 2 cm^{-1} resolution. Lenses were weighed, lyophilized and then mixed with KBr powder (98mg KBr: 2mg lens) to prepare the KBr disks for FTIR analysis. The obtained group spectrum was normalized and analyzed for fingerprint region (1,800:1000 cm^{-1}), which includes the amide I band (1,800:1,600 cm^{-1}).

III. RESULTS

The vibrational FTIR spectra, were directly related to the structural features of molecules were analyzed for the spectral regions: 1800:1000 cm^{-1}which correspond to fingerprint region.

Fingerprint Region

Table 1 summarizes the bands that appeared in the fingerprint region (1800 –1000 cm-1) for all groups

after the first week with compared to the control group. The control group was characterized by seven underlying structural bands as shown in table 3. These seven bands were centered nearly at 1083.79 ± 0.35 cm-1, 1249.03 ± 2.22 cm-1, 1322.44 ± 1.08 cm-1, 1401.57 ± 3.32 cm-1, 1540.85 ± 0.33 cm-1, 1642.78 ± 0.17 cm-1, and 1724.37 ± 1.81cm-1 which correspond to str.C-N, asym.PO2, str.C-N, str.C-C, Amide II, Amide I, and str.C=O respectively.

Table 1. Wavenumber and percentage area of bands at fingerprint region for different groups compared with the control group after the first week.

	Control	CNV.	Avast.	PDT.
str. C=O	1724.37 ± 1.81	1723.14 ± 7.11	1680.23 ± 9.58	1738.45 ± 0.50
	5.11	6.21	14.18	2.48
Amide I	1642.78 ± 0.17	1642.62 ± 0.42	1642.59 ± 0.37	1641.46 ± 0.13
	47.69	54.34	41.32	56.17
Amide II	1540.85 ± 0.33	1543.14 ± 3.82	1530.02 ± 1.18	1541.66 ± 0.71
	20.76	13.69	16.47	7.66
str. C-C	1401.57 ± 3.32	1423.81 ± 4.89	1417.07 ± 1.01	1407.02 ± 1.71
	9.83	10.97	8.79	16.22
str. C-N	1322.44 ± 1.08	1367.76 ± 1.03	1332.39 ± 2.01	1317.11 ± 7.43
	0.64	4.46	6.81	2.71
asym. PO_2	1249.03 ± 2.22	1262.48 ± 3.95	1240.13 ± 1.26	1239.82 ± 3.51
	8.76	5.14	5	6.03
str. C-N	1083.79 ± 0.35	1153.48 ± 8.34	1193.36 ± 4.34	1102.7 ± 24.31
	7.21	5.19	7.43	8.73

First line in each cell indicates the frequency of the band, while second line reflects the percentage area.

Figure 1 summarizes the bands that appeared in the fingerprint region (1800 –1000 cm^{-1}) for all groups after the first week with compared to the control group.

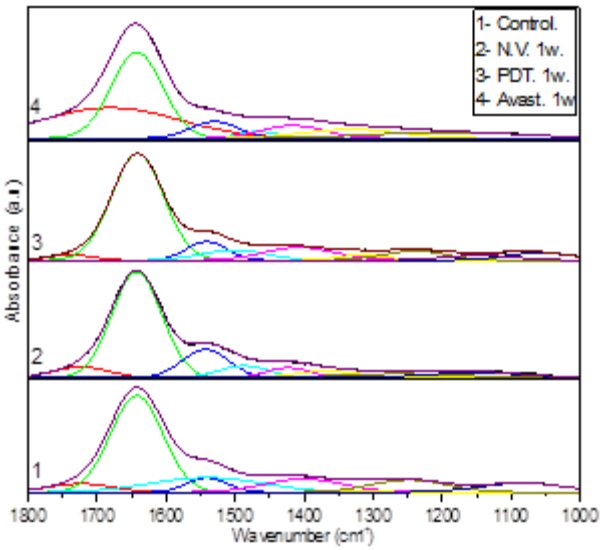

Figure 1. FTIR spectra in the Fingerprint region for all groups after the first week with compared to the control group, showing the estimated underlying peaks. The numbers to facilitate the identification of the bands.

