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Article

Cucurbita argyrosperma seed extracts attenuate angiogenesis in a corneal chemical burn model

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Abstract: Cornea severe inflammation produces opacity or even perforation, scarring, and angiogenesis, resulting in blindness. The cornea can be used to study the effect of new anti-angiogenic chemopreventive agents. We researched the anti-angiogenic effect of two extracts, Methanol (Met) and Hexane (Hex), from the seed of *Cucurbita argyrosperma*, in the inflamed corneas. The corneas of Wistar rats were alkali-injured and treated intragastrically for seven successive days. Clinical manifestation as opacity score, corneal neovascularization (CNV) area, re-epithelialization percentage, and histological evaluation were performed. Inflammatory (COX-2, NF-κB, and IL-1β), and angiogenic (VEGF-A, VEGFR1, VEGFR2) markers were assessed by immunohistochemistry. *Cox-2*, *Il-1β*, and *Vegf-a* mRNA levels were also determined. After treatments, we observed slim corneal thickness with lower opacity scores and low cell infiltration compared to untreated rats. Treatment also accelerated wound healing and decreased CNV area. The staining of inflammatory and angiogenic factors was significantly decreased. These effects are related to a down-expression of *Cox-2*, *Il-1β*, and *Vegf*. These results suggest that intake of *C. argyrosperma* seed can be used to attenuate the angiogenesis secondary to inflammation in corneal chemical damage.

Keywords: *C. argyrosperma*; corneal chemical burn; angiogenesis; corneal neovascularization (CNV); Vascular Endothelial Growth Factor (VEGF); Interleukin-1β (IL-1β); Cyclooxigenase-2 (COX-2); Nuclear Factor-kappaB (NF-κB).

1. Introduction

Angiogenesis, the formation of new blood vessels from the pre-existing ones, inflammation, and oxidative stress are important factors that predispose and promote the progression of degenerative diseases such as tumors and diabetes, and corneal diseases are not an exception. Corneal neovascularization (CNV), caused by viral infections, autoimmune diseases, and chemical burns could progress into defective healing with persistent perforation and ulceration, resulting in blindness or failure in the penetrating keratoplasty when not treated in a timely manner [1]. Under this condition, NF-κB signaling pathway in inflammatory, epithelial, and endothelial cells, is a key step for the transcriptional overexpression of pro-inflammatory and proangiogenic factors, including interleukin-1β (IL-1β), cyclooxygenase 2 (COX-2), and vascular endothelial growth factor A (VEGF-A). COX-2 enzyme increases the synthesis of prostaglandins to modulate cell proliferation, cell death, and tumor invasion in many types of cancer. IL-1β, IL-6 or TNF-α, regulate COX-2, therefore, they are overexpressed during inflammation [2-4].

In the alkali-burn corneal injury, VEGF-A is an important factor of angiogenesis when is expressed in macrophages and epithelial cells. VEGF-A binds to two main tyrosine kinase receptors, VEGFR1 and VEGFR2, on the vascular endothelial cell to promote migration, proliferation, and formation of capillaries, along with monocyte/macrophage migration in the microenvironment injured [1,5,6]. Several therapeutic strategies to reduce CNV are studied based on these observations, topical or subconjunctival treatments, mainly corticosteroids and non-steroidal anti-inflammatory agents [7], have limited use and potential side effects as an impediment of wound healing [8,9]. Anti-VEGF therapy in chemically burned ocular tissues results in a substantial reduction of angiogenesis both animal studies and clinical trials [10,11]. However, to establish its safety and efficacy, controlled and randomized trials to justify their continued use are required. Besides, systemic drug treatment is not recommended because of adverse effects. Thus, it is important the search for new drugs for the systemic treatment of these disorders.

Current data in CNV models shown that natural extracts from plants or the bioactive compounds in its extracts have angiogenic suppressing activity [12-14]. The genus Cucurbita (pumpkin) belongs to one of the 300 genera of the Cucurbitaceae family and it is one of the most popular vegetables eaten in the world. Recently, pumpkin was recognized as a functional food and *Cucurbita pepo*, *C. maxima*, *C. moschata*, *C. andreana*, and *C. ficifolia* are the most cultivated species [15]. Nutritionally, pumpkin seed contains a high amount of polyunsaturated fatty acids as well as proteins, vitamins, several minerals, and other phytochemicals**.** The anti-diabetic, antioxidant, anti-carcinogenic, anti-inflammatory properties of this seed are studied due to its high content natural bioactive compounds, such as carotenoids, tocopherols, and sterols [16-19].

Cucurbita argyrosperma is an economically important species cultivated in Mesoamerica. Isozyme, morphological, and ecological analysis suggest that it was probably domesticated from the Mexican wild squash *C. sororia* [20]. Seed is usually consumed as a snack or as an ingredient in traditional stews, although the scientific findings of its beneficial effects on human health have not been sufficiently proven and the anti-neovascular effects of the secondary metabolites remain unknown. However, conceivably its phytochemical composition could be like the related species, showing anti-inflammatory effects as suggested. Besides, proangiogenic factors such as COX-2, IL-1β, and, VEGF, including to VEGFR1 and -R2, induced by the inflammatory agents has not studied these plants.

The aim of this study was to contribute with new evidence of the effect of seed extracts from *C. argyrosperma* in the inflammatory and angiogenic process attenuation. Here show that hexanic and methanolic extracts from *C. argyrosperma* seed significantly attenuates the expression of proangiogenic factors during the inflammation using CNV model. Also, we observed by clinical manifestation that both extracts significantly diminish corneal neovascularization area. Remarkably, corneal re-epithelialization was higher in the hexane extract treatment than methanol extract.

2. Materials and Methods

2.1 Extract Preparation

The pumpkins of *C. argyrosperma* were harvested in an agricultural field of the Michoacán Province, México and identified by a botanist in the herbarium of The National Polytechnic Institute (IPN). Voucher specimen number 4532 was deposited in the herbarium of the National School of Biological Sciences of IPN. One kilogram of seed was extracted with 3L of hexane (50% v/v) and left to macerate for 8 days at room temperature. Crude extract was filtered for one hour in 8 μM-medium flow filter paper (Whatman®), concentrated using a rotary vacuum evaporator and taken to dryness at 60°C in a vacuum rotator until the complete removal of the solvent, obtaining a viscous residue

(8.38 g/L). The same procedure was applied to the residue, using methanol for a sequential separation of the seed components. Each extract was stored in the dark at 4°C until use.

