Experimental research

Comparative study of the efficacy of bevacizumab and rose bengal photodynamic therapy for treatment of corneal neovascularization

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Abstract

Introduction: To compare the efficacy of bevacizumab and rose bengal photodynamic therapy (RB-PDT) in the treatment of corneal neovascularization (CNV).
Material and methods: The study design included the induction of CNV by suture placement in three groups of New Zealand rabbits: (1) a group with CNV without any treatment; (2) a group treated with subconjunctival injection of bevacizumab (25 mg/eye); (3) a group treated with intravenous injection of rose bengal and exposed to 532 nm photodynamic therapy. For 4 weeks, the animals were followed up by slit-lamp to analyze the extent of CNV, evaluate the corneal protein secondary structure, and determine the oxidative stress index (OSI).
Results: After 4 weeks, traces of neovascularization were observed only in the bevacizumab treated group with grade 0.5. The contents of α-helix and β-sheet were 17% and 61% in CNV, 32%, and 46% in bevacizumab and 40% and 36% in RB-PDT groups vs. 43% and 35% for the control group. Moreover, the percentage changes in the total oxidative status (TOS) for CNV, bevacizumab and RB-PDT groups were 97.1%, 14.6%, and 1.0%, respectively, with respect to the control. The total antioxidant status (TAC) showed no significant changes ($p > 0.05$) for both treated groups. The percentage of changes in OSI was 15.9% and 1.3% in bevacizumab, and RB-PDT treated groups compared with the control group.
Conclusions: Both modes of treatment were effective in the regression of CNV, but RB-PDT was more efficient than bevacizumab by improving the corneal protein secondary structure and the oxidative stress.

Key words: corneal neovascularization, bevacizumab, photodynamic therapy, protein secondary structure, oxidative stress.

Introduction

Corneal avascularity and clarity are critically vital for preserving eyesight, and the evolution of treatments for neovascular ocular diseases is crucial. Corneal neovascularization (CNV) arises as a result of the imbalance between angiogenic factors such as vascular endothelium growth factor (VEGF) and antiangiogenic factors (i.e., angiostatin, endostatin, or pigment epithelium-derived factor) [1, 2]. Many factors, such as in-

flammation, infection, ischemia, loss of the limbal stem cell barrier, and trauma, can induce CNV [1]. Furthermore, it is usually responsible for scarring, edema, deposition of lipid, and inflammation that may reduce visual acuity and damage the immune system of the cornea [3, 4]. Treatments of CNV with anti-inflammatory agents, steroid or non-steroid, laser photocoagulation, and fine-needle diathermy, have limited clinical success and did not precisely target the mediators of angiogenesis [1, 5]. Also, photodynamic therapy (PDT) combined with intravenous verteporfin injection regresses pre-existing CNV [6, 7].

VEGF initiation can induce CNV, and its inhibition can prevent the progression in humans and animal models [4, 8]. Bevacizumab (Avastin; Genentech Inc., San Francisco, CA, USA) is a VEGF inhibitor, which is a full-length monoclonal antibody. It is widely used in combination with cytotoxic chemotherapy in several types of cancer and in the treatment of choroid and iris neovascularization, central retinal vein occlusion, proliferative retinopathy, and neovascular age macular degeneration (AMD) [9–13]. Moreover, subconjunctival and topical bevacizumab has been regarded as a new treatment for CNV by decelerating new blood vessel formation [14, 15].

Rose bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodo-fluorescein disodium or, RB) is a sodium salt ($C_{20}H_4Cl_4I_4O_5$.2Na), used in detecting specific medical problems, such as the cornea, conjunctiva and lid disorders, liver and eye cancer, skin conditions such as melanoma, eczema, and psoriasis [16–18].

This study attempted to assess the improvement of CNV with subconjunctival injection of bevacizumab and rose bengal photodynamic therapy (RB-PDT) through slit-lamp examination, the analysis of corneal protein secondary structure by Fourier-transform infrared spectroscopy (FTIR) and the oxidative stress index.

Material and methods

Animals

Forty-two New Zealand male rabbits (aged 2.5–3 months and weighing 2.5–3 kg) were kept in a standard 12 h light/dark cycle with a balanced diet and free access to water. All experimental procedures were consistent with the principles articulated in the Guide for Care and Use of Laboratory Animals (NIH publication no. 85-23). All animals' eyes were observed by slit-lamp biomicroscope before inducing CNV to indicate no signs of edema or inflammation.

