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The use of aflibercept (VEGF trap) in mitigating sulfur mustard-induced corneal neovascularization in a rabbit model

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ABSTRACT

Sulfur mustard (SM)-induced ocular injury is characterized by an acute inflammatory response that may become chronic or enter a latent phase with delayed pathology. This study aimed to evaluate the efficacy of ziv-aflibercept and aflibercept in preventing and ameliorating corneal neovascularization (NV), respectively, following chemical eye exposure to SM vapor in a rabbit model. Chemical SM ocular insult was induced in the right eye of rabbits. A single application of ziv-aflibercept was administered 2 h or 9 days post-exposure. A single subconjunctival aflibercept treatment in an ocular formulation was administered 4 weeks after SM vapor exposure and subsequent to an initial 1-week treatment with 0.1 % dexamethasone. Clinical monitoring was performed 5–12 weeks post-exposure, and digital corneal pictures were taken to assess the extent of NV. The rabbits were euthanized and the corneas were processed for histological assessment. Treatment with ziv-aflibercept 2 h and 9 days post-exposure moderately reduced insult severity and partially delayed or prevented corneal NV. Aflibercept application 4 weeks post-exposure significantly reduced the extent of NV for 8 weeks. The substantial decrease in existing corneal NV in this group was confirmed by histology. These results reveal the powerful anti-angiogenic efficacy of the VEGF-trap for ameliorating existing NV as opposed to preventing NV development, revealing the ability of this treatment to mitigate corneal NV.

1. Introduction

Sulfur mustard (2,2'-dichlorodiethyl sulfide; SM) is a potent incapacitating chemical warfare agent known for its ability to induce long-term ocular injuries [2,26]. This compound was extensively used during World War I, the Iran-Iraq war and recently in the Syrian civil conflict [44]. SM continues to be a significant threat to military personnel and civilians [16,40] due to its strong incapacitating effect, reactivity with biological materials and cheap and easy manufacturing process [45]. The ocular surface, dermal tissue and respiratory tract are the main target tissues [32], and the ocular surface is the most sensitive, presenting symptoms shortly after exposure [47]. SM exposure leads to a complicated ocular insult with symptoms similar to chemically or thermally induced ocular pathologies. Initially, exposure to SM causes severe ocular inflammation, erosions, endothelial cell density reduction, degeneration of corneal innervation and loss of conjunctival goblet cells. This can resolve and is followed by a delayed or long-term phase in parts of the eye, leading to chronic inflammation, opacity, corneal neovascularization (NV) and epithelial defects [17,18,20,26]. This severe insult may eventually lead to irreversible corneal impairment [9,27]. In the rabbit model, the initial insult partially resolved within 1–2 weeks post-exposure; after a latent period of 1–3 weeks, late pathology characterized by corneal NV, recurrent erosions, and corneal edema developed in 50–70 % of the eyes in a dose-dependent manner [22,25,33,34]. A similar pattern of clinical response was also observed in a mouse model following SM ocular insult [41].

Corneal NV induced by chemical exposure is commonly associated with limbal stem cell deficiency syndrome [13]. These new pathological blood vessels may induce corneal scarring and chronic cell infiltration, ultimately leading to corneal opacification [8,11]. Previously, we showed that topical steroidal treatment reduces the inflammatory reaction during the initial phase and the incidence of late pathology. When

Abbreviations: ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; NV, neovascularization; NZW, New Zealand White rabbit; PlGF, placental growth factor; SM, sulfur mustard; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor.

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used as a symptomatic treatment for existing NV, the NV-reducing benefit of topical steroids is sustained with continued application [23,25]. Steroids are thought to prevent or reduce corneal NV by inhibiting proinflammatory cytokine synthesis and endothelial cell proliferation and migration [7]. Doxycycline, an MMP inhibitor, treatment was also partially beneficial in reducing the SM induced acute and late pathology and only when administered as a prolonged continues treatment [20].

Several critical components determine corneal clarity, including equilibrium between anti-and pro-angiogenic factors; this equilibrium may be dysregulated following corneal injury [5,7]. One of the most potent pro-angiogenic mediators is vascular endothelial growth factor (VEGF), which was shown to be upregulated during SM-induced late pathology [23].

Ziv-aflibercept/aflibercept is a new soluble high-affinity VEGF trap for all isoforms of VEGF-A [38], VEGF-B [19] and placental growth factor (PlGF) [14,31] enabling it to ameliorate angiogenesis. An additional benefit of these novel antiangiogenic compounds is their prolonged course of action compared to other antiangiogenic treatments, such as ranibizumab [49].

Previously, we showed that multiple bevacizumab treatments reduced existing corneal NV, although this effect was partial and temporary [17,23]. The reduced effect of antiangiogenic treatments, such as bevacizumab, pegaptanib or ranibizumab, can be explained by their VEGF-A specificity [8,38]. Previously, it has been shown that one subconjunctival ziv-aflibercept symptomatic treatment drastically decreased the extent of NV after SM ocular exposure in our rabbit model [17]. Although the beneficial effect of aflibercept on existing NV is well established, a few questions remain unsolved regarding the prophylactic benefit of this treatment. To evaluate the benefit of postexposure anti-NV prophylactic treatment, eyes were treated with ziv-aflibercept 2 h or 9 days post-exposure before NV manifestation. In addition, since topical steroid treatment is recommended for use in humans following SM-induced acute insult [18], we evaluated the interaction between steroid treatment and aflibercept. For this reason, we also evaluated the benefit of a sequential treatment starting with topical steroidal treatment 1 h post-exposure (QID for 6 days) and with a single subconjunctival aflibercept (FDA-authorized ocular formulation) treatment following NV growth at 4 weeks post-exposure.

2. Materials and methods

2.1. Chemicals

2,2'-Dichlorodiethyl sulfide (sulfur mustard; SM) was synthesized at a purity of > 95 %. Commercial eye drops of Localin® (oxybuprocaine hydrochloride 0.4 % (w/v)) (Fischer Pharmaceutical Labs, TA, Israel) and Zaltrap® (100 mg/4 ml, ziv-aflibercept) (Regeneron, Tarrytown, NY, USA; the product is not approved by the FDA for corneal treatment) were used. Commercial EYLEA® (40 mg/ml aflibercept for ocular injection; the product is not yet approved by the FDA for corneal treatment) was used to treat existing NV (Bayer Pharma AG, Berlin, Germany). Commercial dethamycin (0.5 % neomycin sulfate with 0.1 % dexamethasone) was purchased from Vitamed Pharmaceutical Industries, Binyamina, Israel.

