Therapeutic Effects of Extracts from *Spirulina platensis* versus Bevacizumab on Inflammation-Associated Corneal Neovascularization

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**Abstract**

**Aim:** The aim of this work was to study histological and immunohistochemical changes resulting from corneal alkali burn and to assess these changes after treatment with either polysaccharide extract from *Spirulina platensis* (PSP) or Bevacizumab.

**Methods:** Alkali burn was induced in corneas by direct application of a filter paper ring (2.5 mm diameter) saturated with 1 N NaOH and applied to the center of the rat corneas for 30 seconds. PSP (extracted from dry powder of *Spirulina platensis*) and avastin were administered topically on the corneas 4 times daily for 7 days starting 7 days after induction of corneal alkali burn. The therapeutic effects of PSP and avastin were evaluated daily using slit-lamp. At the end of the therapy, corneas were harvested for H and E staining, Masson trichrome staining, immunohistochemical study for assessment of inflammation and corneal neovascularization (CorNV).

**Results:** Topical application of either PSP extract or Avastin had significant therapeutic effects on corneal injuries induced by alkali burn. This was demonstrated by decreasing the CorNV, total and differential corneal thickness and inflammatory cellular responses. They increased the epithelial thickness. PSP showed better results than Avastin in this regards.

**Conclusion:** The naturally occurring *Spirulina platensis* (PSP) is more cost effective in treatment of corneal neovascularization and decrease inflammation and fibroblast activity in a rat model of corneal neovascularization induced by alkali burn as compared to Avastin.

**Keywords:** *Spirulina platensis*, Bevacizumab; Avastin; Corneal Neovascularization; Inflammation; Immunohistochemical changes

**Introduction**

Chemical trauma of the eye can range from mild irritation to the severe damage of ocular surfaces such as the conjunctiva, cornea, and anterior segment. Such injury has the possibility to result in permanent vision loss. Chemical burns of the cornea cause both superficial and deep neovascularization [1-3] which can lead to significant diminution of vision due to scar formation and lipid deposition [4]. Regarding the effect of strong alkalis, ocular tissues have a limited ability to be protected against such burns, which denature proteins and saponify lipids.

Neovascularization is a poorly understood pathologic response of the cornea against chronic inflammation which caused by infection, sterile corneal ulceration, chemical or thermal trauma, or the immune rejection of corneal grafts. Following chemical burns, inflammatory cells such as polymorphonuclear leukocytes, and mesenchymal cells such as myofibroblasts, activated keratocytes, macrophages, and neovascularization factors are stimulated. Some of the factors responsible for the encouragement of inflammation include vascular endothelial growth factor (VEGF), platelet-activating factor, transforming growth factors, basic fibroblast growth factor, and tumor necrosis factor-α [5-7]. It has been reported previously that VEGF is up regulated in corneas with inflammation and vascularization and that it is a significant angiogenic factor in corneal neovascularization [8,9].

Bevacizumab (avastin) is a monoclonal antibody that binds to VEGF-A and all its isoforms, its amino acid sequence contains both human IgG (93%) and murine antibody (7%). It has been accepted as an antiangiogenic pharmaceutical for the treatment of certain types of cancers and it has also been used to treat ocular neovascularization [10]. In numerous studies it has been reported that topical or subconjunctival Bevacizumab application results in the inhibition or regression of corneal neovascularization [11-13].

*Spirulina platensis* (PSP) has been proven to possess multiple bioactivities including inhibition of tumor invasion and antiviral, antioxidant, chemoprotective and radioprotective properties [14-16]. To our knowledge, this study demonstrates for the first time that PSP has a strong inhibitory effect on inflammation-induced corneal neovascularization in comparison to Avastin which suggest the potential use of PSP in the treatment of inflammatory neovascularization-related corneal diseases.