Corneal neovascularization

Six rabbits (n = 12 eyes) served as a control group, and the remaining 36 rabbits (n = 72 eyes) were systematically anesthetized by intramuscular injection with Xylaject (0.2 ml/kg) and ketamine hydrochloride (0.6 ml/ kg). Proparacaine eye drops (Alcaine; Alcon, Fort Worth, TX, USA) were used for topical anesthesia. Three interrupted silk sutures (7-0, Sofsilk; Syneture, Quebec, Canada) at mid-stromal depth about 1 mm from the limbus were applied (Figure 1 B). Anti-inflammatory eye drops (Diclofenac sodium 1%, Bausch & Lomb Incorporated, USA) were used three times daily for four days to prevent eye inflammation and pain. CNV was examined using a slit-lamp with standard magnification (25×) and photographed. The extent of CNV was evaluated by a semi-quantitative method using a scoring system with grades 0-4 to describe the maximum extent of the CNV [19].

Bevacizumab injection and photodynamic therapy

Twelve rabbits with CNV were left without treatment, and the remaining 24 rabbits were anesthetized and divided into two groups. In one group (n = 24 eyes) rabbits were subjected to subconjunctival injection of 5 mg (200 µl/eye) of bevacizumab (25 mg/ml, Avastin, Roche Pharma AG, Germany) [20]. The other 12 rabbits (n = 24 eyes) were injected in the central ear vein with 40 mg/kg of RB (Sigma Chemical Co., St. Louis, MO). After 15 min, ten shots from a 532 nm doubled frequency Nd-YAG laser (Quantel-Medical, Vitra, France) were applied to the vascularized portion of the cornea in a pulsed mode, a spot size of 60 µm, pulse duration of 0.2 ms and power of

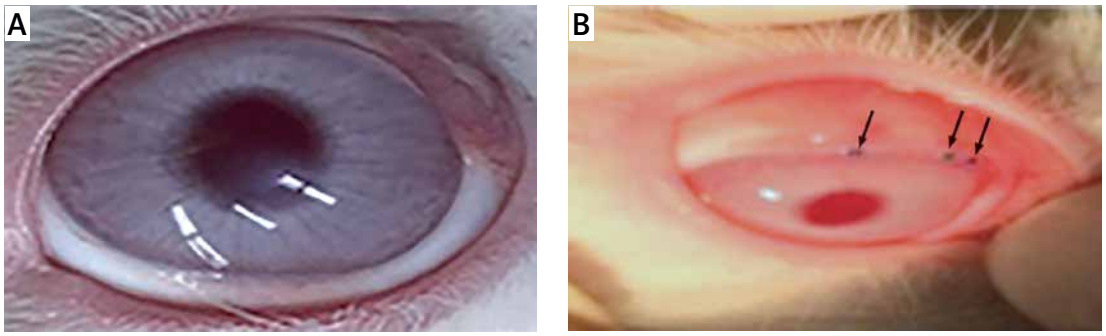

Figure 1. Slit-lamp photography for control cornea (**A**) and after sutures (**B**)

150–200 mW/cm^2, as described previously [21]. For 4 successive weeks, 3 rabbits were selected from each group (n = 6 eyes), sacrificed, and the corneas were removed for analysis.

Fourier transform infrared spectroscopy analysis for corneal protein secondary structure

Parts of control and NV corneas were lyophilized and pressed with potassium bromide (KBr) with (98 mg KBr: 2 mg cornea) to form the disks for FTIR analysis. The recording of FTIR spectra was obtained in the range of 1700–1600 cm^{-1} for the amide I band using a Thermo Nicolet iS5 FTIR spectrometer (Thermo Electron Scientific Instruments LLC, Madison, WI USA) and analyzed using Origin Pro 9.0 software (Origin Lab Corporation; Northampton, MA 01060, USA).

The rest of the corneal samples from all groups were weighed and homogenized in 20 mM ice-cold Tris-HCl buffer at pH 7.4 (10-fold volume) using a cell homogenizer (Tübingen 7400, Germany) and centrifuged at 10,000 rpm for 20 min (Awel centrifuge MS 20, Awel International, Blain, France). The supernatant was used for measurements of total antioxidant status (TAS), total oxidative status (TOS), and oxidative stress index (OSI).

