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CROSS-LINKED CARBOXYMETHYL CELLULOSE AND SILK PROTEINS FOR DRY EYE DISEASE MANAGEMENT AND CORNEAL WOUND HEALING: *IN VIVO* AND *IN VITRO* RESULTS

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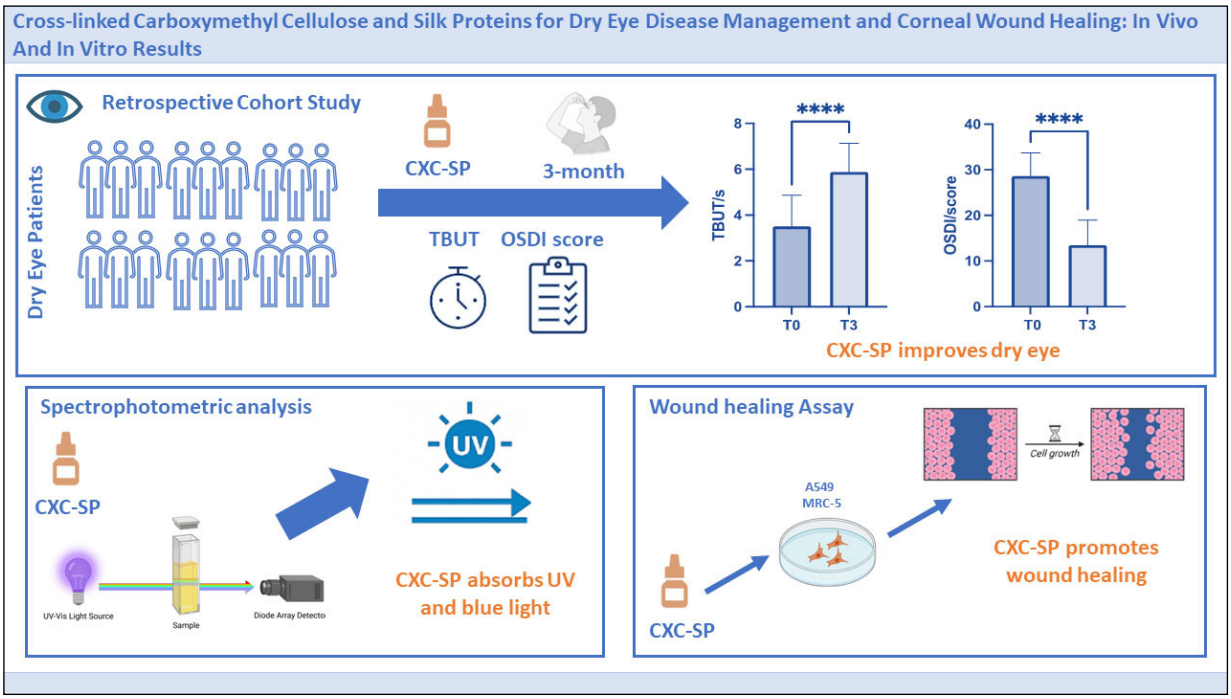
ABSTRACT – *Objective:* This retrospective cohort study aims to uncover the potential clinical effectiveness of a novel ophthalmic solution containing cross-linked carboxymethyl cellulose (CX-CMC) and silk proteins (SP) in dry eye disease, evaluating its efficacy in improving Tear Breakup Time (TBUT) and Ocular Surface Disease Index (OSDI) over a 3-months treatment and in absorbing ultraviolet (UV) and blue light and promoting wound healing.

Materials and Methods: Two different populations were retrieved from the database; the first group consisted of 20 dry-eye patients who underwent a 3-month treatment with an ophthalmic solution containing CX-CMC and SP. The second group was composed of 10 dry-eye patients who did not want to use any eye lubricant or other treatment. The outcome measures, TBUT and OSDI, were analyzed before (T0) and after (T3) treatment. In addition, the ophthalmic solution was investigated for its capacity to absorb UV and blue light and to promote wound healing. Specifically, UV and blue absorbance were tested by means of a UV-VIS spectrophotometer, while the wound healing test was conducted using two distinct cell lines to assess the efficacy of the solution in facilitating cicatrization.