2.2. Animals

New Zealand White (NZW) female rabbits (weighing 2–3 kg) were purchased from Charles River Laboratories International Inc. (Quebec, Canada). Animals were housed in individual cages in a temperature-controlled environment, and a 12 h light/dark cycle (lights on at 6 a.m.) was maintained. Rabbits were provided food and tap water ad libitum during the study and were maintained in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals, Eighth Edition, National Academy Press, Washington DC, 2010 and in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures began at least 4 days following acclimatization. The experimental protocols were approved by the IIBR animal care and use committee (IACUC; approval number RB-17–18 approved during 2017 and RB-19–17, approved during 2019) and were designed to prevent or minimize any unnecessary pain and stress. Since no significant difference was seen between males and females regarding SM induced ocular insult and the beneficial effect of steroid treatment in the rabbit model [36], in the current study only female rabbits were used. Female rabbits are less aggressive, enabling clinical evaluation with no sedation.

2.3. Experimental model

The experimental model has been previously described [26]. Briefly, SM vapor pressure was built up after a 10 μl (over 95 % purity) application of SM to a filter disc held in glass goggles fastened to the right eye of each animal for 4 min. All animals received a vapor exposure of SM at a target concentration of 535 ± 64 μg/liter/min. Eyes were not washed following SM exposure. This exposure protocol (with no eye wash) results in moderate to severe ocular insult. The exposure setup (4 min duration at 21 °C) was controlled to produce 100 % injured eyes during the acute phase and 50–70 % eyes with delayed injuries. A clinical follow-up was carried out up to 11 weeks later using slit-lamp microscopy and pachymetry (see details below).

2.4. Treatment protocols

Experiment 1: Ziv-aflibercept (25 mg/ml) was used as a single subconjunctival injection of 80 μl (2 mg) to half of the exposed eyes (n = 10) 2 h after SM exposure before symptoms of the acute phase were detected.

We examined the ability of aflibercept to prevent NV formation before the acute phase in this group. Clinical monitoring continued up to 5 weeks after exposure.

Experiment 2: Ziv-aflibercept (25 mg/ml) was applied as a single subconjunctival injection of 80 μl (2 mg) to half of the exposed eyes (n = 23) 9 days after SM exposure. At this time, the symptoms of the acute phase had decreased, and the symptoms of the late pathology were not yet observed. We examined the ability of aflibercept to prevent NV formation when applied at the end of the acute phase in this group. Clinical monitoring continued until 7 weeks after exposure.

Experiment 3: Eyes were exposed to SM (n = 38), and 2/3 (n = 26) were treated with 50 μl of dexamethasone QID for 6 days, starting 1 h post-exposure. Four weeks post-exposure, half of the eyes (n = 8) in which significant NV developed despite steroidal treatment were treated with a single subconjunctival injection of aflibercept (2 mg/50 μl; 40 mg/ml). We examined the ability of aflibercept to ameliorate existing NV in this group. The three experimental groups of SM-induced neovascularized corneas included a) nontreated (n = 12), b) dexamethasone only treatment (n = 8) and c) dexamethasone followed by aflibercept treatment (n = 8). Clinical follow-up continued up to 11 weeks post-exposure.

Subconjunctival treatment was performed under general anesthesia (i.m. injection of a mixture of 10 % ketamine [50 mg/kg] and 2 % xylazine [10 mg/kg], 1:1, 1 ml/kg) and Localin® (oxybuprocaine 0.4 %) for local anesthesia. For this procedure, a 28 G needle was inserted into the superior space between the conjunctiva and sclera, and the conjunctiva was held with forceps. Fluid was slowly injected to create a ballooning effect. Following this procedure, the eyelids were closed, and the animals were monitored until recovery.

2.5. Clinical evaluation

Detailed slit-lamp (Keeler Symphony 40 H, 5 step, Windsor, United Kingdom) examinations were performed twice a week during the first week post-exposure and once a week thereafter. The clinical

observations were scored, documented by digital photography and analyzed semiquantitatively using our clinical scoring scale described elsewhere [26]. Briefly, the clinical scoring scale for each observation was as follows: hair loss, 0–0.5; eyelid edema, 0–3; eyelid ulcer, 0–0.5; conjunctival edema, 0–4; corneal opacity, 0–4; corneal bullae, 6/9; degenerative sediment, 4/7/11; first-week erosions, 0.5–3.5; second-week erosions, add 3 to the prior score; peripheral NV, 6/7; extended NV to corneal center, 9/10; and iris congestion, 0.5. Erosions were stained by applying fluorescein (BioGlo™) to the ocular surface and identified and documented by employing a slit-lamp cobalt blue filter. Ultrasound pachymetry was used to measure corneal thickness as previously described [17]. The NV severity score was semiquantitatively analyzed using digital photographs and calculated using the NV clinical score, as described earlier by Kadar 2001, multiplied by the NV coverage. The NV severity score estimates provided values near the actual NV length measurements and were used for simplicity.

2.6. Histology

At different time points following exposure, animals were euthanized by an overdose of pentobarbitone sodium (200 mg/ml,1.5 ml; Pental® from CTS chemical industries Ltd, Kiryat-Malachi, Israel) via ear vein administration.

The eyes were enucleated, fixed in 4% neutral-buffered paraformaldehyde, and processed for paraffin embedding as described elsewhere [17].

2.7. Statistical analysis

GraphPad Prism 9.2 (GraphPad Software, La Jolla, CA, USA) software was used for the.

statistical analyses. Each experiment was conducted independently. The results of this study are presented as the mean ± SEM. Statistical significance was set at P value > 0.05. Statistical analysis of unpaired parametric (corneal thickness) and nonparametric (clinical severity scores and NV extent) repeated measures was performed by two-way ANOVA (mixed model for multiple comparisons) with Bonferroni post hoc test, determining the significance of all semiquantitative data.

3. Results

3.1. Effect of early subconjunctival ziv-aflibercept treatment on SM-induced ocular insult

3.1.1. Clinical evaluation

The eyes exposed to SM vapor with or without subconjunctival ziv-aflibercept treatment 2 h post-exposure developed acute ocular injuries, including eyelid, corneal and conjunctival inflammation (Fig. 1A, B), with corneal erosions (Fig. 1C, D). These eyes experienced no beneficial effect of subconjunctival ziv-aflibercept treatment. One week after SM exposure, inflammation eased, and corneal erosions were not observed. Two weeks after exposure and partial healing, the eyes developed corneal NV (late pathology), expanding and progressing with time toward the central cornea. The decrease in the magnitude of NV observed in the ziv-aflibercept-treated group vs. the nontreated group