Measurement of total antioxidative status

The TAS was determined by the interaction of antioxidants in 20 µl of the resultant supernatant with 0.5 ml of hydrogen peroxide (H_2O_2). The amount of antioxidants in the samples was determined enzymatically through the conversion of 3,5,dichloro-2-hydroxy benzenesulfonate to a colored product (Biodiagnostic, Giza, Egypt). This enzymatic reaction was measured using a spectrophotometer at 505 nm (Evo 600, Thermo Fisher Scientific, Madison, WI USA) [22].

Measurement of total oxidative status

The TOS of the corneal samples was measured using a colorimetric method (Biodiagnostic, Giza, Egypt). The oxidative molecules present in 50 µl of the supernatant react with 3,5-dichloro-2-hydroxy benzene sulfonate (0.5 ml, 1.0 mM/l) and 4-aminoantipyrine (0.5 ml, 2.0 mM/l) in the presence of peroxidase (> 2000 U/l) to form a chromophore measured at 510 nm that correlated with the sum of oxidative molecules existing in the corneal samples [23]. The OSI value was documented as follows: OSI = TOS (mM/g tissue)/TAS (mM/g tissue) [6, 24].

Statistical analysis

A commercial statistical software program (SPSS 17.0 for Windows; SPSS, Inc, Chicago, Illinois, USA) was used for statistical analysis. The results were analyzed using Student's t-test, expressed as the mean ± standard deviation (SD), and assumed statistically significance at a level of $p < 0.05$.

Results

Slit-lamp examination

Slit-lamp examination for control rabbit showed clear cornea without any vascularization, as illustrated in Figure 1 A and with sutures (1 B). After removing sutures, the existence of CNV was classified according to their extension from the limbus to the cornea surface from grade 0 to 4 (Figure 2). The CNV showed marked progression with grade 3 after 1 week and 2 weeks and significantly increased to grade 4 (severe) after the 3rd and 4th weeks. The extent of CNV treated with bevacizumab showed a dramatic decrease in the grades with scores of 3 (marked), 2 (moderate), 1 (mild), and 0.5 (traces) after 1, 2, 3, and 4 weeks, respectively. In contrast, with RB-PDT, the extent of CNV showed better improvement and indicated approximately complete recovery after 4 weeks with grade 0 (nil).

Amide I region

The FTIR spectra for the corneal samples were directly correlated with the structural properties of the amide I region at 1700–1600 cm^{-1}. The curve enhancement procedure resolves the contour of the control amide I band into three structural components (Figure 3) that centered at 1678 cm^{-1}, 1647 cm^{-1}, and 1613 cm^{-1} corresponding to β-turn, α-helix, and β-sheet, respectively. Table I summarizes the wavenumbers for the bands that appeared in the amide I region during 1, 2, 3, and 4 weeks for all groups.

The area percentages of the β-turn components relative to the total band area are illustrated in Table I and Figure 4. The CNV group showed slight changes after two and 3 weeks with values of 19% ($p < 0.05$) and 18% ($p < 0.05$) compared with 22% for the control group. The α-helix showed a significant change ($p < 0.05$) in the vibrational frequency (1659 cm^{-1}) after 4 weeks, and β-sheet showed a significant change ($p < 0.05$) after the third and fourth week (Table I) with values of 1627 cm^{-1} and 1628 cm^{-1}. Furthermore, the content of the α-helix in the control was 43%, which showed a progressive reduction due to CNV to 39%, 23%, and 17% after 2, 3, and 4 weeks, respectively (Figure 5). Moreover, there was a noticeable increase in the contents of β-sheet from 35% for the control to 43%, 58%, and 61% after 2, 3, and 4 weeks, respectively.