The aim of this work was to study histological and immunohistochemical changes resulting from corneal alkali burn and to assess these changes after treatment with either polysaccharide extract from *Spirulina platensis* (PSP) or Bevacizumab.
Material and Methods

Experimental animals

In this study, 40 adult male albino rats of Sprague Dawley strain weighing 200-250 g and averaging 16 weeks old were utilized. They were obtained from the Animal house Ain shams University faculty of medicine. Animals were maintained according to the principle and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Food and tap water were available ad libitum. In the windowless animal area automatic temperature (21 ± 1°C) and lighting controls (12 h light/12 h dark cycle) were done. Humidity ranged from 55% to 60%. All animals received human care according to the principles defined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Corneal alkaline burn injury

Under general anesthesia with intraperitoneal Xylazine hydrochloride (5 mg/kg) (Rompun, Bayer, Turkey) and ketamine hydrochloride (50 mg/kg) (Ketalar, Eczacibasi, Turkey), the alkali burn CorNV model was established by instillation of topical anesthesia with a drop of 0.5% proparacaine hydrochloride (Alcaine eye drops; Alcon Inc., Fort Worth, TX) and direct application of a filter paper (2.5 mm diameter) was soaked with 1 N NaOH and applied to the corneal center for 30 s.

The burn stimulus response was scored by slitlamp inspection, as grade 0 (no blister), +1 (small blister), +2 (medium blister), or +3 (large blister), according to lesions detected on the corneal surface [18].

Following cauterization, the burn stimulus scores were immediately calculated and treatment was begun with gentamicin eye drops four times a day for one week.

Experimental design

The animals were classified into four groups, ten rats each, as the following:

- **Group 1 (control group):** 10 rats, were divided in to 2 sub-groups
  - **Sub group 1A:** 5 rats (10 eyes) to whom balanced saline solution (BSS) was administered topically on the corneas 4 times daily for 14 days
  - **Sub group 1B:** to assess drug toxicity without alkali burn: 5 rats (10 eyes) will be subdivided as the following:
    - Their (Right) Rt eye received Bevacizumab (Avastin) topically 4 times daily starting 7 days after induction of corneal alkali burn for 7 days
    - **Sub group 2B:** Their Lt eye received PSP extract was administered topically 4 times daily starting 7 days after induction of corneal alkali burn for 7 days
  
- **All experimental animals were carried out following the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Drug preparation and treatment protocol

- **Preparation of polysaccharide extract from Spirulina platensis (PSP):** PSP was prepared as described previously [19]. In brief, dry powder of was incubated with 95% (V/V) ethanol overnight, torrefied, and re-incubated in NaOH (pH 10.0) solution at 80 for 4-6 hours, then centrifuged and collected the supernatant, adjusted pH to 7.0, precipitated with 5% trichloracetic acid (TCA) at 4 overnight, centrifuged and collected the supernatant, then precipitated with 5% TCA for another 3 hours, centrifuged and the supernatant was precipitated with ethanol (1/5:V/V) at 4 overnight. The precipitate was washed twice with acetone and then was lyophilized in a freeze dryer, and stored at -20°C. This precipitate, mainly containing polysaccharides (PSP), was dissolved in normal saline and filtered with 0.22 μm filtration membrane and the stock polysaccharides concentration was measured using anthrone-sulfuric acid method. For topical application, the stock PSP was adjusted to a concentration of 2 mg/ml in sodium chloride eye drops compound.

- **Preparation of Bevacizumab eye drops:** Bevacizumab eye drops were prepared in the hospital pharmacy by dilution with normal saline (0.9%) under sterile situations to a concentration of 10 mg/ml and were kept at 4°C. The eye drops were stored at -20°C for up to 14 days; after opening, they were used within 1 day and kept at 4°C during that time. All procedures were performed by the same investigator.

Clinical monitoring: Assessment of corneal inflammation and neovascularization: A slit-lamp microscopy examination was performed under anesthesia and analgesia, on 1st day to assess the burn stimulus response, on the 7th day to evaluate the corneal neovascularization and on the 14th days to evaluate the therapeutic effects of PSP extract and Bevacizumab. Then we evaluated inflammation and neovascularization by image analysis as it is an objective tool using the stained sections [20,21].