2.2 Animal model

Twenty-eight male Wistar rats weighing 200-250 g were used. Water and standard food were available *ad libitum*. The care and management of experimental animals were performed according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the standards described by ARVO (Association for Research in Vision and Ophthalmology), and Official Mexican Standard NOM-062-ZOO-1999.

2.3 Experimental design

Each extract was dissolved in 0.5 ml (water) and Tween-80 (20%), which was also used as the vehicle (Veh). Rats were randomly divided into four groups (n=7 each): Non-chemically burned healthy corneas (Non-CB) treated only with the vehicle; chemically burned (CB) corneas treated with the vehicle (CB-Veh); hexanic extract treatment (CB-Hex); and methanolic extract treatment (CB-Met). Groups were intragastrically injected with 400 mg/kg of Hex/Met extracts or 0.5 ml of the vehicle in a single dose, at the same time daily (10:00 am). 48h later, animals were intraperitoneally anesthetized with pentobarbital sodium (0.5 mg/kg), inhaled sevoflurane, and one drop of ophthalmic tetracaine, to perform the chemical cauterization of the cornea. Central corneas from the right eyes were burned by applying a 3-mm-diameter filter paper saturated with 1M NaOH solution for 30 seconds and immediately washing with 10 ml of saline solution. To avoid infection, a drop of ophthalmic ciprofloxacin was applied every 24 hours until the end of the study. Animals were euthanized with an overdose of pentobarbital.

2.4 Clinical manifestation

Corneal opacity, epithelial defects, and the CNV area were clinically evaluated eight days after CB. Corneal opacity was scored using a scaling system from 0 to 4: 0=no opacity, completely clear cornea; 1=slightly hazy, iris and lens visible; 2=moderately opaque, iris and lens still detectable; 3=severely opaque, iris and lens hardly visible; and 4=completely opaque, with no visibility of the iris and lens [21].

The measurement of the CNV area (mm2) was performed *in vivo* using a ruler under a microscope and photographed. The software program Image-Pro Plus version 6.0 software (by Media Cybernetics, Inc., Rockville, MD, USA) was used. Inferonasal quadrant was selected to calculate the neovascularized area, according to previous reports [22].

To evaluate corneal wound re-epithelialization, we used corneal fluorescein staining. Briefly, the lateral conjunctival sac was stained using fluorescein sodium ophthalmic strips. Corneas were examined using a slit lamp biomicroscope with cobalt blue light. Injured epithelial tissues retain the fluorescein staining, meanwhile, the lack of stain indicates a re-epithelialization. The re-epithelialization percentage was evaluated from corneal central burning, considering that a total area of approx. 7 mm^2 is 100 percent (3 mm disc 1M NaOH-embedded).

2.5 Histological evaluation

Enucleated eyes (n=4 by group) were immediately fixed in neutral formalin. Cut tissue slides (3-5 mm) were made, anteroposterior, and included the optic nerve. Slides were dehydrated in graded alcohols and embedded in paraffin. Histological sections of 2μm were processed and stained with hematoxylin-eosin (H&E). We measured the corneal thickness and cell infiltration in the peripheral region (500 μm beyond the limb area) using light microscopy (axioscope 2 plus, Carl Zeiss). The percentage of the infiltration was calculated in a masked fashion based on the density in the corneal stroma of CB-Veh group.

Other 2µm-sections were dewaxed and rehydrated up to antigen recovery solution (ImmunoDNA Retriever 20X with Citrate; BioSB). Slides were then loaded into a Shandon Sequenza chamber (Thermo Fisher Scientific, Inc). We used the procedure described for the polymer-based immunodetection system (PolyVue® mouse/rabbit DAB detection system, Diagnostic BioSystems, Pleasanton, CA, USA). We applied 100 µl of IL-1β (Cat. No. sc-7884), NF-κB p65 (sc-8008), COX-2 (sc-1746), VEGF-A (sc-7269), VEGFR1 (sc-31173). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). VEGFR2 antibody (MAB3571) was purchased from R&D systems. All dilutions were at 1:200 and incubated overnight at 4°C. Later, the enhancers Polyvue Plus and HRP were added and incubated with DAB plus/chromogen substrate and counterstained hematoxylin. An Axio Imager.A2 microscope with an integrated camera (axiocam ICc5; Carl Zeiss Microscopy GmbH, Germany) was used for histological observation and image capture. Micrographs of the peripheral region of the cornea (3 fields per side and 500 mm above the limb, at 200X of magnitude) were taken to measure the mean staining intensity of these markers. Images were analyzed with the Image-Pro Plus software version 6.0.

2.6 Quantitative-reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from corneal tissue using TRIzol™ Reagent (Invitrogen, Boston, MA, USA) (n=3 by group). One microgram of DNase I-treated RNA (Roche Applied Science, Germany) was reverse transcribed with SuperScript® II Reverse Transcriptase system. Quantification of mRNA was carried out using qPCR with SYBR green and the following primers: *Cox-2* (*5'-CTGAGGGGTTACCACTTCCA-3'*; and *5'-CTTGAACACGGACTTGCTCA-3*); *Il-1β* (*5'-AGGCTTCCTTGTGCAAGTGT-3'* and *5'-TGAGTGACACTGCCTTCCTG-3'*); *Vegf-a* (*5'-GCCCATGAAGTGGTGAAGTT-3'* and *5'-ACTCCAGGGCTTCATCATTG-3'*); and *Gapdh* (*5'-CTCATGACCACAGTCCATGC-3'* and *5'-TTCAGCTCTGGGATGACCTT-3'*). The cycling protocol was as follows: denaturation (95°C for 10 min), 45 cycles of amplification (95°C for 15 s, 59°C for 15 s, and 72 ° C for 20 s), and a final extension at 72°C. A melting curve analysis was also performed to ascertain the specificity of the amplified product. The expression for each gene was normalized to G*apdh*. Expression was quantified as fold-change using the ΔΔCt method.