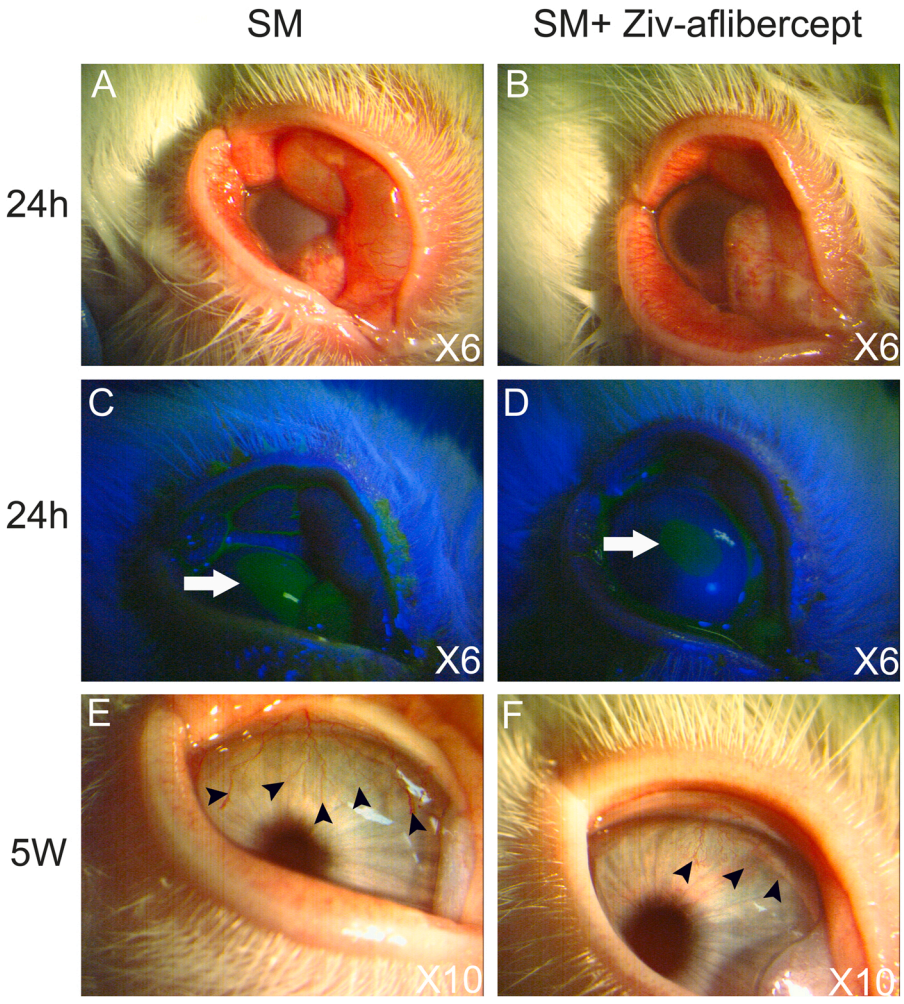

Fig. 1. The effect of early ziv-aflibercept treatment on ocular insult following SM exposure. Two hours after SM exposure, half of the eyes received a single 2 mg (80 μl) subconjunctival ziv-aflibercept treatment (B, D, F), and the results were compared to those of the untreated group (A, C, E). Bright-field (A, B) and erosion (C, D) representative slit-lamp pictures at 24 h showed no differences between the groups. Five weeks post-exposure and treatment, NV extent was less dominant in the ziv-aflibercept-treated group vs. the untreated group (E, F). Pictures were obtained by using a slit-lamp microscope with a magnitude of X6 (A-D) or X10 (E, F). Erosions are indicated by white arrows, and corneal NV is indicated by black arrowheads.

may be observed in the representative picture 5 weeks after SM exposure and treatment (Fig. 1E, F).

Ocular insult and pathological corneal NV formation were observed in both the ziv-aflibercept-treated and nontreated groups. However, the clinical severity score of the treated group was significantly ($p < 0.05$) lower than that of the nontreated group at the 3- and 5-week time points post-treatment (Fig. 2A). Consistent with this, the extent of NV in the treated group was significantly lower (~2-fold) than that in the nontreated group at 2 weeks post-treatment (Fig. 2B).

3.1.2. Histological evaluation

Naïve eyes were stained with H&E (Fig. 3A) or Masson's trichrome (Fig. 3D; red for keratin and blue for collagen) and presented normal intact corneal layers with a minor presence of inflammatory cells. Histological observations (H&E staining) at 5 weeks following exposure (no treatment) showed cell infiltration, an irregular epithelial layer and corneal pathological NV growth (Fig. 3B). Active vascularization (identified by the presence of erythrocytes) was observed in the stroma using specific staining (Fig. 3E). Eyes treated 2 h after SM exposure with ziv-aflibercept presented reduced stromal cell infiltration and NV (Fig. 3C) with numerous empty blood vessels (Fig. 3F).

3.2. Effect of subconjunctival ziv-aflibercept treatment administered 9 days post-exposure on SM-induced ocular insult

3.2.1. Clinical evaluation

Eyes were exposed to SM, and 9 days later, after the initial phase eased and before NV manifested (Fig. 4A, B), half of the eyes were administered one subconjunctival ziv-aflibercept (2 mg/80 μl) treatment. Two weeks post-exposure, corneal NV was detected in both the untreated (Fig. 4C) and treated groups (Fig. 4D). Seven weeks post-exposure, a decrease in NV extent in the ziv-aflibercept-treated group vs. the nontreated group was observed, as shown in the representative pictures (Fig. 4E, F).

Ocular insult and pathological corneal NV formation following SM exposure were observed in both the treated and nontreated groups at the end of the acute phase, 9 days post-exposure. However, the semiquantitative clinical severity score was significantly higher in the untreated group than in the ziv-aflibercept-treated group at 2, 3, 4 and 5 weeks post-treatment ($p < 0.05$), showing that the treatment did not ease the ocular insult but did prevent its deterioration, as seen in the nontreated group (Fig. 5A). In addition, the semiquantitative NV extent score of the treated group was significantly lower (~2-fold; $p < 0.05$) than that of the nontreated group at 5, 6 and 7 weeks post-treatment (Fig. 5B).

3.2.2. Histological evaluation

Naïve eyes stained with H&E (Fig. 6A) presented normal intact corneal layers. Histological observations (H&E staining) of exposed nontreated eyes at 7 weeks post-exposure revealed inflammatory cell infiltration (red arrowheads), an irregular epithelial layer and corneal pathological NV (black arrowheads; Fig. 6B). Eyes treated 9 days after SM exposure with ziv-aflibercept exhibited reduced stromal cell infiltration and NV in addition to reduced corneal edema (Fig. 6C).

3.3. Effect of subconjunctival aflibercept administered 4 weeks post-exposure in addition to topical dexamethasone treatment administered during the first week after SM exposure

3.3.1. Clinical evaluation

Finally, we evaluated the ability of the FDA-approved intraocular formulation of aflibercept to ameliorate existing corneal NV. Aflibercept treatment was applied 4 weeks post-exposure following one week of topical dexamethasone treatment (used to reduce acute inflammation). Eyes were exposed to SM, and starting 1 h post-exposure, 2/3 of the eyes were treated with 0.1% dexamethasone (QID) for 6 days. Four weeks after exposure, half of the eyes in the dexamethasone group that developed NV received no further treatment, and half received subconjunctival aflibercept therapy (2 mg/50 μl) (Fig. 7A-C).