Histological analysis

The rats were sacrificed on the 15th day using a high dose of Pentothal sodium (Pentothal, Abbott, Italy). The globes were then enucleated and fixed in 10% buffered formalin for 24 h. Corneas were then removed from the limbus and 5 μm thick paraffin sections were set and stained with hematoxylin and eosin (Figure 1) and maissen trichrome (MTC) (Figure 2), then were examined using light microscopy. Sections were evaluated by image analysis to demonstrate the corneal thickness, the amount of neovascularization, the quantity of inflammation, and the fibroblast activity. Image analysis was performed by an examiner who does not know the coding of the study groups to avoid bias.
Immunohistochemistry

Assessment of angiogenesis by immunohistochemistry of vascular endothelial growth factor (VEGF) and assessment of fibroblast activity using α-smooth muscle actin.

As paraffin sections were stained with avidin-biotin peroxidase for demonstration of cells immunoreactive to VEGF and α-smooth muscle actin and counterstained with HX (Figures 4 and 5). Antibodies against VEGF and α-smooth muscle actin were purchased from (Santa Cruz Biotechnology, USA).

Image analysis

Sections were examined by using Leica DM2500 microscope with built in camera (Weltzelar, Germany). All images were digitally acquired using an image analyzer Leica Q win V.3 program (Weltzelar, Germany) installed on a computer in histology department faculty of medicine Ain Shams University.

Ten different non-overlapping fields from ten different stained sections of ten different rats were examined in each group for measuring each of the following:

- Corneal epithelial layer thickness
- Substantia propria (stroma) layer thickness
- Descemet’s membrane (DM) layer thickness
- The total corneal thickness
- Mean Mononuclear cells infiltration count
- Active fibroblast cell infiltration count
- Corneal neovascularization (area% of brown color) using VEGF

All measurements were taken at high power field of magnification (400X), all data are collected, revised and subjected for Statistical Analysis.

Statistical analysis

Statistical analysis was done using (Statistica software, version 8). Quantitative variables were expressed as mean ± SD and qualitative variables were given as numbers and percentage. Student's t-test was used to assess the statistical differences regarding the quantitative variables whereas ANOVA test was used for the qualitative ones.

Results

By slit-lamp microscopy examination revealed that the mean burn stimulus score was (2.25 ± 0.79 µm) for group 2, (2.10 ± 0.64 µm) for group 3A and (2.15 ± 0.59 µm) for group 3B. No statistical differences were found between the different groups (p=0.52).

Group 1 B show no deleterious effect was seen on PSA on the cornea, exactly similar to avastin results.

Histological results

In hematoxylin and eosin stained sections, the superficial layer of stroma show aggregation of polymorphonuclear cell infiltrations noticed in group 2 with new vessel formation. These changes were few in group 3A and absent in group 3B (Figure 1). In Masson’s trichrome (MTC) sections, abnormal disorganized loose collagen fibers present in corneal stroma with new vessels in group 2, this disorganization was less observed in group 3A while group 3B apparently similar to the control group, also there is difference in the corneal thickness between the different studied groups (Figure 2). Immunohistochemical stained sections for assessment of neovascularization using VEVF showed positive immune reaction in group 2, group 3A presented weak reactions while group 3B showed negative reaction (Figure 4). α-smooth muscle actin stained sections showed positive brown stained fibroblasts in group 2, weak staining in group 3A and negative reaction in group 3B (Figure 5).
Table 1: Different corneal layer thickness (µm) in the different four studied groups.

As Fuentes-Julián et al. (2015) stated that one of the signs of inflammation is corneal edema with increased thickness, We studied the different corneal layer thickness in histological H and E (Figures 1 and 2) staining sections by Image analysis and we found that [22]:

Epithelial thickness in group 1 was statistically higher than in group 2 (p ≤ 0.001), and lower than in group 3A (p ≤ 0.001). However it was not statistically different from group 3B (p=0.16). Also group 3A was statistically higher than in group 2 (p ≤ 0.001) and group 3B was statistically higher than in group 2 (p ≤ 0.001). Also group 3A was statistically higher than in group 3B (p=0.02) (Table 1)