2.7 Statistical analysis

We used GraphPad Prism software (La Jolla, CA, USA) (version 5.0). Values are mean with standard deviation (mean ± SD). In all cases, we used unifactorial analysis of variance followed by Tukey's *post hoc* analysis.

3. Results

3.1 Amelioration of corneal wound repair

We evaluated corneal wound healing mediated by the extracts in the alkali-burn corneal model. Figure 1a shows that treated groups had a significant reduction in corneal opacity score and CNV area compared to CB-Veh ($p<0.05$ and $p<0.001$, respectively). However, CNV in CB-Met was lower that CB-Hex treatment ($p<0.05$). Furthermore, CB-Met does not show significant differences in the percentage of re-epithelialization compared to CB-Veh, inverse to CB-Hex treatment (Figure 1b).

H&E stained slides (Figure 1c), non-CB group had an average corneal thickness, 336.7±39.5µm, with integral epithelial layers and a dense stroma, consisting of keratocytes. There are neither inflammatory cells nor blood vessels. Conversely, corneal integrity in CB-Veh group was severely impaired, with the loss of the epithelial cell layers, an extreme denaturation of stromal collagen fibers, and an average cell infiltration of 81±11.9%. Corneal thickness was 592.3±112.1µm. Whereas, CB-Hex tissues display a 40±8.1% infiltration, with a corneal thickness of 427.2±113.7µm ($p<0.001$ compared to CB-Veh). In CB-Met group, there is a significant decrease in cell infiltration (33.2±5.9%) and thickness of 295.2±62.67µm ($p<0.001$ compared to CB-Veh), but corneal thickness in CB-Met no shown difference with Non-CB.

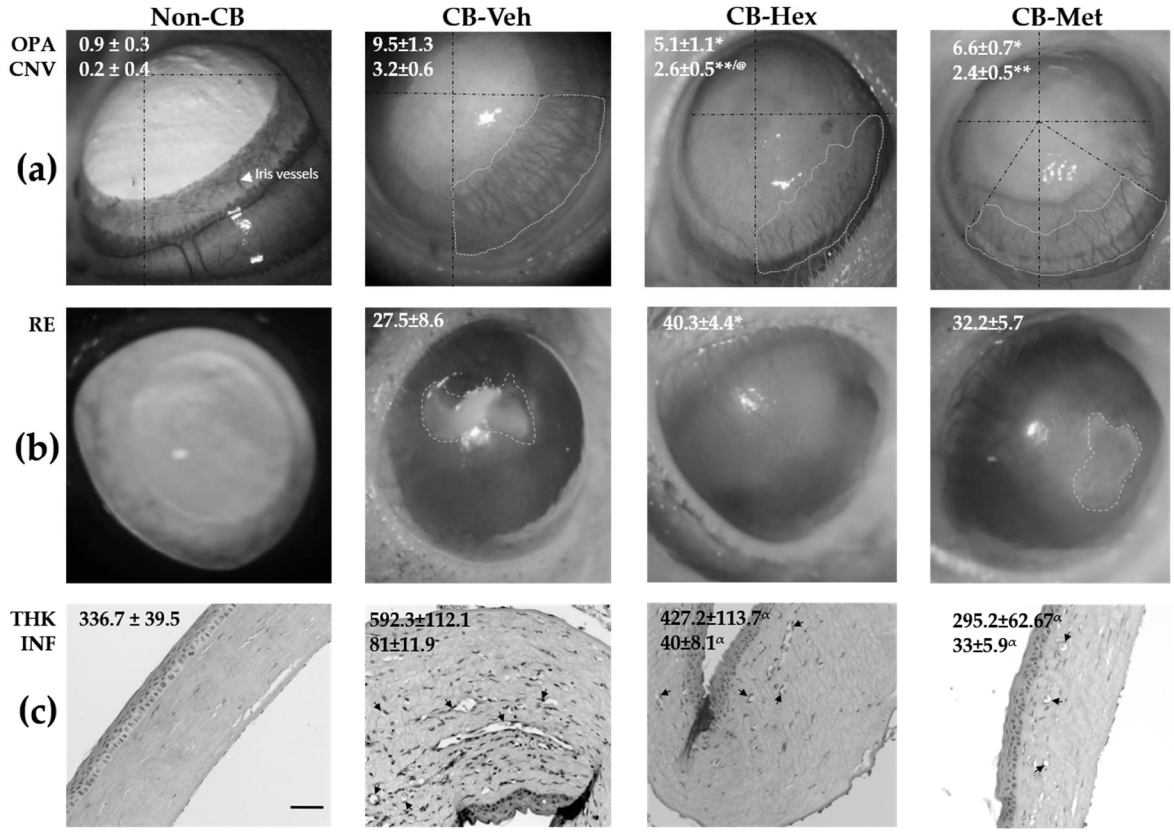

Figure 1. Methanol (Met) and hexane (Hex) extracts of *C. argyrosperma* seed in alkali-injured corneas (CB) compared to the untreated group (CB-Veh). **(a)** average of opacity score (Opa) and corneal neovascularization area (CNV) in mm². **(b)** re-epithelialization percentage (RE) and **(c)** corneal thickness in microns (THK) and infiltration cell percentage (INF). Average value±SD; *$p<0.05$; **$p<0.01$, and $^{\alpha}p<0.001$ compared to CB-Veh. $^{@}p<0.05$ compared to CB-Met. Gray dotted lines show studied area and black lines are geometrical axis. Arrows indicate the lumen of stromal blood vessels. (Scale bar = 100 μm).