Results: The ophthalmic solution effectively restored tear film stability, as evidenced by the improved TBUT values and reduced dry eye disease symptoms evaluated through the OSDI score. Furthermore, it demonstrated absorption capabilities within the UV and blue wavelength range. In terms of wound recovery, the ophthalmic solution supported cell motility compared to untreated cells.

Conclusions: The ophthalmic solution containing CX-CMC and SP was effective in improving dry-eye signs and symptoms in patients and in absorbing UV and blue light. Furthermore, the ophthalmic solution promoted wound healing *in vitro*. Collectively, the results suggest that CX-CMC and SP eye drops could serve as a promising tool for treating dry eye disease of various origins.

KEYWORDS: Tear film, Dry eye, UV protection, Silk, CMC, Cornea.



Graphical Abstract. A cross-linked carboxymethyl cellulose and silk protein ophthalmic solution improves tear film stability and dry eye symptoms after 3 months of treatment. *In vitro* analyses show UV/blue light absorption and enhanced cell migration, supporting a multifunctional role in ocular surface protection and corneal wound healing.

INTRODUCTION

Dry eye is a common ocular surface disorder characterized by an inadequate or aberrant tear film, affecting both the conjunctiva and cornea[1]. From a pathophysiological standpoint, Dry Eye Disease (DED) can be classified into two subtypes: aqueous-deficient dry eye and evaporative dry eye, which represent 80% of DED manifestations[1,2].

The prevalence of DED ranges from 5% to 50%, depending on the geographic location[3,4], marking it as a growing global medical concern. DED is associated with several risk factors, including age, sex, environmental conditions, prolonged screen use, and diabetes. Its pathogenesis involves a vicious cycle of tear film instability and inflammation, primarily driven by meibomian gland dysfunction, hormonal changes, and autoimmune disorders such as Sjögren's syndrome[5,6]. Moreover, DED alters the tear film, leading to reduced tear production and, ultimately, damage to the corneal epithelium and loss of conjunctival goblet cells, caused by friction from eyelid movement over the ocular surface. This, in turn, promotes inflammation and stimulates the production of inflammatory cytokines. These events play a pivotal role in the pathogenesis of DED, resulting in a cascade of damage to the ocular surface[2].

Diagnosis of DED relies on symptom evaluation through questionnaires [e.g., Ocular Surface Disease Index (OSDI), Dry Eye Questionnaire-5] and clinical tests such as non-invasive Tear Break-up Time (NIBUT), tear osmolarity measurements, and ocular surface staining with fluorescein or lissamine green[6,7].

The main goal of DED management is to restore ocular surface and tear film homeostasis. The first-line therapy for all degrees of severity is the use of artificial tears, which increase tear film stability, reduce evaporation, alleviate ocular surface stress, improve optical quality and contrast sensitivity, and ultimately enhance quality of life[8-11].

Artificial tears typically contain water-soluble polymers that act as lubricants, reducing friction and irritation. These polymers also enhance viscosity and improve mucoadhesion, thereby increasing tear film retention on the ocular surface[12]. Among them, carboxymethyl cellulose (CMC)-based artificial tears have demonstrated superior efficacy in controlling DED symptoms compared with other formulations[13-15]. The viscosity and mucoadhesive properties of CMC are key to prolonging eye drop residence time[16]. By binding to matrix proteins, CMC forms a protective epithelial layer that supports physiological epithelial cell growth, reduces epithelial defects and wounds[17,18], and lowers inflammatory markers[14,19]. Cross-linked CMC has been widely used in biomedical and pharmaceutical applications, offering technical and clinical advantages over its linear counterpart[17,20,21]. Cross-linked materials are, indeed, well known for their stability, since they are less susceptible to degradation[22].

In the context of water-soluble polymers, silk has a long history of use in medicine, specifically in ophthalmology, as a scaffold for the treatment of several eye disorders[23,24]. Silk is obtained from the cocoon of *Bombyx mori*, and its fibers consist of two main components: fibroin and sericin[23]. Both fibroin and sericin have been widely studied in a plethora of diseases, including eye-associated ones, and have shown an excellent safety and effectiveness profile. Silk has been shown to stabilize the tear film, creating a favorable environment for ocular surface recovery. Studies[25] in murine models of DED have highlighted its ability to improve corneal health and restore hydration by promoting tear film stability. Similarly, silk protein-based bioadhesive gels offer prolonged ocular surface retention, making them particularly effective in addressing chronic dryness and inflammation[26,27].