Eleven weeks post-exposure (7 weeks after aflibercept treatment), a decrease in the extent of NV in the aflibercept-treated group vs. the dexamethasone-treated or nontreated groups was observed, as demonstrated in the representative slit-lamp pictures (Fig. 7D, E, F).

A significant decrease in the clinical severity grading score during the initial and delayed ocular insult following SM exposure was noted in the dexamethasone-treated group compared to the untreated group, although this treatment did not prevent NV formation (Fig. 8A-C).

A further significant reduction in the total clinical severity score (Fig. 8D), corneal thickness (Fig. 8E) and extent of NV (Fig. 8F) was observed in the aflibercept group compared to the nontreated group 1 week post-treatment.

3.3.2. Histological evaluation

Histological observations (H&E staining) of nontreated eyes exposed to SM at 11 weeks following exposure revealed inflammatory cell infiltration (red arrowhead), an irregular epithelial layer and corneal pathological NV (black arrowhead; Fig. 9B). Dexamethasone-treated eyes exhibited similar pathological changes but to a lesser degree than

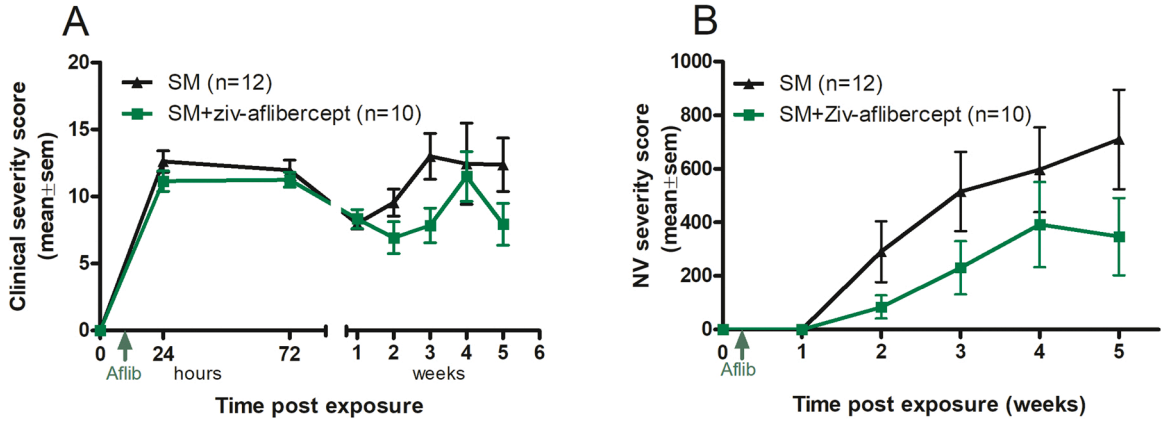

Fig. 2. Effects of early subconjunctival ziv-aflibercept treatment on the clinical insult and corneal NV formation. Semiquantitative assessment of the clinical manifestation in rabbit corneas up to 5 weeks following SM exposure and a single ziv-aflibercept subconjunctival treatment (2 mg/80 μl) based on the clinical scoring scale (A). Semiquantitative assessment of the NV extent in ziv-aflibercept-treated and SM-exposed corneas (B). For the clinical severity score assessment, differences between groups were significant at $p < 0.05$ at 3 and 5 weeks. For the NV severity score assessment, differences between groups were significant at $p < 0.05$ at 2, 3 and 5 weeks. The time of treatment is noted by a green arrow.

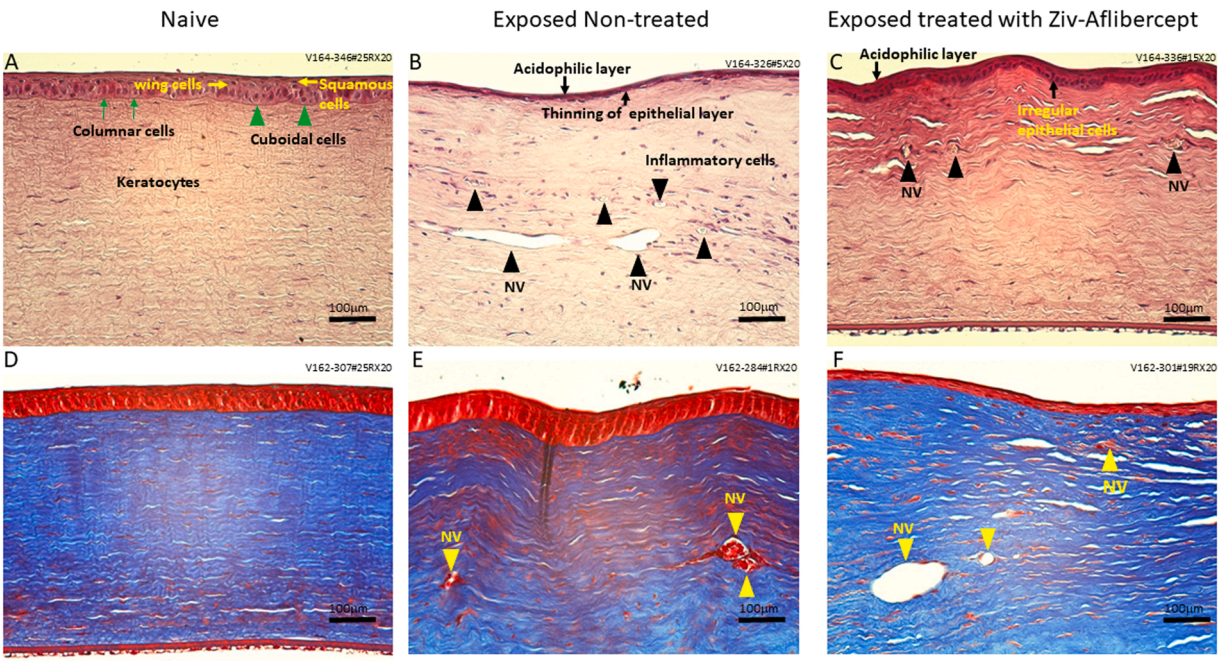

Fig. 3. Histological evaluation of corneal tissues after SM exposure and early ziv-aflibercept treatment. Naïve (A, D), untreated (B, E) and ziv-aflibercept-treated (C, F) corneal representative images taken 5 weeks following SM exposure were analyzed for morphological changes, NV appearance and inflammation. H&E staining (A, B, C) and Masson's trichrome staining (D, E, F). Yellow or black arrowheads denote blood vessels. Magnitude X20, scale bar, 100 µm.