2 Anti-inflammatory effect

Several inflammatory cytokines implicated in alkali-induced corneal injury are regulated by the nuclear internalization of active NF-κB. Hence, we glance for the staining location of NF-κB in the corneal lesions. In Non-CB, NF-κB was restricted in the cytoplasm of epithelial cells in the basal layer (Figure 2a). In CB-Veh and both extracts-treated groups, NF-κB was distributed in the nuclear compartment of endothelial and inflammatory cells. However, staining density decreased in CB-Hex (41.13±9.6) and CB-Met (32.73±8.1) compared to CB-Veh (73.31±10.4; $p<0.0001$ both). Additionally, we performed measurements of the staining intensity for IL-1β (Figure 2b) and COX-2 (Figure 2c). In Non-CB there was a low staining intensity for IL-1β (9.28±2.6) compared to CB-Veh (75.95±12.16; $p<0.0001$), showing a distribution along the corneal stroma, as well as endothelial cells. Meanwhile, the intensities in CB-Hex (42.16±9.14) and CB-Met (38.21±7.9) were lower than CB-Veh group ($p<0.0001$). Staining intensity for IL-1β between CB-Met and CB-Hex have no differences ($p>0.5$). Likewise, the intensity for COX-2 was significantly different when comparing CB-Veh (102.6±13.08) to CB-Hex (68.79±10.73) and CB-Met (37.15±7.18) ($p<0.0001$). Non-CB has a detectable expression of 7.49±3.48. Staining intensity for IL-1β and COX-2 in the cornea was also confirmed at the level of mRNA (Figure 3), *Il-1β* expression for CB-Met (2.31±0.30) and CB-Hex (1.89±0.11) was decreased compared to CB-Veh (3.03±0.35; $p<0.05$ and $p<0.01$, respectively) (Figure 3a). *Cox-2* in CB-Hex (2.22±0.10) and CB-Met (1.91±0.15) was also diminished compared to Veh (3.15±0.31; $p<0.01$) (Figure

3b). *The Il-1ß expression* had not differences between CB-Met and CB-Hex ($p>0.5$), on the other hand, there was for *Cox-2* ($p<0.05$).

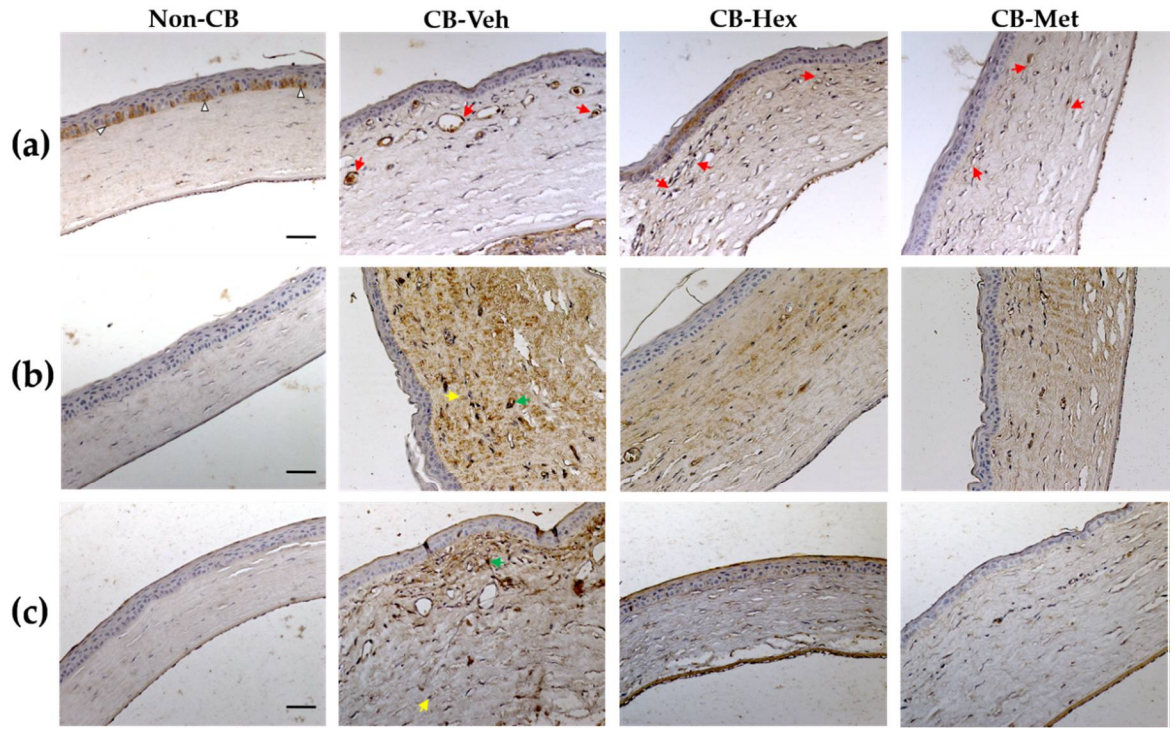

Figure 2. Micrograph of Met and Hex extracts of *C. argyrosperma* seed in CB compared to non-CB-Veh. **(a)** Nuclei stained with anti-NF-κB p65 (red arrows). **(b)** Staining intensity for IL-1β along the corneal thickness. **(c)** COX-2 staining in studied groups. Arrowheads indicate the cytoplasmic distribution of NF-κB p65. Yellow and green arrows represent the minimum and maximum, respectively, staining intensity considered for software analysis. (Scale bar = 100 μm).

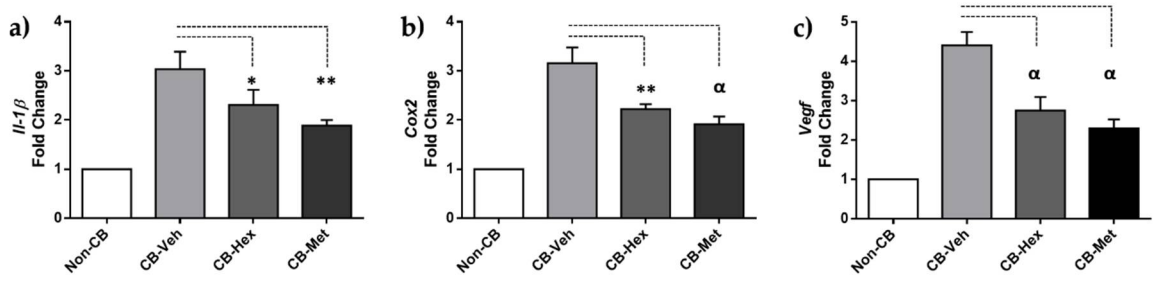

Figure 3. qRT-PCR for *Il-1β* **(a)**, *Cox-2* **(b)**, and *Vegf-a* **(c)**. Bars are the expression levels in each group. *$p\leq0.05$, **$p\leq0.01$, and $^{\alpha}p\leq0.001$ compared to CB-Veh.