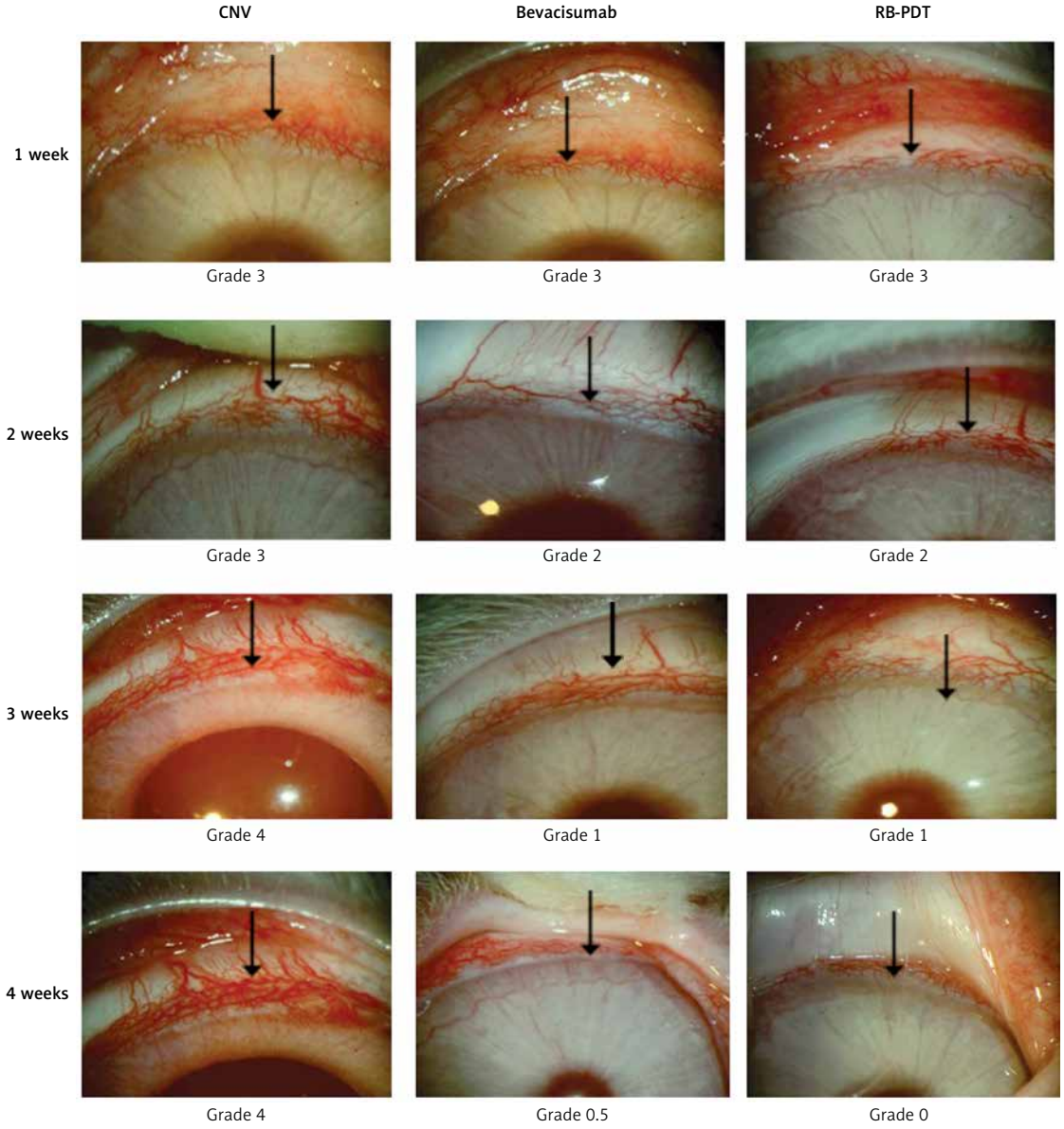

Figure 2. Slit-lamp photography for CNV group, group treated with bevacizumab, and group exposed to RB-PDT, showing the extent of neovascularization from grade 0-4. Grade 0 (nil), grade 1 (mild), grade 2 (moderate), grade 3 (marked) and grade 4 (severe)

Treatment of CNV with bevacizumab showed a noticeable improvement in the wavenumber of α-helix (Table I) compared with the control. There was a progressive reduction in the content of α-helix with values of 41%, 35%, 34% and 32% (Figure 5) associated with a gradual increase in the β-sheet content with values of 40%, 42%, 44%, 46% ($p < 0.01$) after 1, 2, 3, and 4 weeks, respectively, as shown in Figure 6.

Furthermore, pronounced improvement was observed in the vibrational frequencies and area % of amide I region after RB-PDT, which was better than that in the bevacizumab treated group (Table I). The α-helix structure (Figure 5) showed considerable improvement in the content that started after 3 weeks and 4 weeks of RB-PDT with values of 31% and 40% ($p < 0.05$), while the β-sheet contents (Figure 6) were changed with values of 42% ($p < 0.05$), 59% ($p < 0.01$), 46% ($p < 0.01$%) and 36% ($p > 0.05$) after 1, 2, 3, and 4 weeks, respectively.