According to table 1, there was an increase in the vibrational frequency in the asym. PO_2, str.C-N, str.C-C, and Amide II band in corneal neovascularization (CNV) group after the first week. No noticeable change in the band position of Amide I or str.C=O bands was found in this group relative to the corresponding control ones. For the treated groups, there was no change in most bands but there was a slight increase in vibrational frequency of str.C-N and str.C=O bands. By contrast, there has been a decrease in the vibrational frequency in the Amide II band in the group which treated by bevacizumab (Avastin) after the first week.

As shown in the table 2, there was an increase in the vibrational frequency in the str.C-N, str.C-C, and str.C=O in corneal neovascularization (CNV) group after the second week. No noticeable change in the band position of Amide I, Amide II, and str.C-N bands was found in this period for all groups relative to the corresponding control ones.

Table 2. Wavenumber and percentage area of bands at fingerprint region for different groups compared with the control group after the second week.

	Control	CNV.	Avast.	PDT.
str. C=O	1724.37 ± 1.81	1691.57 ± 4.21	1728.80 ± 1.78	1734.88 ± 3.38
	5.11	11.13	12.01	4.61
Amide I	1642.78 ± 0.17	1645.04 ± 1.82	1641.35 ± 0.09	1642.89 ± 0.35
	47.69	47.17	42	53.74
Amide II	1540.85 ± 0.33	1540.04 ± 8.91	1538.91 ± 0.81	1540.93 ± 0.71
	20.76	11.33	17.56	13.59
str. C-C	1401.57 ± 3.32	1447.16 ± 5.31	1445.11 ± 1.28	1462.52 ± 2.61
	9.83	12.8	9.7	9.62
str. C-N	1322.44 ± 1.08	1332.17 ± 8.61	1356.97 ± 1.72	1367.26 ± 1.31
	0.64	6.93	6.48	9.96
asym. PO_2	1249.03 ± 2.22	1233.54 ± 1.58	1245.88 ± 7.47	1236.35 ± 5.01
	8.76	4.25	7.98	3.3
str. C-N	1083.79 ± 0.35	1081.81 ± 0.62	1096.22 ± 1.23	1086.27 ± 6.99
	7.21	6.39	4.27	5.18

First line in each cell indicates the frequency of the band, while second line reflects the percentage area.

Figure 2 summarizes the bands that appeared in the fingerprint region after two weeks of the study.

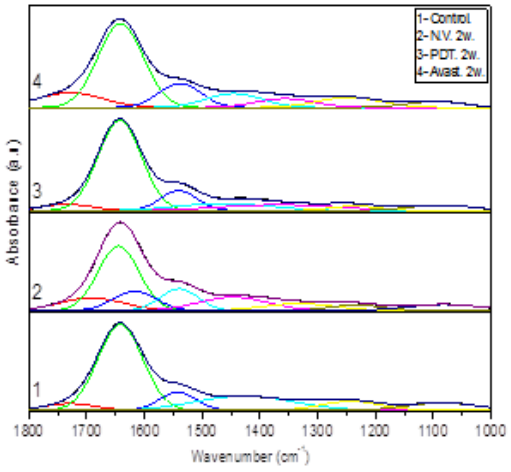

Figure 2. FTIR spectra in the Fingerprint region for all groups after two weeks with compared to the control group, showing the estimated underlying peaks. The numbers to facilitate the identification of the bands.

After the third week, some changes were observed in all groups not only in vibrational frequency but also in percentage area for different bands for both groups (Figure 3).

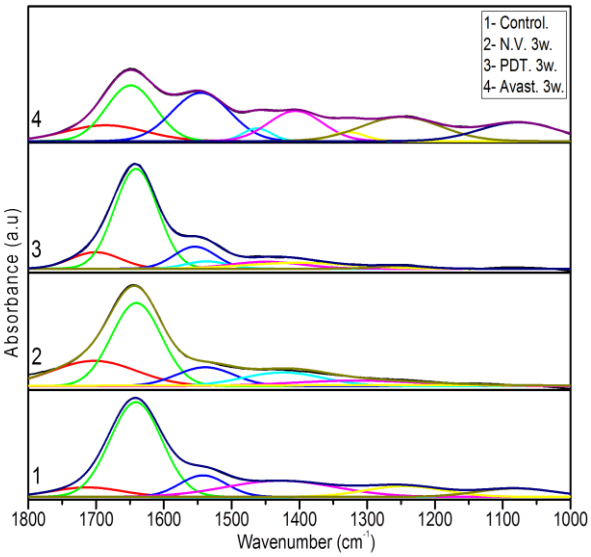

Figure 3. FTIR spectra in the Fingerprint region for all groups after three weeks with compared to the control group, showing the estimated underlying peaks. The numbers to facilitate the identification of the bands.