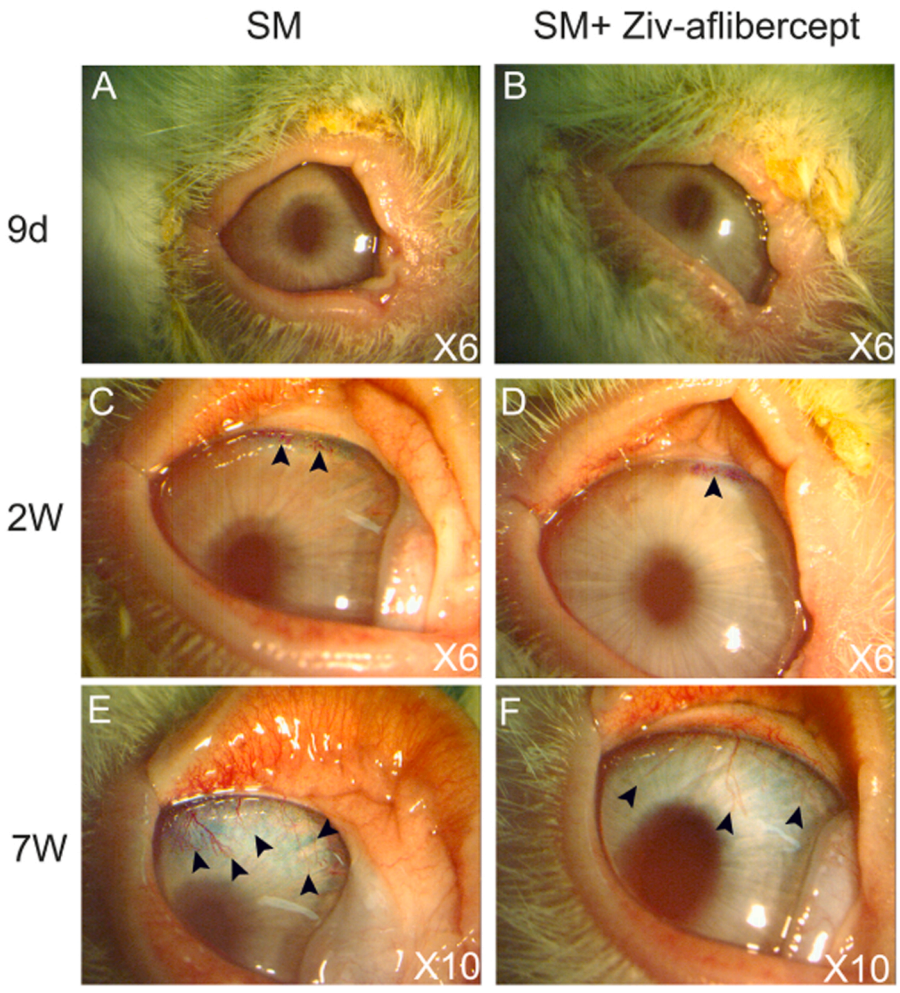

Fig. 4. The effect of ziv-aflibercept treatment 9 days post-exposure on ocular insult following SM exposure. Nine days after SM exposure, half of the eyes were not treated (A, C, E), and they were compared to the single 2 mg (80 µl) subconjunctival ziv-aflibercept-treated group (B, D, F). Representative slit-lamp pictures at 9 days show a reduction in insult severity following the acute phase (A, B). Two weeks post-exposure, NV sprouting was observed in the nontreated (C) and treated groups (D). Seven weeks post-exposure, NV growth was more dominant in the nontreated group (E) than in the ziv-aflibercept-treated group (F). Pictures were obtained by using a slit-lamp microscope with a magnitude of X6 (A-D) or X10 (E, F). Corneal NV is indicated by black arrowheads.

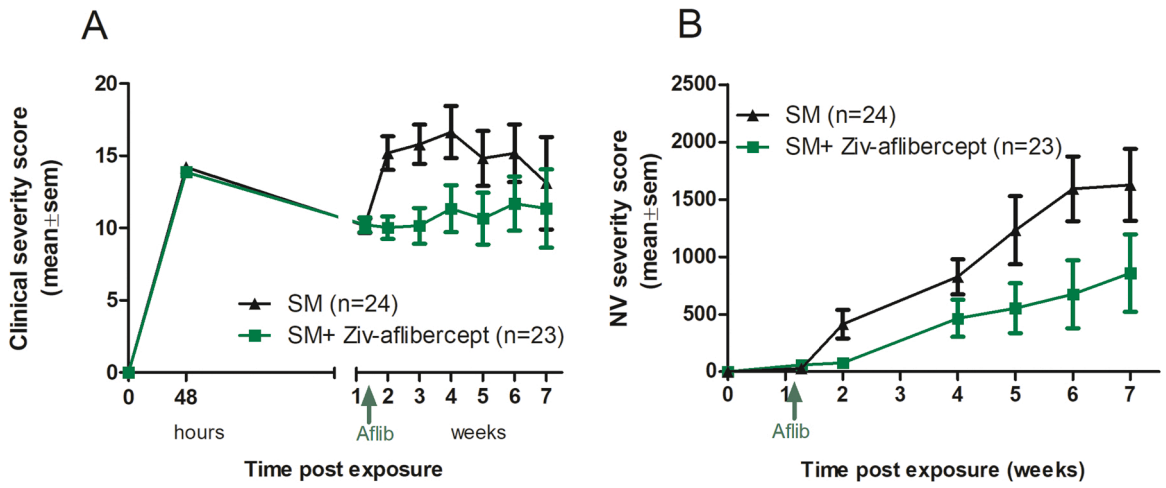

Fig. 5. Clinical efficacy evaluation following treatment administered 9 days post-exposure. (A) Semiquantitative assessment of ocular surface clinical manifestations up to 7 weeks following SM exposure and ziv-aflibercept single subconjunctival treatment (2 mg/80 μl) based on our scoring scale. (B) Semiquantitative assessment of the NV extent in ziv-aflibercept-treated and nontreated SM-exposed corneas. For the clinical severity score assessment, differences between groups were significant at $p < 0.05$ at 2, 3, 4 and 5 weeks. For the NV severity score assessment, differences between groups were significant at $p < 0.05$ at 5, 6 and 7 weeks. The time of treatment is noted by a green arrow.