Oxidative stress markers

The TOS was significantly increased in the CNV to 40.6 ±0.8 mM/g tissue ($p < 0.001$) after 4 weeks compared with 20.6 ±0.2 mM/g tissue for the control, with a percentage change of 97.1% (Table II). Treatment with bevacizumab induced a significant improvement in the TOS after four weeks (23.6 ±0.16 mM/g tissue), with a percentage change of 14.6%, ($p < 0.01$). Interestingly, the improvement in TOS was more pronounced after treatment with

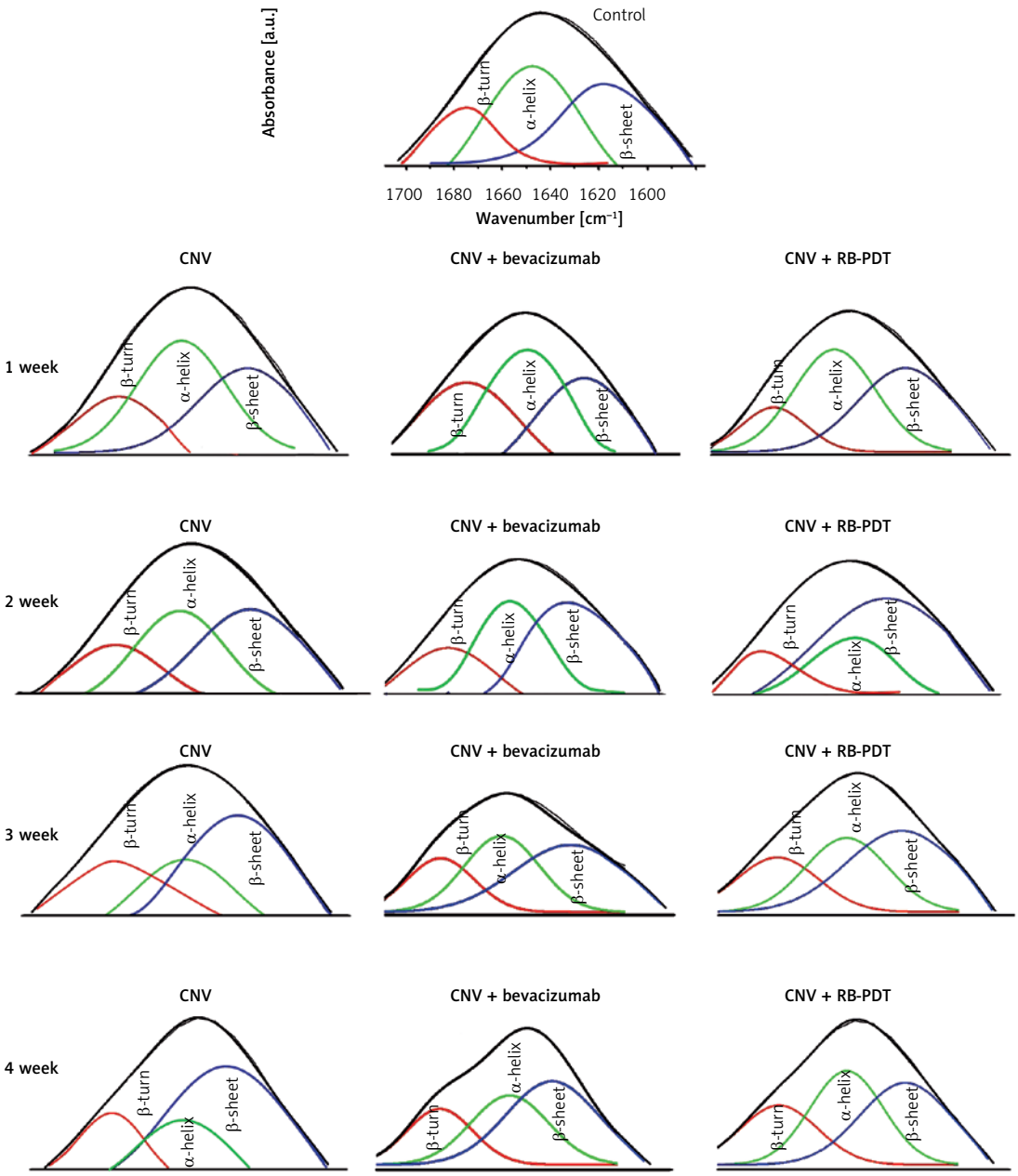

Figure 3. FTIR spectroscopy for rabbit's cornea in the amide I region (1700–1600 cm^{-1}) showing three underlying components for β-turn, α-helix and β-sheet for control group, CNV group, group treated with bevacizumab, and group exposed to RB-PDT

RB-PDT than bevacizumab ($p > 0.05$), with a percentage change of 1.0%.