Stromal thickness in group 1 was statistically lower than in group 2 (p ≤ 0.001), group 3A (p ≤ 0.001) and group 3B (p=0.02). Also group 2 was statistically lower than in group 3A (p≤0.001) and group 2 was statistically lower than in group 3B (p ≤ 0.001). Also group 3A was statistically higher than in group 3B (p ≤ 0.001) (Table 1)

DM thickness in group 1 was statistically lower than in group 2 (p ≤ 0.001), but there is no statistical different between group 1 and group 3A (p=0.09) and group 3B (p=0.91). Also group 2 was statistically higher than in group 3A (p ≤ 0.001) and group 2 was statistically higher than in group 3B (p ≤ 0.001). Also group 3A was statistically higher than in group 3B (p=0.04) (Table 1)

Finally, the total corneal thickness in group 1 was statistically lower than in group 2 (p ≤ 0.001), but there is no statistical different between group 1 and group 3A (p=0.10) and group 3B (p=0.11). Also group 2 was statistically higher than in group 3A (p ≤ 0.001) and group 2 was statistically higher than in group 3B (p ≤ 0.001). Also group 3A was statistically higher than in group 3B (p=0.04) (Table 1)

Corneas of eyes with alkali burn, possessed more new vessels, while that treated with Bevacizumab group, and that treated Spirulina platensis (PSP) group with statistical significant difference between group 2 and group 3A (p≤0.03), group 2 and group 3B (p ≤ 0.001) and group 3A and group 3B (p=0.01) (Table 2).

Histological H and E staining showed that the corneas of eyes with alkali burn, showed more mononuclear cells infiltration in the corneal stroma than did corneas treated with Bevacizumab and PSP, with statistical significant difference between group 2 and group 3A (p ≤ 0.001 ), group 2 and group 3B (p ≤ 0.001) and group 3A and group 3B (p=0.01) (Table 2).
**Table 2: Corneal neovascularization and different inflammatory cell infiltration in the different four studied groups.**

<table>
<thead>
<tr>
<th></th>
<th>1 vs 2</th>
<th>1 vs 3A</th>
<th>1 vs 3B</th>
<th>2 vs 3A</th>
<th>2 vs 4</th>
<th>3A vs 3B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neovascularization</td>
<td>0.20 ± 0.11</td>
<td>3.62 ± 0.52</td>
<td>1.26 ± 0.16</td>
<td>0.99 ± 0.42</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>(percentage/high power field)</td>
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<tr>
<td>Mononuclear cells infiltration (per high power field [Magnification, ×400])</td>
<td>0.00 ± 0.00</td>
<td>3.85 ± 1.31</td>
<td>2.25 ± 2.55</td>
<td>0.50 ± 0.51</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Active fibroblasts cell infiltration (per high power field [Magnification, ×400])</td>
<td>5.90 ± 3.63</td>
<td>22.00 ± 2.81</td>
<td>6.50 ± 2.12</td>
<td>5.60 ± 3.50</td>
<td>0.00000</td>
<td>0.5</td>
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**Discussion**

**Baseline assessment**

The mean burn stimulus score was the same between the studied groups has no statistical difference (p=0.48) and (p=0.67). This makes it plausible to compare between these groups after therapy.

**Post therapy assessment**

CorNVs, as assessed immunohistochemically for vascular endothelial growth factor, was significantly lower in treated groups compared to untreated one, as CorNV is a significant sight-threatening event that usually leads to loss of central vision or even results in blindness [19].

Inflammatory reactions were assessed through assessment of thickness of different corneal layers shown in histological Masson’s trichrome staining sections as well as quantification of inflammatory cells shown in H and E and α smooth muscle acting stained staining sections [22].

Total corneal thickness was significantly lower in Bevacizumab (Avastin) treated group and *Spirulina platensis* [PSP] treated group compared to untreated alkaline burn group.

Similarly, epithelial thickness was significantly higher in Bevacizumab (Avastin) treated group and *Spirulina platensis* [PSP] treated group compared to untreated alkaline burn group.

Moreover, stromal thickness was significantly lower in Bevacizumab (Avastin) treated group and *Spirulina platensis* [PSP] treated group compared to untreated alkaline burn group.