Considering corneal wound healing, silk-derived proteins enhance epithelial cell migration, adhesion, and proliferation, contributing to faster corneal recovery. These effects have been demonstrated in both *in vitro* models and animal studies[27,28]. Furthermore, silk-derived protein films with nanotopographic features have been shown to enhance corneal epithelial regeneration by mimicking extracellular matrix properties, further underscoring their therapeutic potential[29].

To date, there is no literature to support the potential use of an ophthalmic solution based on cross-linked CMC and silk proteins for DED management. Here, we report our experience with a cross-linked CMC and silk protein-based ophthalmic formulation in improving Tear Breakup Time (TBUT) and Ocular Surface Disease Index (OSDI) over a 3-month treatment period in patients with DED. In addition, we present *in vitro* data on the formulation's ability to absorb UV and blue light and to promote wound healing.

MATERIALS AND METHODS

Study design and participants

This was an observational, retrospective, cohort study designed to evaluate the clinical outcomes obtained on DED patients between the ages of 59 and 93 years. All the patients included in this study were diagnosed with DED, and the data were collected from patients who were seen during routine clinical examinations as part of standard clinical practice. Data from subjects affected by pathologies such as diabetes, glaucoma, or subjects using lubricant eye drops in the last 30 days were excluded.

Treatment

Within the study cohort, two subgroups were examined: one that agreed to use eye drops for the treatment of DED symptoms, and another that chose not to use any lubricant eye drops or alternative treatments. In the first subgroup, we identified twenty individual patients (40 eyes; mean age 72.04 ± 9.01, 75% female, 25% male) who were treated with an iso-osmolar ophthalmic solution containing cross-linked CMC (0.1%) and SP (0.05%) (CXC-SP) (CORD-EV, Ophtagon Srl, Rome, Italy). Patients received two drops three times daily for three months, a dosage treatment consistent with that approved by the regulatory authorities for this product. In the second cohort subgroup, which served as a control group, 10 patients were enrolled, matched for gender and age. Product sterility was achieved *via* a validated filtration with a 0.22 µm filter. The solution was preservative-free since the device used was able to filter the air entering the bottle, granting sterility up to 60 days post-opening.

Tear film break-up time (TBUT)

To evaluate the efficacy of the CXC-SP solution, the TBUT was assessed before (T0) and after (T3) 3 months of treatment. In particular, the TBUT test was performed using a sterile staining solution based on riboflavin 5-phosphate (SERVIMED, Rome, Italy). During the test, the dye is instilled into the tear film. The patient is asked not to blink, and the time between the last blink and the first appearance of a dry spot on the cornea is measured under cobalt blue light. A TBUT of less than 10 seconds is generally considered abnormal, indicating tear film instability and possible dry eye disease[30].

Ocular surface disease index

Similar to TBUT, the OSDI was assessed before (T0) and after (T3) 3 months of treatment. In particular, OSDI was scored on a scale of 0 to 100, with higher scores indicating more severe disease and a negative change from baseline indicating improvement[31].

The OSDI score, which consists of 12 questions assessing symptoms related to ocular discomfort, visual function, and environmental triggers, was administered with an inclusion threshold of ≥13, indicating the presence of clinically relevant symptoms. Any adverse events or complications that occurred during the study were recorded to evaluate the safety of the treatment.

Spectrophotometric measurements

The analysis of the absorbance as a function of wavelength was measured using a spectrophotometer model UVmini-1280 (Shimadzu, Kyoto, Japan) in the wavelength range of 200-500 nm. Samples were placed in disposable UV-VIS 1 cm

cuvettes. To remain in the working range of the spectrophotometer, the ophthalmic solution had to be diluted 10-fold. Therefore, two solutions containing CXC-SP were analyzed:

- Solution A: CXC-SP diluted 1:50 (used as blank)
- Solution B: CXC-SP diluted 1:10

The spectra were analyzed using the blank correction function, which eliminates background peaks due to water absorbance and thus provides a better baseline.