In contrast, the TAS after the first 2 weeks of CNV induction was increased to 190 ±4 ×10^{-4} mM/g tissue ($p < 0.05$) and 215 ±7 × 10^{-4} mM/g tissue ($p < 0.01$), with a percentage change of 10.4% and 25.0% compared with the control. These increments were followed by apparent decreases after the 3rd and 4th week, with values of 200 ±6 × 10^{-4} mM/g tissue (16.03%, $p < 0.01$) and 138 ±4 × 10^{-4} mM/g tissue (–19.0%, $p < 0.01$). Moreover, both bevacizumab and RB-PDT groups showed no significant changes ($p > 0.05$) in TAS after 4 weeks, with percentage changes of –1.2% and –2.3%, respectively.

OSI for the control was 1198 ±23 and defined as the ratio of the TOS level to TAS, as summarized in Figure 7. In the CNV group the OSI was shifted towards very high oxidative status with values of 1547 ±48, 1544 ±42, 1990 ±44 and 2942 ±50 after 1, 2, 3 and 4 weeks, respectively. At the end of the 4th week, both bevacizumab and RB-PDT groups showed high efficacy compared with the control group, with percentage changes of 15.9% and 1.3%, respectively.

Table I. Wavenumbers (cm^{-1}) of the resolved contour for amide I region for control cornea, CNV, CNV + bevacizumab, and CNV + RB-PDT after 1, 2, 3, and 4 weeks

Groups	Periods	Amide I region (1800–1600 cm^{-1})		
		β-turns [cm^{-1}]	α-helix [cm^{-1}]	β-sheet [cm^{-1}]
Control	–	1678 22%	1647 43%	1613 35%
CNV	1 week	1673 ±6 21%	1647 ±1 45%	1616 ±2 35%
	2 weeks	1677 ±7 19%	1649 ±2 39%	1619 ±7 43%
	3 weeks	1673 ±1 18%	1645 ±8 23%	1627 ±5* 58%
	4 weeks	1680 ±6 23%	1659 ±7* 17%	1628 ±2* 61%
CNV + bevacizumab	1 week	1669 ±10 19%	1645 ±3 41%	1620 ±5 40%
	2 weeks	1676 ±3 23%	1649 ±3 35%	1619 ±6 43%
	3 weeks	1676 ±5 22%	1646 ±4 34%	1622 ±1 44%
	4 weeks	1673 ±5 22%	1646 ±4 32%	1627 ±6* 46%
CNV + RB-PDT	1 week	1673 ±4 19%	1648 ±2 44%	1619 ±2 42%
	2 weeks	1673 ±2 17%	1648 ±7 24%	1625 ±4 59%
	3 weeks	16 75 ±5 23%	1646 ±4 31%	1623 ±2 46%
	4 weeks	1674 ±4 24%	1646 ±9 40%	1615 ±3 36%

**Statistically significant.*

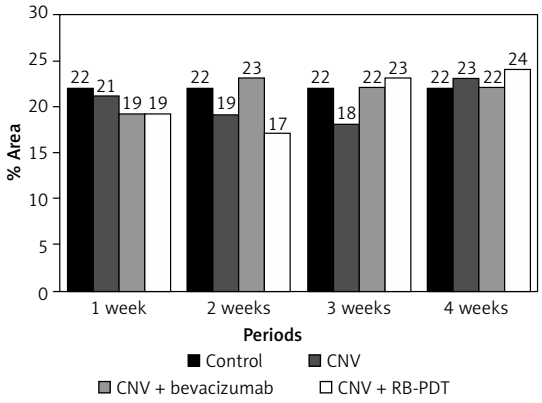

Figure 4. Content of β-turn in amide I of rabbit corneal protein for control group, CNV group, group treated with bevacizumab, and group exposed to RB-PDT

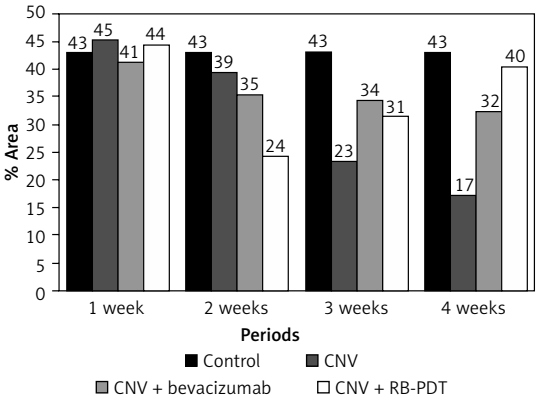

Figure 5. Content of α-helix in amide I of rabbit corneal protein for control group, CNV group, group treated with bevacizumab, and group exposed to RB-PDT

Discussion

In the current study, the alteration in corneal protein secondary structure was evaluated by FTIR analysis for the amide I region. The CNV group showed a progressive reduction in α-helix content (17%) associated with an elevation in the content of β-sheet to 61% after 4 weeks.