DM thickness was significantly lower in Bevacizumab (Avastin) treated group and *Spirulina platensis* [PSP] treated group (compared to untreated alkaline burn group.)
Fibroblasts cellular infiltration was found to be significantly lower in Bevacizumab (Avastin) treated group and *Spirulina platensis* [PSP] treated group compared to untreated alkaline burn group.

Mononuclear cells infiltration was found to be significantly lower in Bevacizumab (Avastin) treated group and *Spirulina platensis* [PSP] treated group compared to untreated alkaline burn group.

The previous results demonstrate the beneficial outcome of Bevacizumab (Avastin) and *Spirulina platensis* [PSP] on inflammatory process and regression of CorNVs induced by alkali burn.

In fact, Bevacizumab (avastin) effect was previously studied by Ozdemir et al. (2014), who demonstrated that topical administration of Bevacizumab inhibits corneal neovascularization and decreases inflammation and fibroblast activity in a rat model of corneal neovascularization induced by alkali burn [23]. Oner et al. (2012), stated that topical use of Bevacizumab is effective and safe in controlling corneal neovascularization [24]. Hashemian et al. (2011), found that topical Bevacizumab can prevent CorNV in rats [25]. Also Kim et al. (2013), proved that topically administered Bevacizumab has standing anti-angiogenic effect on corneal neovascularization following chemical injury in rats [26].

On the other hand, *Spirulina platensis* [PSP] was studied by Yang et al. (2009), who found that polysaccharide extract from *Spirulina platensis* is a potent inhibitor of CorNVs and that it may be of value in the therapy of corneal diseases including neovascularization and inflammation. Similarly, Yang et al. (2012), stated that polysaccharide extract from *Spirulina platensis* [PSP] inhibited alkali burn-induced inflammation and CorNV more effectively than AME extract at the studied doses, thus may be used for the treatment of corneal diseases involving neovascularization and inflammation.

Chen et al. (2004), demonstrated the possible anti-inflammatory effects of PSP on alkali-burned corneas. The inflammation factors as SDF1 and TNF-α are known to be one of the key regulators of inflammation and can mediate angiogenesis. In corneas, the infiltration of SDF1-positive inflammatory cells and the expression of SDF1 and TNF-α RNA were significantly depressed by PSP [27].

Yang et al. (2009), stated that PSP suppressed the expression of VEGF, matrix metalloproteinase (MMP2, and MMP9) and stimulated the expression of PEDF, suggesting that PSP may inhibit CorNVs by down regulating the expression of angiogenic factors and up regulating the expression of the anti-angiogenic factors. The anti-angiogenic effects of PSP were mediated by interference with the proliferation, migration, and tube formation of vascular endothelial cells in vitro.

Adisakkwattana et al. (2013), reported that hydroxyl cinnamic acid and their derivatives of PSP extract induce corneal epithelial wound healing by inhibiting protein tyrosine phosphatases (PTP)-1β gene [28]. Also Bodet et al. (2008), stated that narigenin is an effective inhibitor of the pro-inflammatory cytokine (IL-1β, IL-6, IL-8 and TNF-α) response stimulated by lipopolysaccharide in both macrophages and in whole blood [29].

Comparison of the two treated groups demonstrated superiority (not only non-inferiority basis) of *Spirulina platensis* [PSP] over Bevacizumab [avastin] therapy. This is shown by comparison between Bevacizumab [avastin] treated group and *Spirulina platensis* [PSP] treated group. This showed significantly lower stromal thickness, DM thickness, total corneal thickness, CorNVs and active fibroblasts and mononuclear cell infiltration.

Another point in favor of *Spirulina platensis* [PSP] over Avastin was its potential to bring down the stromal thickness near to normal control values more than Avastin.
Conclusion

The naturally occurring Spirulina platensis (PSP) is more cost effective in treatment of corneal neovascularization and decrease inflammation and fibroblast activity in a rat model of corneal neovascularization induced by alkali burn as compared to Avastin.

Contributors


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Competing interests

There is no conflict of interest of any of the authors with any establishment having a relation to this present work.

Ethics approval

Institutional Review Board.

Data sharing statement

All data collected here are contained within the manuscript.

References