3.3 Anti-angiogenic effect

Due to the anti-inflammatory effect in CB-Hex and CB-Met, we assessed whether it was related to an attenuation of CNV, by determining the staining intensity of VEGF-A and its receptors (Figure 4). In CB-Veh, VEGF-A was in the cytoplasm and nucleus of the epithelial, endothelial and other infiltrated cells with an intensity of 102.02±14.04. Decreased on VEGF-A intensities were observed for CB-Hex (71±9.11) and CB-Met (61.3±9.59) ($p<0.001$). Besides, staining intensity between VEGF-A in CB-Met and CB-Hex show differences ($p<0.05$) and in Non-CB, this was about 16.25±5.25 (Figure 4a). *Vegf-a* expression was also confirmed (Figure 3c). *Vegf-a* for CB-Hex (2.74±0.34) and CB-Met (2.29±0.23) was decreased compared to CB-Veh (4.40±0.34; $p<0.05$ and $p<0.01$, respectively).

Relevantly, staining for VEGFR1 was distributed in endothelial and inflammatory cells of CB-Veh corneas (54.4±6.8) and was higher as compared to CB-Hex (33.13±5.8; p<0.0001) and to CB-Met (25.47±3.7; *p<0.0001*) (Figure 4b). VEGFR2 immunostaining was localized in the membrane region in endothelial cells and in CB-Veh (23.06±3.5) was higher compared to CB-Hex (15.67±2.6; *p<0.0001*) and CB-Met (10.95±2.1; *p<0.0001*) (Figure 4c).

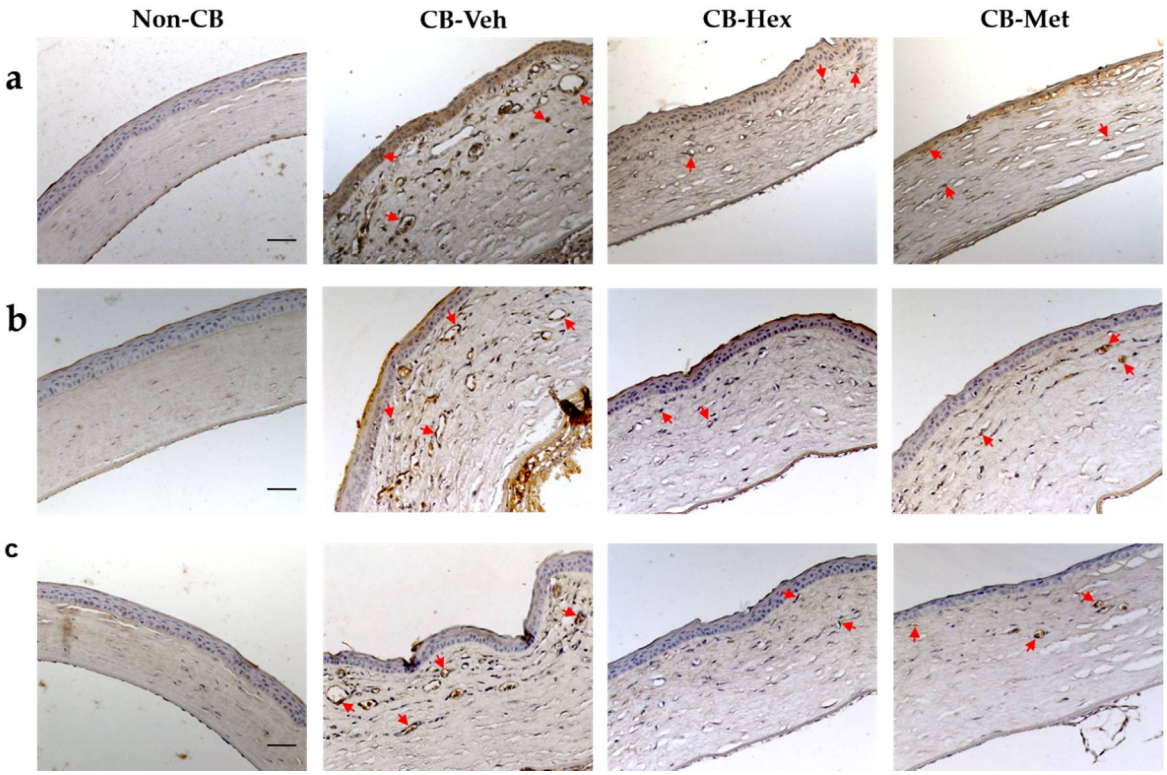

Figure 4. Immunolocalization of VEGF-A and its receptors, VEGFR and VEGFR2, in treated groups. **(a)** Staining intensities for VEGF-A. **(b** and **c)** Membranous staining in endothelial cells for VEGFR1 and VEGFR2, respectively (red arrows). Red arrows represent staining intensity considered for software analysis. (Scale bar = 100 μm).

4. Discussion

The cornea is a transparent, avascular an immune privileged tissue. However, the inflammatory response and growth of neovessels induced by infections, autoimmunity, and chemical burn may cause vision loss and high rejection rate of corneal allografts if not treated effectively [1,23]. Currently, it has been demonstrated by histopathological and clinical findings in animal models that anti-neovascular topical treatments are successful to avoid CNV [8,24]. Nonetheless, its regulatory mechanisms are just starting to be understood. To examine this phenomenon, experimental models combined with new therapeutic strategies have been planned, primarily aimed to preserve corneal transparency through epithelial integrity by attenuating the inflammatory and neovascularization responses.