There are two types of β-sheet, called intermolecular β-sheet (< 1620 cm^{-1}) and intramolecular β-sheet (1630–1620 cm^{-1}) [25]. It has been proposed that the insolubility of protein is relat-

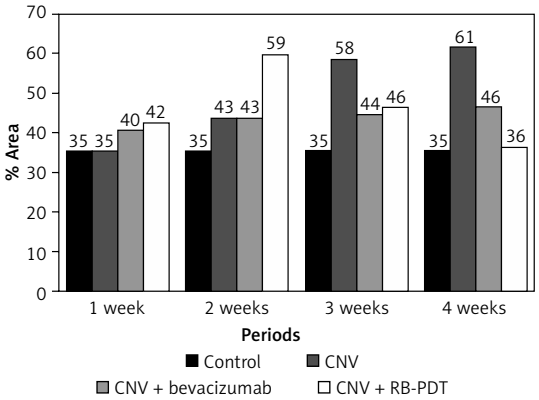

Figure 6. Content of β-sheet in amide I of rabbit corneal protein for control group, CNV group, group treated with bevacizumab, and group exposed to RB-PDT

ed to the quantity of β-sheet, the increase in the β-sheet structures, and the increase in protein insolubility [27].

Thus, the obtained results indicate the existence of intermolecular β-sheets after 1 and 2 weeks of CNV (at wavenumbers of 1616 and 1619 cm^{-1}) which changed to intramolecular β-sheet interactions after the 3rd and fourth week (at wavenumbers of 1627 and 1628 cm^{-1}). The establishment of an intramolecular hydrogen-bonded β-sheet structure indicated the formation of more folded insoluble proteins, protein aggregation, or the creation of new protein with different structural configurations.

On the other hand, the induction of CNV caused corneal hypoxia, thus increasing the activity of the VEGF protein that initiates angiogenesis and is thought to be a negotiator in the progression of neovascularization [5, 26]. Moreover, VEGF protein will disturb the metabolism of extracellular matrix and increase the expression of the urokinase-type plasminogen activator gene (uPA) in corneal epithelium. This uPA plays a part in cell immigration, cell bonding, tissue reorganization, and structural changes in tissue layers [27].

The present study indicated a gradual improvement in the content of α-helix and β-sheet during the next 4 weeks in both groups as observed by slit-lamp examination. This improvement was not wholly achieved in the bevacizumab group, as shown by the presence of traces of vascularization (grade 0.5) on the cornea after 4 weeks (Figure 2), and consequently agrees with the previous report, which indicated that bevacizumab alone could not eliminate established blood vessels [28]. Nevertheless, the injection of bevacizumab seemed to be effective in suppressing new vessel formation as observed by slit-lamp examination (Figure 2). This result agrees with previous successful results from using anti-VEGF therapy in many experimental animal models to reduce chemically induced CNV and improve the visual function and corneal clarity [15, 29]. Furthermore, the improvement is better in RB-PDT than the bevacizumab group, indicating the complete deterioration of CNV (grade 0). In the bevacizumab group, the percentage change in α-helix content was 25.2% ($p < 0.01$) and in β-sheet was 31.4% ($p < 0.001$) while in the RB-PDT group, the percentage change in α-helix was 6.9% ($p < 0.05$) and in β-sheet contents was 5.8% ($p < 0.05$) after 4 weeks with respect to the control group, respectively.