After the third week, some changes were observed in all groups not only in vibrational frequency but also in percentage area for different bands for both groups.

Table 4 summarizes the bands that appeared in the fingerprint region after four weeks of the study for different groups. During this week, there was a clear improvement in the photodynamic therapy (PDT) group compared to the control group.

Table 3. Wavenumber and percentage area of bands at fingerprint region for different groups compared with the control group after the third week.

	Control	CNV.	Avast.	PDT.
str. C=O	1724.37 ± 1.81	1702.71 ± 4.74	1685.92 ± 2.25	1700.61 ± 35.81
	5.11	12.55	10.17	10.72
Amide I	1642.78 ± 0.17	1640.43 ± 3.08	1648.81 ± 4.48	1640.93 ± 2.71
	47.69	53.47	41.87	49.91
Amide II	1540.85 ± 0.33	1539.03 ± 2.2	1545.29 ± 1.41	1554.11 ± 9.2
	20.76	11.22	18.46	16.15
str. C-C	1401.57 ± 3.32	1424.59 ± 6.81	1405.71 ± 5.69	1448.91 ± 2.4
	9.83	10.42	10.98	10.5
str. C-N	1322.44 ± 1.08	1316.46 ± 1.23	1328.38 ± 7.82	1402.45 ± 3.12
	0.64	4.33	2.46	8.04
asym. PO_2	1249.03 ± 2.22	1256.98 ± 3.41	1249.76 ± 3.54	1250.98 ± 3.51
	8.76	4.56	9.37	1.48
str. C-N	1083.79 ± 0.35	1083.52 ± 3.72	1076.58 ± 2.48	1087.37 ± 2.79
	7.21	3.45	6.69	3.2

First line in each cell indicates the frequency of the band, while second line reflects the percentage area.

In Avast group, seven bands were formed as in the control group with a slight difference in the vibrational frequency of some bands compared to the control group which centered nearly at 1081.37 ± 0.39 cm-1, 1249.23 ± 0.62 cm^{-1}, 1316.70 ± 6.19 cm^{-1}, 1427.10 ± 3.17 cm^{-1}, 1547.4 ± 2.0 cm^{-1}, 1638.82 ± 0.62 cm-1, 1692.98 ± 2.18 cm-1 which correspond to str. C-N, asym.PO2, str.C-N, str.C-C, Amide II, Amide I, and str.C=O respectively.

Table 4: Wavenumber and percentage area of bands at fingerprint region for different groups compared with the control group after four weeks.

	Control	CNV.	Avast.	PDT.
str. C=O	1724.37 ± 1.81	1726.29 ± 1.73	1692.98 ± 2.18	1705.53 ± 1.82
	5.11	10.38	6.83	11.31
Amide I	1642.78 ± 0.17	1641.64 ± 0.12	1638.82 ± 0.62	1640.51 ± 1.67
	47.69	54.09	49.94	45.95
Amide II	1540.85 ± 0.33	1542.44 ± 0.12	1547.4 ± 2.0	1541.55 ± 2.87
	20.76	12.33	19.28	18.76
str. C-C	1401.57 ± 3.32	1429.94 ± 2.6	1427.10 ± 3.17	1469.37 ± 1.67
	9.83	5.91	10.62	2.7
str. C-N	1322.44 ± 1.08	1312.07 ± 10.2	1316.70 ± 6.19	1418.22 ± 5.68
	0.64	2.95	1.33	11.95
asym. PO_2	1249.03 ± 2.22	1237.19 ± 6.49	1249.23 ± 0.62	1247.59 ± 2.45
	8.76	6.47	8.4	5.22
str. C-N	1083.79 ± 0.35	1085.71± 0.51	1081.37 ± 0.39	1081.80 ± 1.08
	7.21	7.87	6.6	4.11

First line in each cell indicates the frequency of the band, while second line reflects the percentage area.