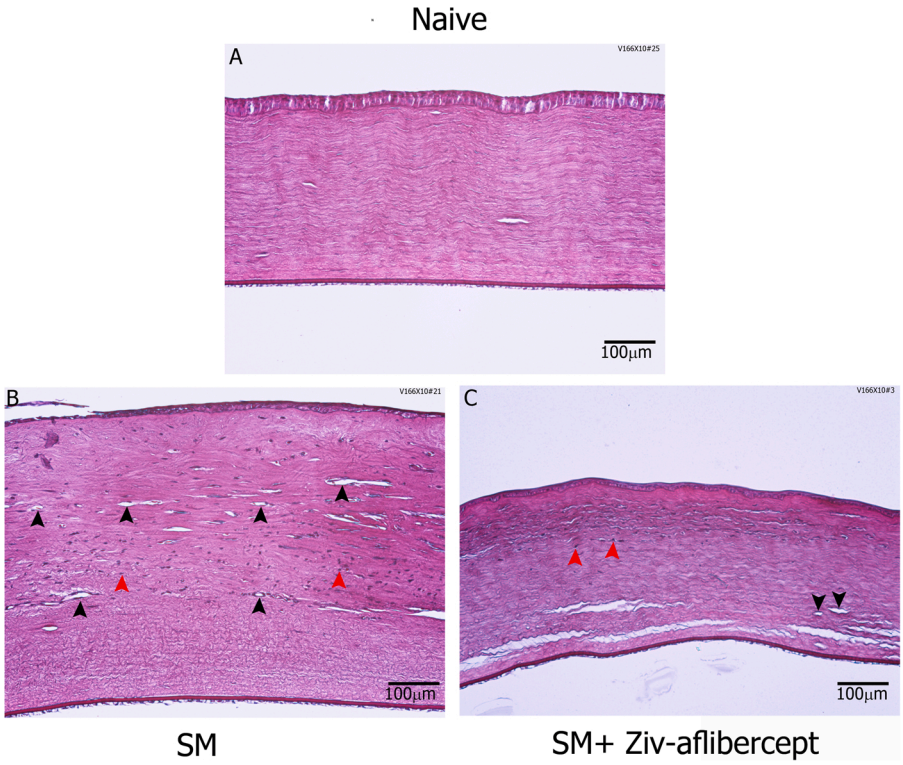

Fig. 6. Histological assessment of corneal tissues after SM exposure and ziv-aflibercept treatment administered 9 days post-exposure. Naïve (A), nontreated (B) and subconjunctival ziv-aflibercept (2 mg/80 μl)-treated (C) corneal representative sections taken 7 weeks following SM exposure were evaluated for morphological changes, NV appearance and inflammation. H&E staining was used for all sections. Reduced cell infiltration and NV was observed in the ziv-aflibercept-treated group vs. the untreated group. Red arrowheads denote inflammatory cells, and black arrowheads denote blood vessels. Magnitude X10, scale bar represents 100 μm.

the nontreated group (Fig. 9C). However, eyes treated with aflibercept 4 weeks post-exposure (only in NV-presenting eyes) in addition to the first week of dexamethasone treatment exhibited a drastic reduction in corneal insult with no inflammatory cells and no NV (Fig. 9D), similar to the naïve eyes (Fig. 9A).

4. Discussion

NV is a major vision-endangering corneal pathology that may evolve after chemical injury, SM exposure and many other ocular pathologies [17,30,42]. Due to the increased incidence of corneal NV worldwide [1, 28,30], substantial effort has been invested in developing new technologies to alleviate and prevent NV. New drugs originally developed as antitumor therapies have been adopted as antiangiogenic therapies in ophthalmic pathologies involving pathological NV; thus, new technologies should emerge in the future. This study continues our previous study that showed the extraordinary ability of a single subconjunctival treatment with the antiangiogenic drug ziv-aflibercept to reduce existing corneal NV following ocular chemical insult [17]. The present study describes the potential for ziv-aflibercept as a prophylactic means to obstruct NV development and the role of aflibercept as a symptomatic treatment against corneal NV that developed despite steroid treatment during the first week after SM exposure. Administration of ziv-aflibercept 2 h post-exposure induced an inflammatory cell

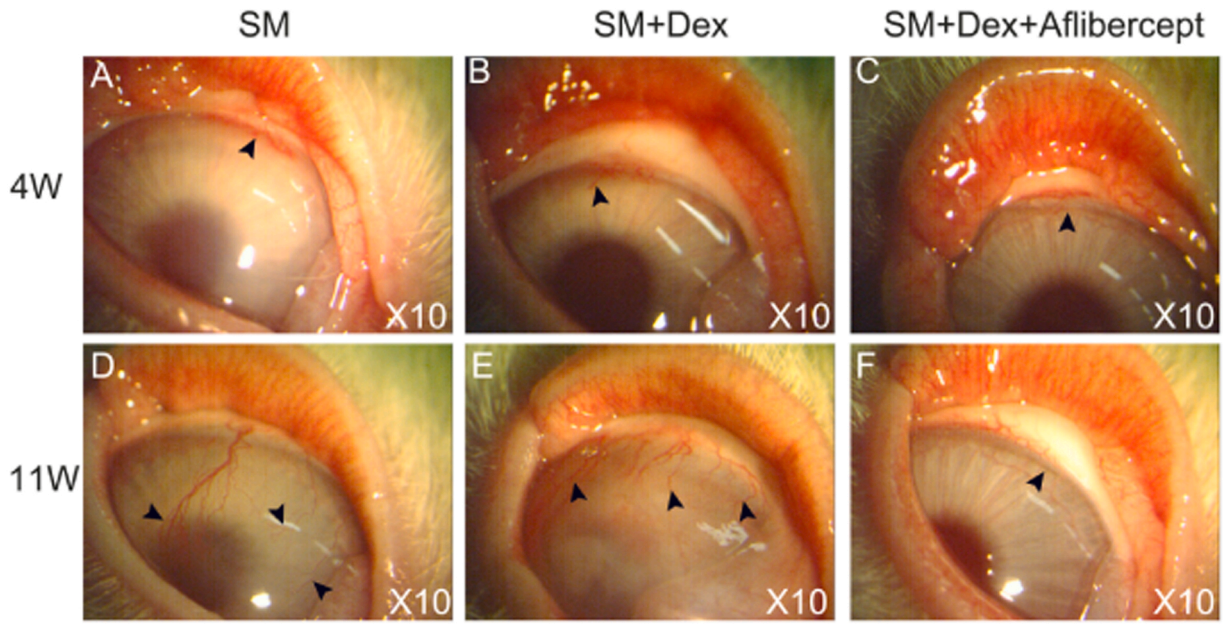

Fig. 7. The effect of aflibercept administration 4 weeks post-exposure and one-week dexamethasone treatment on long-term ocular insult following SM exposure-slit-lamp evaluation. SM-exposed eyes were divided into 3 groups: untreated (A, D), dexamethasone only treatment (B, E) and dexamethasone followed by aflibercept treatment (C, F). Representative pictures from all groups showing NV sprouting before aflibercept treatment was administered (A, B, C). Representative pictures from all groups showing NV corneal spread 11 weeks post-exposure and 7 weeks after aflibercept treatment (D, E, F). Pictures were obtained by using a slit-lamp microscope with a magnitude of X10. Corneal NV is indicated by black arrowheads.