In PDT, the interaction of the RB with surrounding molecules after irradiation by green light la-

Table II. Total oxidative capacity (TOC) and total antioxidant capacity (TAC) for the control cornea, CNV, CNV + bevacizumab, and CNV + RB- PDT after 1, 2, 3, and 4 weeks

Groups	Period	TOC		TAC	
		Mean ± SD [mM/g tissue]	% change	Mean ± SD [10^{-4} mM/g tissue]	% change
Control	–	20.6 ±0.2	–	172 ±5	–
CNV	1 week	29.4 ±0.6*	42.7	190 ±4*	10.4
	2 weeks	33.2 ±0.2*	61.2	215 ±7*	25.0
	3 weeks	39.8 ±0.3*	93.2	200 ±6*	16.3
	4 weeks	40.6 ±0.8*	97.1	138 ±4*	–19.0
CNV + bevacizumab	1 week	31.3 ±0.12*	51.9	183 ±1*	6.4
	2 weeks	35.1 ±0.04*	70.3	196 ±4*	14.0
	3 weeks	27.2 ±0.07*	32.0	181 ±1*	5.2
	4 weeks	23.6 ±0.16*	14.6	170 ±3	–1.2
CNV + RB-PDT	1 week	30.4 ±0.17*	47.6	186 ±9*	8.0
	2 weeks	34.8 ±0.14*	68.9	210 ±2*	22.1
	3 weeks	28.1 ±0.04*	36.4	180 ±5	4.7
	4 weeks	20.38 ±0.06	–1.0	168 ±3	–2.3

**Statistically significant.*

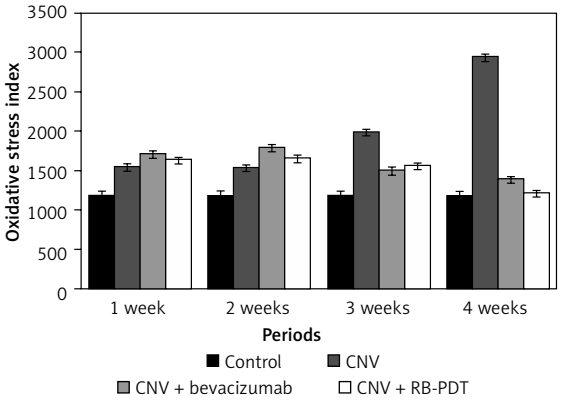

Figure 7. Oxidative stress index (OSI) of rabbit cornea for control group, CNV group, group treated with bevacizumab, and group treated with RB-PDT

ser (532 nm) induces photooxidative reactions, producing intermediate free radicals. These intermediates will react with oxygen to form hydroxyl radicals, superoxide ions, peroxides, and/or reactive oxygen species (ROS), the most substantial reactive species in PDT-intermediated cytotoxicity, which selectively occlude the target vessels by the formation of thrombus [21]. RB-PDT can induce severe endothelium damage leading to numerous biochemical alterations, such as DNA damage, protein cross-linking, protein cleavage, and phagocytic activity [30]. Therefore, the restoration in α-helix (Figure 5) and β-sheet (Figure 6) indicates an excellent regression rate and successful PDT.

The pathology and physiology of many ocular diseases have been associated with oxidative stress [31]. Oxidative stress ensues when the amounts of vitamins and antioxidant enzymes are decreased, and the amounts of protein and lipid oxidation products are increased [32]. Consequently, antioxidative defense systems, for instance, intracellular superoxide dismutase (SOD), glutathione peroxidase, ferritin, ceruloplasmin, albumin, catalase, ascorbic acid, reduced glutathione, β-carotene, and food-derived antioxidant, will overcome the ROS potential toxicity.

In this study, CNV induced a significant decrease in the TAS (–19%) and an increase in TOS (97.1%) after 4 weeks, causing a change in OSI by 145.5%. Furthermore, both bevacizumab and RB-PDT stimulate the antioxidant defense system after 1 and 2 weeks because they induce more oxidative stress and strong cytotoxicity to neovascular endothelium. Moreover, gradual improvements in TAS and TOS after the third and fourth weeks were obtained. Furthermore, OSI moved towards an antioxidative state in both bevacizumab and RB-PDT, with marked variation in their percentage changes with values of 15.9%, and 1.3% concerning the control, indicating the inhibition of vascularization and the curing of the cornea with time related to cell death.

Based on all the above-obtained data, CNV was controlled to a reasonable extent in the bevacizumab-treated group. In comparison, RB-PDT is considered a powerful cytotoxic technique, which efficiently activates the treatment of CNV by inducing a better recovery in corneal protein secondary structure and the oxidative state than in the bevacizumab-treated group. Furthermore, the analysis of protein secondary structure and oxidative stress markers provides useful indicators for assessing the degree of efficacy of both therapeutic methods against CNV.

Conflict of interest

The authors declare no conflict of interest.

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