Chemically burned corneas has long been used for this purpose because is accompanied by the recruitment/migration of neutrophils and macrophages with the resultant damage to the normal tissue structure, conducted by the release of oxidative derivatives, cytokines, and chemokines, matrix metalloproteinases (MMPs), and growth factors, including to VEGF can influence corneal angiogenesis [25-27]. Topical treatments with monoclonal antibody anti-VEGF- or their receptor VEGFR2 suppress the mechanism of action of VEGF in endothelial cell. Whereas dexamethasone

inhibits CNV mediated by suppressing the activity of NF-κB, decreasing the expression of IL-1β and COX-2, and VEGF [7,28]. In the same way, treatments with extracts of Propolis and *Diospyros kaki*; as well as other purified phytochemicals (Naringenin and EGCG) shown a decrease CNV through down-regulation of VEGF-A, IL-1β, IL-6, and metalloproteases, promoting corneal wounds healing [12-14].

In the present study, we aim to evidence the ability of the hexane and methanol extracts of *C. argyrosperma* seed to reduce CNV after an inflammatory stimulus induced by chemical burn in rat cornea. To this goal, we examined the nuclear localization of NF-κB p65, an important marker for the overexpression of VEGF-A, IL-1β and COX-2 in damaged corneas. Receptor VEGFR2 in endothelial cell of corneal stromal neovessels as an active marker of the angiogenesis was studied. Angiogenesis initiate when VEGFR2 is activated by tyrosine phosphorylation by VEGF-A binding. Consecutively, downstream pathways are activated such as p38 MAPK and ERK1/2, producing a strong mitogenic and survival process [29,30]. In contrast, such a mitogenic signal is not equally induced by VEGFR1. Although VEGFR1 binds to VEGF-A with higher affinity than VEGFR2, the induction of VEGFR1 phosphorylation is low and its downstream signaling is still poorly explored [31]. VEGFR1 possesses anti-angiogenic activity by avoiding the union between VEGF-A and VEGR2. Corneal studies show that the proteolytic enzyme, MMP14, can shave the extracellular domain of VEGFR1, converting it into the receptor soluble (VEGFRs) that acts as a decoy for VEGF-A [32]. Nevertheless, VEGFR1 indirectly induces angiogenesis by stimulating the migration of monocytes and macrophages directed towards the damaged microenvironment. For these reasons, this study evaluates both receptors because they are expressed predominantly in endothelial cells in the angiogenic environment: (1) VEGFR1 as a marker in the infiltration of monocytes and neovessels and (2) VEGFR2 as an active marker of angiogenesis. Our results show that the staining intensity and the expression of *Vegf-a* decreased along with the staining of VEGFR2 and VEGFR1 in endothelial cells of extract-treated corneas. Additionally, the decrease of *Il-1β* and *Cox-2* expressions were also observed, suggesting that hexanic- and methanolic-extract components can attenuate these pro-angiogenic factors, likely through a lack of nuclear activation of NF-κB, which was also observed.

Treatment with hexanolic extract of *C. argyrosperma* seed shows a repairing of the corneal damage associated with a decrease in the expression of inflammatory and angiogenic factors. *C. pepo* seed extracts, containing majorly linoleic (ALA), linolenic acids (LA), tocopherols, and sterols, showed effective healing skin wounds with a complete re-epithelialization, organization of collagen fibers, and absence of inflammatory cells [18]. Particularly, treatment with ALA, in cultures of corneal epithelial cells and when is topically applied to a dry eye animal model, has an anti-inflammatory activity by decreasing the release and the expression of inflammatory factors (TNF-a, IL-6, IL-1β, and IL-8) regulated by NF-κB pathway [33,34]. In addition, LA decreased corneal fluorescein staining and was associated with a significant decrease in the number of CD11b(+) cells [35].

Although we are still characterizing the bioactive compounds in the methanolic extract, we can speculate that the content of phytochemicals is similar to the *C. pepo* seed [16]. Some of these components include flavonoids as quercetin, luteolin, and apigenin which at low concentration intake has a protective anti-inflammatory effect on human retinal pigment epithelial damage by hypoxia, inhibiting VEGF and the factors related to its activation [36]. For example, quercetin inhibits the production of inflammatory factors in VEGF-stimulated retinal photoreceptor cells, associated with inactivation of NF-κB as a consequence of the blockage of mitogen-activated protein kinases (MAPK) and protein kinase B (Akt) phosphorylation [37]. Nonetheless, *C. argyrosperma* may differ in the content and types of flavonoids from *C. pepo*., This would bring us closer to a better understanding of the mechanisms of attenuated angiogenesis by the phytochemicals contained in the methanol extract, which in turn could act synergistically, either directly or indirectly, in VEGF-A regulation.

Corneal inflammation eventually causes vision loss due to CNV. Corneal alkali-injury not only raised to NF-κB, IL-1β, and COX-2 expression, but also significantly increase VEGF and their receptors VEGFR1 and VEGFR2 in endothelial cells. This work demonstrates for the first time, that methanolic or hexanic extracts of *C. argyrosperma* seed (400 mg/kg/7d) improves the healing of corneal wound injured by a chemical agent and may contribute to the anti-inflammatory properties of the phytochemicals in its composition. In addition, a significant reduction of the CNV was related to the attenuation of proangiogenic factors. Significantly, our results indicate that C. *argyrosperma* Hex-extract is better than Met-extract to reduce the corneal re-epithelialization time, improving the healing process and thus preventing the entrance of microorganisms and inflammatory mediators into the deeper layers, probably through the inhibition of the NF-κB pathway during at least seven days after corneal alkali-burn.

Consequently, ingestion of the seed can be an option to prevent the corneal angiogenesis. As well, it might benefit wound healing or inhibit neovascularization in other degenerative pathologies. Further pharmacological and phytochemical studies are required to identify its constituents and accurately assess this activity.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Azar, D.T. Corneal angiogenic privilege: angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis, and wound healing (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc* **2006**, *104*, 264-302.

2. Kuwano, T.; Nakao, S.; Yamamoto, H.; Tsuneyoshi, M.; Yamamoto, T.; Kuwano, M.; Ono, M. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2004**, *18*, 300-310, doi:10.1096/fj.03-0473com.