Figure 4 summarizes the bands that appeared in the fingerprint region after four weeks of the study for different groups. During this week, there was a clear improvement in the photodynamic therapy (PDT) group compared to the control group. In Avast group, seven bands were formed as in the control group with a slight difference in the vibrational frequency of some bands compared to the control group which centered nearly at 1081.37 ± 0.39 cm^{-1} 1249.23 ± 0.62 cm^{-1}, 1316.70 ± 6.19 cm-1, 1427.10 ± 3.17 cm^{-1}, 1547.4 ± 2.0 cm^{-1}, 1638.82 ± 0.62 cm^{-1}, 1692.98 ± 2.18 cm^{-1} which correspond to str. C-N, asym.PO^2, str.C-N, str.C-C, Amide II, Amide I, and str.C=O respectively.

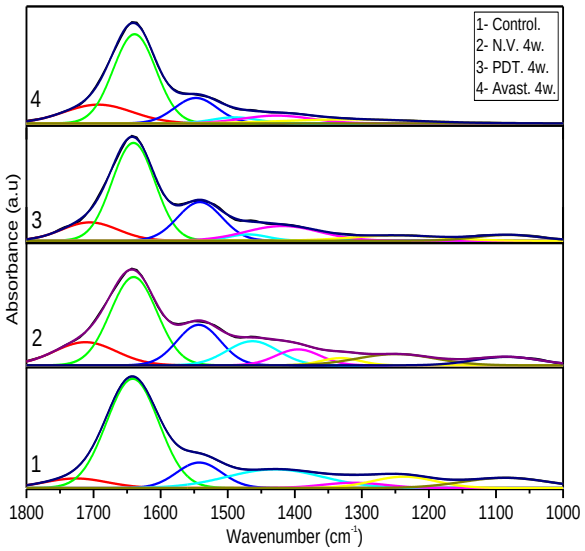

Figure 4. FTIR spectra in the Fingerprint region for all groups after four weeks with compared to the control group, showing the estimated underlying peaks. The numbers to facilitate the identification of the bands.

IV. DISCUSSION

The purpose of this study is to report the efficacy of treatment of experimentally corneal NV in rabbits by using photodynamic therapy as compared with subconjunctival injection of Avastin. In the present study, the change in corneal protein after induction of NV is evaluated. The results indicate elevated levels of corneal protein percentage above the baseline of the control (28.25%.) Furthermore, decreasing in protein content is observed during the next four weeks after exposure to PDT and treatment with Avastin due to regression of corneal NV. These results suggest that, the induction of corneal NV caused corneal hypoxia, inducing the formation of new vascular growth by increasing the activity of VEGF (**The Association for Research in Vision and Ophthalmology ARVO, 2008**). This process is associated with an enhanced formation of blood vessels containing blood component, leading to elevated levels of protein content of the cornea and therefore changing in its SDS-electrophoresis pattern. Furthermore, once rose bengal is activated by argon laser in the presence of oxygen, highly reactive, short-lived singlet oxygen and ROS are generated. Laser

activation of rose bengal resulted in local damage to neovascular endothelium, vessel occlusion, leading to gradual improvement in protein content and in electrophoresis patterns during the next four weeks after PDT, indicating the suppression of vessels growth and the survival of corneal tissue. It is well known that various antioxidants have an additive effect, protecting the organism from free radicals according to **Wayner et al., 1987** [29].

V. CONCLUSION

Corneal angiogenic factors include vascular endothelial growth factor (VEGF), with studies showing that VEGF activation can induce corneal NV, and that inhibition of VEGF can block new vessel formation in animal cornea model. Anti-VEGF therapy is considered as a possible tool for controlling NV. Bevacizumab (Avastin) showed an inhibitory effect on CNV in rabbits' eyes. Photodynamic therapy (PDT) with a photosensitizer can induce microvascular thrombosis with minimal damage to the surrounding normal tissue. Activation of the photosensitizer (rose bengal) by argon laser energy releases highly reactive, cytotoxic, short-lived singlet oxygen and other reactive oxygen radicals. This leads to endothelial cell damage and vascular leakage, coagulation, and thrombocyte aggregation, which trigger neovascular vessel occlusion. PDT with rose bengal is also effective in decreasing the corneal blood vessels. Final results of all measurements showed a gradual improvement over the different periods and a significant similarity in the efficacy of both argon laser and vascular chemotherapy of the rabbit cornea using avastin.

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