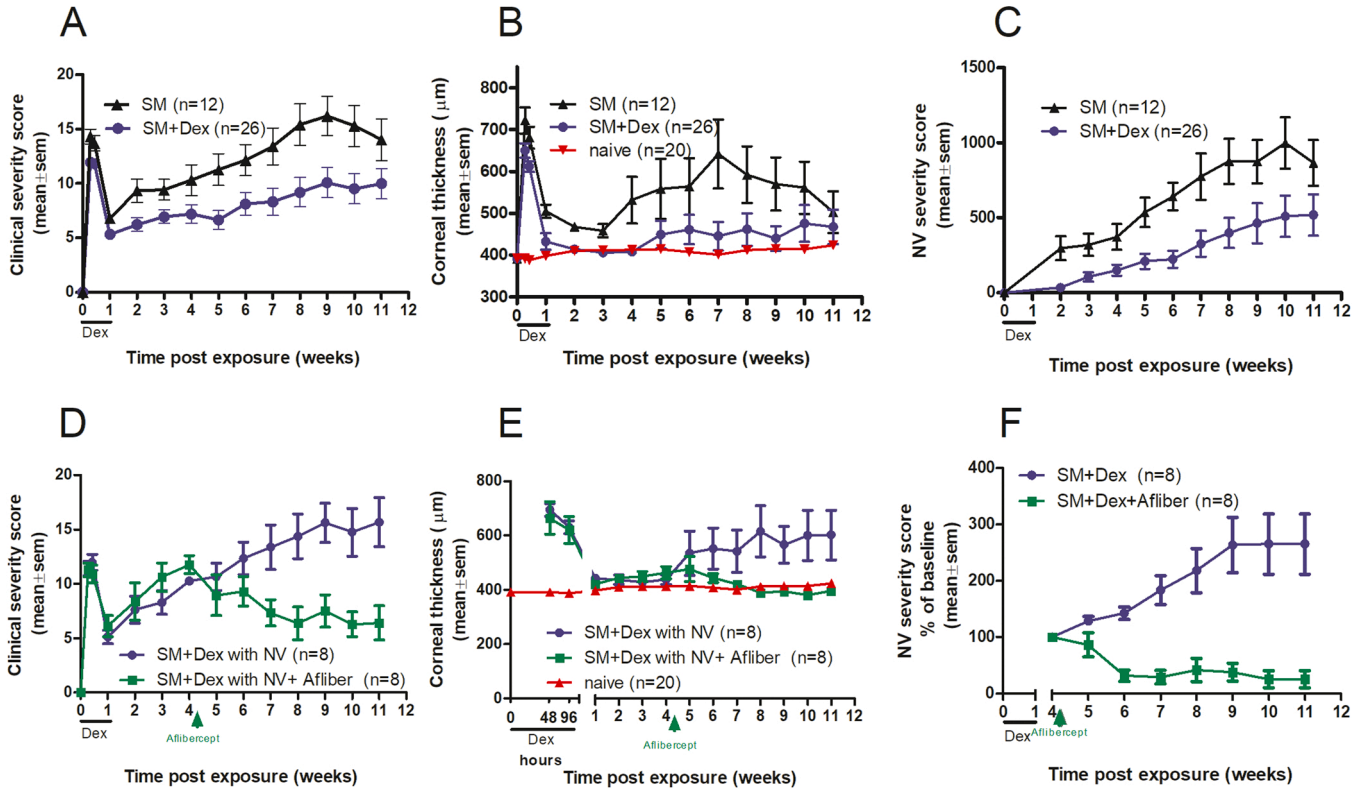

Fig. 8. Clinical evaluation of aflibercept treatment (4 weeks) in addition to dexamethasone treatment during the first week after SM exposure. Comparison between the untreated and one-week dexamethasone (dex)-treated groups after SM exposure at the 11 weeks follow-up (A, B, C). Semiquantitative scoring of the ocular insult based on our clinical score (A), corneal thickness (edema) measurement (B) and semiquantitative scoring of the NV extent in the dexamethasone-treated group vs. the nontreated eyes (C). Effect of aflibercept (Afliber; 2 mg/80 μl) treatment administered 4 weeks post-exposure in addition to the one-week dexamethasone treatment vs. dexamethasone treatment alone (D, E, F). Semiquantitative analysis of the ocular insult (D), corneal thickness (edema) measurement (E) and semiquantitative analysis of the NV extent (F). Dexamethasone and aflibercept treatment time points are denoted at the lower side of each graph. Statistical differences between the SM vs. SM + Dex groups (A, B, C) were significant at $p < 0.01$ at 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 weeks. Statistical differences between the SM + Dex vs. SM + Dex + aflibercept groups (D, E, F) in all graphs were significant at $p < 0.01$ at 7, 8, 9, 10 and 11 weeks.

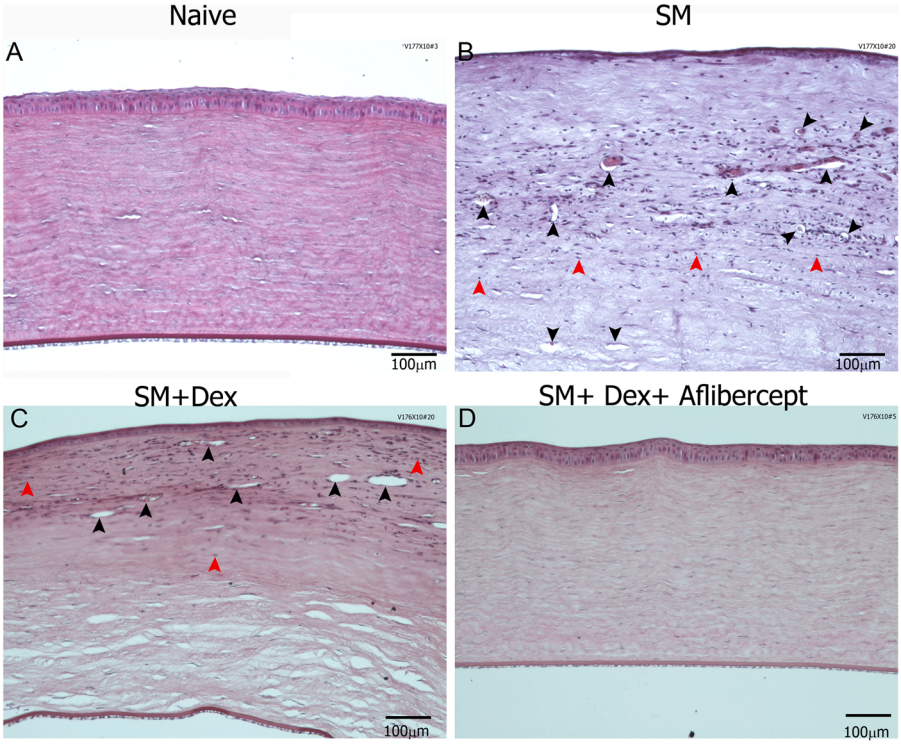

Fig. 9. Corneal histological evaluation following aflibercept treatment (4 weeks) in addition to dexamethasone treatment during the first week after SM exposure. Naïve eyes (A), SM-exposed nontreated eyes (B), SM-exposed eyes treated with 0.1% dexamethasone (QID) during the first week only (C) or with an added aflibercept (2 mg/50 μl) treatment administered 4 weeks post-exposure only in corneas presenting NV (D). Corneal representative images documented 11 weeks after SM exposure and treatments were analyzed for morphological changes, NV appearance and inflammation. H&E staining was used for all sections. A reduction in cell infiltration and NV was present in the dexamethasone group vs. the untreated group, and complete resolution was observed in the group receiving an additional aflibercept treatment. Red arrowheads denote inflammatory cells, and black arrowheads denote blood vessels Magnitude X10; scale bar represents 100 μm.