3. Nakao, S.; Kuwano, T.; Tsutsumi-Miyahara, C.; Ueda, S.; Kimura, Y.N.; Hamano, S.; Sonoda, K.H.; Saijo, Y.; Nukiwa, T.; Strieter, R.M., et al. Infiltration of COX-2-expressing macrophages is a prerequisite for IL-1 beta-induced neovascularization and tumor growth. *The Journal of clinical investigation* **2005**, *115*, 2979-2991, doi:10.1172/jci23298.

4. Sobolewski, C.; Cerella, C.; Dicato, M.; Ghibelli, L.; Diederich, M. The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies. *Int J Cell Biol* **2010**, *2010*, 215158, doi:10.1155/2010/215158.

5. Cursiefen, C.; Chen, L.; Borges, L.P.; Jackson, D.; Cao, J.; Radziejewski, C.; D'Amore, P.A.; Dana, M.R.; Wiegand, S.J.; Streilein, J.W. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *The Journal of clinical investigation* **2004**, *113*, 1040-1050, doi:10.1172/jci20465.

6. Sivak, J.M.; Ostriker, A.C.; Woolfenden, A.; Demirs, J.; Cepeda, R.; Long, D.; Anderson, K.; Jaffee, B. Pharmacologic uncoupling of angiogenesis and inflammation during initiation of pathological corneal neovascularization. *J Biol Chem* **2011**, *286*, 44965-44975, doi:10.1074/jbc.M111.294967.

7. Nakao, S.; Hata, Y.; Miura, M.; Noda, K.; Kimura, Y.N.; Kawahara, S.; Kita, T.; Hisatomi, T.; Nakazawa, T.; Jin, Y., et al. Dexamethasone inhibits interleukin-1beta-induced corneal neovascularization: role of nuclear factor-kappaB-activated stromal cells in inflammatory angiogenesis. *The American journal of pathology* **2007**, *171*, 1058-1065, doi:10.2353/ajpath.2007.070172.

8. Gupta, D.; Illingworth, C. Treatments for corneal neovascularization: a review. *Cornea* **2011**, *30*, 927-938, doi:10.1097/ICO.0b013e318201405a.

9. Sheppard, J.D.; Comstock, T.L.; Cavet, M.E. Impact of the Topical Ophthalmic Corticosteroid Loteprednol Etabonate on Intraocular Pressure. *Advances in therapy* **2016**, *33*, 532-552, doi:10.1007/s12325-016-0315-8.

10. Al-Debasi, T.; Al-Bekairy, A.; Al-Katheri, A.; Al Harbi, S.; Mansour, M. Topical versus subconjunctival anti-vascular endothelial growth factor therapy (Bevacizumab, Ranibizumab and Aflibercept) for treatment of corneal neovascularization. *Saudi journal of ophthalmology : official journal of the Saudi Ophthalmological Society* **2017**, *31*, 99-105, doi:10.1016/j.sjopt.2017.02.008.

11. Zhou, C.; Robert, M.C.; Kapoulea, V.; Lei, F.; Stagner, A.M.; Jakobiec, F.A.; Dohlman, C.H.; Paschalis, E.I. Sustained Subconjunctival Delivery of Infliximab Protects the Cornea and Retina Following Alkali Burn to the Eye. *Invest Ophthalmol Vis Sci* **2017**, *58*, 96-105, doi:10.1167/iovs.16-20339.

12. Keshavarz, M.; Mostafaie, A.; Mansouri, K.; Shakiba, Y.; Motlagh, H.R. Inhibition of corneal neovascularization with propolis extract. *Archives of medical research* **2009**, *40*, 59-61, doi:10.1016/j.arcmed.2008.10.004.

13. Oguido, A.; Hohmann, M.S.N.; Pinho-Ribeiro, F.A.; Crespigio, J.; Domiciano, T.P.; Verri, W.A., Jr.; Casella, A.M.B. Naringenin Eye Drops Inhibit Corneal Neovascularization by Anti-Inflammatory and Antioxidant Mechanisms. *Invest Ophthalmol Vis Sci* **2017**, *58*, 5764-5776, doi:10.1167/iovs.16-19702.

14. Yang, S.J.; Jo, H.; Kim, K.A.; Ahn, H.R.; Kang, S.W.; Jung, S.H. Diospyros kaki Extract Inhibits Alkali Burn-Induced Corneal Neovascularization. *Journal of medicinal food* **2016**, *19*, 106-109, doi:10.1089/jmf.2014.3404.

15. Kocyan, A.; Zhang, L.B.; Schaefer, H.; Renner, S.S. A multi-locus chloroplast phylogeny for the Cucurbitaceae and its implications for character evolution and classification. *Molecular phylogenetics and evolution* **2007**, *44*, 553-577, doi:10.1016/j.ympev.2006.12.022.

16. Seun F. Akomolafe; Ganiyu Oboh; Sunday I. Oyeleye; Olorunfemi R. Molehin; Opeyemi B. OgunsuyiPeiretti, P.G. Phenolic Composition and Inhibitory Ability of Methanolic Extract from Pumpkin (Cucurbita pepo L) Seeds on Fe-induced Thiobarbituric acid reactive species in Albino Rat's Testicular Tissue In-Vitro. *Journal of Applied Pharmaceutical Science* **2016**, *6*, 115-120, doi:10.7324/JAPS.2016.60917

17. Yadav, M.; Jain, S.; Tomar, R.; Prasad, G.B.; Yadav, H. Medicinal and biological potential of pumpkin: an updated review. *Nutrition research reviews* **2010**, *23*, 184-190, doi:10.1017/s0954422410000107.

18. Bardaa, S.; Ben Halima, N.; Aloui, F.; Ben Mansour, R.; Jabeur, H.; Bouaziz, M.; Sahnoun, Z. Oil from pumpkin (Cucurbita pepo L.) seeds: evaluation of its functional properties on wound healing in rats. *Lipids in health and disease* **2016**, *15*, 73, doi:10.1186/s12944-016-0237-0.

19. Medjakovic, S.; Hobiger, S.; Ardjomand-Woelkart, K.; Bucar, F.; Jungbauer, A. Pumpkin seed extract: Cell growth inhibition of hyperplastic and cancer cells, independent of steroid hormone receptors. *Fitoterapia* **2016**, *110*, 150-156, doi:10.1016/j.fitote.2016.03.010.