reduction as seen in histological Section 5 weeks post-exposure (Fig. 3C), in agreement with the results seen in a rat model of ocular chemical insult during the acute phase [43], although no clinical beneficial effect was seen during the first 2 weeks after SM exposure (Fig. 2A). However, this treatment prevented an increase in the clinical score evaluation from the 2 weeks' time point and presented fewer defects in histological sections at 5 weeks vs. the group receiving no treatment. The relatively low effect of ziv-aflibercept on the clinical score during the first 2 weeks may be attributed to its comparatively weak anti-inflammatory capacity or SM-induced endothelial layer functional damage, which is not affected by ziv-aflibercept treatment [24,35]. The decrease in the accumulating inflammatory cells observed 5 weeks post-exposure suggests that ziv-aflibercept treatment may induce more rapid corneal healing or even prevention or reduction of pathological NV formation and, in the long term, enhanced rehabilitation of the cornea. In vivo evaluation of corneal inflammatory cell infiltration at various time points after SM exposure and aflibercept administration may contribute to the mechanistic understanding of the treatment benefits. Inflammatory cellular reduction was observed in histology Sections 5, 7 and 11 weeks (Figs. 3, 6, 9) following the 2 h or 9 day post-exposure prophylactic ziv-aflibercept treatment or the 4 weeks symptomatic treatment, respectively, in comparison with the group receiving no treatment and the dexamethasone-treated group. These data are consistent with prior results demonstrating a decrease in $CD68^+$ macrophages in rats or $CD45^+$ in mice after corneal chemical alkali insult or suture followed by ziv-aflibercept topical treatment [29,43]. This decrease in cell infiltrate may be a result of NV regression due to ziv-aflibercept or aflibercept treatment, narrowing the main means of inflammatory cell transit into the cornea. Additionally, this finding may be associated with a decrease in proinflammatory VEGF-A activity [6,46], thereby directly reducing the inflammatory response.

The central factors involved in angiogenesis are VEGF-A, basic fibroblast growth factor (bFGF), IL-8, PlGF, TGF-β and VEGF-B. The use of anti-VEGF treatments against corneal NV following SM exposure was based on studies that revealed the high efficacy of these drugs in reducing corneal NV [8,48] and the elevated expression and involvement of VEGF in angiogenesis in corneal pathologies in general [51] and after SM exposure [23]. The rationale for using drugs that reduce pro-angiogenic factors is to prevent or reduce pathological blood vessel formation by stopping the processes involved in their development. In the case of corneal epithelial insult, VEGF elevation may be observed mainly in the basal epithelial layer [50] but also in the endothelial layer [51]. A previous study [23] showed the therapeutic potential of bevacizumab, an anti-VEGF-A antibody, which suppresses NV during the late phase following SM exposure, similar to studies showing its benefit in other animal models of NV and human NV cases [4,12]. Recently, ziv-aflibercept was shown to be much more effective than the previous generation of anti-VEGF treatments for suppressing existing blood vessels in ocular pathologies, including those arising after SM exposure [17,3,39].

Early treatment 2 h or 9 days following exposure and prior to blood vessel sprouting was partially effective in preventing and reducing corneal NV, consistent with other studies in which treatment was applied following corneal chemical insult but before NV formation [15,43]. Furthermore, treatment of existing NV induced a significant reduction in NV extent and prevented further NV growth (Fig. 8). The mechanisms for time-dependent treatment effectiveness are currently unknown. We postulate that a reduction in PlGF or VEGF-A and B by aflibercept treatment during the first stages of insult may conversely induce a massive and long-term secretion of other pro-angiogenic factors, such as bFGF, IL-8 or TGF-β, thus inducing only a reduction in NV sprouting and elongation without complete regression, as observed following symptomatic treatment. Future experiments evaluating VEGF-A, VEGF-B and PlGF levels and other pro-angiogenic factors in corneal tissue following exposure and treatment may shed light on the mechanisms of action. Furthermore, future experiments may provide data regarding the optimal treatment time and indicate if additional treatment would be beneficial.

Steroidal treatment is known to reduce inflammation, elevate cytokine levels [21] and decrease VEGF levels [37]. Despite this mechanism of action, we have shown here that corneal NV developed regardless of whether steroidal treatment was applied during the first week following SM exposure. This finding is consistent with previous reports [23] showing that dexamethasone treatment moderately reduced the extent

of corneal NV when given during the acute phase following SM exposure and as a symptomatic treatment after NV appearance. This finding could be explained by the fact that steroids can reduce VEGF levels (in a dose-dependent manner) but not the levels of other pro-angiogenic factors [10]; alternatively, NV development could be attributable to a possible VEGF elevation due to treatment termination 1 week post-exposure. Additional treatment with aflibercept 3 weeks post-dexamethasone likely reduced residual VEGF in the cornea, resulting in NV regression. Our previous study showed that ziv-aflibercept treatment effectively reduces pathological corneal NV with no need for initial steroidal treatment [17]. Nevertheless, this combined treatment starting with steroids enables a reduction in inflammation during the acute phase following the ocular chemical insult and prevents or reduces NV development in part of the eyes during the late phase. The addition of aflibercept decreased existing NV, prevented further NV development, and showed no unwanted interaction with the early steroid treatment. This treatment protocol of initial steroid treatment followed by aflibercept subconjunctival treatment is currently the best optional treatment in treating SM induced corneal delayed pathology and is easy to implement in a of mass casualty scenario.

5. Conclusions

To summarize, the results of this research demonstrate that one subconjunctival ziv-aflibercept treatment applied 2 h or 9 days after ocular SM exposure performed an anti-inflammatory role and exhibited a moderate effect in preventing NV formation starting 2 weeks post-exposure. Furthermore, combined sequential treatment with dexamethasone following the initial inflammatory response and aflibercept after the development of corneal NV resulted in a reduced inflammatory reaction and a long-term dramatic decrease in the extent of existing corneal NV. To conclude, aflibercept treatment may not only be beneficial for ameliorating SM-induced corneal NV but may also be highly valuable for mitigating chemical ocular injuries in general.

Author statement

The experimental protocols were approved by the Israel Institute for Biological Research animal care and use committee (IACUC; approval number RB-17–18 approved during 2017 and RB-19–17, approved during 2019) and were designed to prevent or minimize any unnecessary pain and stress.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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