20. Sanchez-de la Vega, G.; Castellanos-Morales, G.; Gamez, N.; Hernandez-Rosales, H.S.; Vazquez-Lobo, A.; Aguirre-Planter, E.; Jaramillo-Correa, J.P.; Montes-Hernandez, S.; Lira-Saade, R.; Eguiarte, L.E. Genetic Resources in the "Calabaza Pipiana" Squash (Cucurbita argyrosperma) in Mexico: Genetic Diversity, Genetic Differentiation and Distribution Models. *Frontiers in plant science* **2018**, *9*, 400, doi:10.3389/fpls.2018.00400.

21. Yoeruek, E.; Ziemssen, F.; Henke-Fahle, S.; Tatar, O.; Tura, A.; Grisanti, S.; Bartz-Schmidt, K.U.; Szurman, P. Safety, penetration and efficacy of topically applied bevacizumab: evaluation of eyedrops in corneal neovascularization after chemical burn. *Acta ophthalmologica* **2008**, *86*, 322-328, doi:10.1111/j.1600-0420.2007.01049.x.

22. Rogers, M.S.; Birsner, A.E.; D'Amato, R.J. The mouse cornea micropocket angiogenesis assay. *Nature protocols* **2007**, *2*, 2545-2550, doi:10.1038/nprot.2007.368.

23. Dana, M.R.; Streilein, J.W. Loss and restoration of immune privilege in eyes with corneal neovascularization. *Invest Ophthalmol Vis Sci* **1996**, *37*, 2485-2494.

24. Roshandel, D.; Eslani, M.; Baradaran-Rafii, A.; Cheung, A.Y.; Kurji, K.; Jabbehdari, S.; Maiz, A.; Jalali, S.; Djalilian, A.R.; Holland, E.J. Current and emerging therapies for corneal neovascularization. *The ocular surface* **2018**, *16*, 398-414, doi:10.1016/j.jtos.2018.06.004.

25. Choi, H.; Phillips, C.; Oh, J.Y.; Stock, E.M.; Kim, D.K.; Won, J.K.; Fulcher, S. Comprehensive Modeling of Corneal Alkali Injury in the Rat Eye. *Current eye research* **2017**, *42*, 1348-1357, doi:10.1080/02713683.2017.1317817.

26. Shakiba, Y.; Mansouri, K.; Arshadi, D.; Rezaei, N. Corneal neovascularization: molecular events and therapeutic options. *Recent Pat Inflamm Allergy Drug Discov* **2009**, *3*, 221-231.

27. Edelman, J.L.; Castro, M.R.; Wen, Y. Correlation of VEGF expression by leukocytes with the growth and regression of blood vessels in the rat cornea. *Invest Ophthalmol Vis Sci* **1999**, *40*, 1112-1123.

28. Nakao, S.; Zandi, S.; Lara-Castillo, N.; Taher, M.; Ishibashi, T.; Hafezi-Moghadam, A. Larger therapeutic window for steroid versus VEGF-A inhibitor in inflammatory angiogenesis: surprisingly similar impact on leukocyte infiltration. *Invest Ophthalmol Vis Sci* **2012**, *53*, 3296-3302, doi:10.1167/iovs.11-8114.

29. Kim, Y.M.; Hwang, S.; Kim, Y.M.; Pyun, B.J.; Kim, T.Y.; Lee, S.T.; Gho, Y.S.; Kwon, Y.G. Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. *J Biol Chem* **2002**, *277*, 27872-27879, doi:10.1074/jbc.M202771200.

30. Gee, E.; Milkiewicz, M.; Haas, T.L. p38 MAPK activity is stimulated by vascular endothelial growth factor receptor 2 activation and is essential for shear stress-induced angiogenesis. *J Cell Physiol* **2010**, *222*, 120-126, doi:10.1002/jcp.21924.

31. Koch, S.; Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harbor perspectives in medicine* **2012**, *2*, a006502, doi:10.1101/cshperspect.a006502.

32. Han, K.Y.; Chang, J.H.; Lee, H.; Azar, D.T. Proangiogenic Interactions of Vascular Endothelial MMP14 With VEGF Receptor 1 in VEGFA-Mediated Corneal Angiogenesis. *Invest Ophthalmol Vis Sci* **2016**, *57*, 3313-3322, doi:10.1167/iovs.16-19420.

33. Erdinest, N.; Shmueli, O.; Grossman, Y.; Ovadia, H.; Solomon, A. Anti-inflammatory effects of alpha linolenic acid on human corneal epithelial cells. *Invest Ophthalmol Vis Sci* **2012**, *53*, 4396-4406, doi:10.1167/iovs.12-9724.

34. Erdinest, N.; Shohat, N.; Moallem, E.; Yahalom, C.; Mechoulam, H.; Anteby, I.; Ovadia, H.; Solomon, A. Nitric oxide secretion in human conjunctival fibroblasts is inhibited by alpha linolenic acid. *Journal of inflammation (London, England)* **2015**, *12*, 59, doi:10.1186/s12950-015-0104-1.

35. Rashid, S.; Jin, Y.; Ecoiffier, T.; Barabino, S.; Schaumberg, D.A.; Dana, M.R. Topical omega-3 and omega-6 fatty acids for treatment of dry eye. *Archives of ophthalmology (Chicago, Ill. : 1960)* **2008**, *126*, 219-225, doi:10.1001/archophthalmol.2007.61.

36. Chen, R.; Hollborn, M.; Grosche, A.; Reichenbach, A.; Wiedemann, P.; Bringmann, A.; Kohen, L. Effects of the vegetable polyphenols epigallocatechin-3-gallate, luteolin, apigenin, myricetin, quercetin, and cyanidin in primary cultures of human retinal pigment epithelial cells. *Molecular vision* **2014**, *20*, 242-258.

37. Lee, M.; Yun, S.; Lee, H.; Yang, J. Quercetin Mitigates Inflammatory Responses Induced by Vascular Endothelial Growth Factor in Mouse Retinal Photoreceptor Cells through Suppression of Nuclear Factor Kappa B. *International journal of molecular sciences* **2017**, *18*, doi:10.3390/ijms18112497.