Cell lines and culture conditions

To evaluate the efficacy of CXC-SP in promoting wound healing, we selected two distinct cell lines: the A549 (lung carcinoma epithelial cells; ATCC accession number CCL-185) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) whereas the MRC-5 (fetal lung fibroblast cells immortalized with SV-40; ATCC accession number CCL-171) cell line was obtained from the Coriell Institute (Coriell Institute, Camden, NJ, USA). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (D6429, Corning, NY, USA) supplemented with 10% fetal bovine serum (35-079-CV, Corning, NY, USA), 100 µg/mL penicillin and streptomycin (30-002-CI, Corning, NY, USA), and 2.0×10^{-3} M L-glutamine (25-005-CI, Corning, NY, USA). A549 and MRC-5 were incubated at 37°C in 5% CO_2.

The two cell lines for the wound healing experiments were selected based on lineages present in the eye (i.e., epithelial cells and fibroblasts), which are capable of proliferating without the addition of growth factors, and for which a well-established and extensive literature on proliferation and migration kinetics was available. These characteristics will ensure reproducibility; indeed, A549 and MRC-5, unlike primary cell lines, do not rely on hormones and growth factors to replicate.

Wound healing assay

The wound healing assay was performed using the Ibidi Culture-Inserts 2 Well (81176, Ibidi cell focus, Fitchburg, WI, USA). These inserts are characterized by 2 silicon wells separated by a 500 µm gap, creating a cell-free region. After placing the insert in the center of a 24-multiwell plate, 70 µL of complete medium containing 4.5 × 105 cells (either A549 or MRC-5) was added to each of the two silicon wells. After 24 hours, the Culture-Insert 2 Well was gently removed using sterile tweezers. Cells were then treated with 1.0 mL of CXC-SP solution; cells treated with 1.0 mL of complete medium supplemented with 5% fetal bovine serum were used as a control group. The imaging acquisition started immediately after wounding application and continued for 30 hours, or at least until the cells reached full confluence. Experiments were performed in triplicate for each cell line and treatment.

Image acquisition

Image acquisition was performed at 10/0.22× magnification using a Leica DM IL LED microscope (Leica, Wetzlar, Germany) paired with a Samsung camera. The illumination was adjusted before the plate was placed on the microscope stage, and the acquisition positions were configured under the control of the camera immediately after the scratch was made. The resulting images were then processed in Adobe Photoshop (San Jose, CA, USA) to derive area values for the analysis of wound closure dynamics.

Methods for quantifying migration

Quantification of cell migration in the wound healing assay was performed using the area method[32]. The migration rate is indirectly evaluated as the percentage of wound area at a specific time point according to the equation:

$$A^t = \times 100$$

Where A^t is the wound area at time t and A^0 is its initial area

Wound healing: velocity calculation

To determine the average velocity of the wound edges, we first calculated the displacement between consecutive data points and divided it by the length of the wound. Next, we divided this result by 2 to find the average velocity of cells on each side of the wound.

Data analysis

Population data, TBUT, and OSDI results are presented as mean ± standard deviation (SD). Wound healing results are also expressed as mean ± SD, based on four independent experiments each performed in triplicate. The Kolmogorov-Smirnov statistical test was used to evaluate the goodness-of-fit of the datasets. Statistical significance between means was evaluated using either a *t*-test or a Wilcoxon test as appropriate (GraphPad InStat 3.1 Software Inc., San Diego, CA, USA), with significance considered at a *p*-value of ≤ 0.05. In particular, for both TBUT and OSDI, a non-Gaussian distribution was assumed; therefore, a non-parametric test has been used.

RESULTS

Cohort results

CXC-SP improves TBUT and OSDI

The age range of the analyzed subjects was 59 to 93, resulting in a mean age of 72.04 ± 9.01 (mean ± SD) years (95% CI 68-76). DED was mainly due to reduced tear production because of age. In the first subgroup, at the end of the 3-month treatment period (T3), both TBUT and OSDI parameters showed a significant improvement compared to T0. At T3, the TBUT score increased by approximately 40.7%, showing an improvement in the tear film stability (Figure 1a). Similarly, the mean OSDI score improved at T3, decreasing by approximately 52.9% (Figure 1b). At the same time, the second subgroup (20 eyes) that refused to use an eye drop had a TBUT of 3.95 ± 2.2 and an OSDI of 30.5 ± 2.9 at the baseline, with no significant change at follow-up.

In vitro results

CXC-SP absorbs UV and blue light

A spectrophotometric measurement was performed to evaluate the ability of the ophthalmic solution to absorb UV and blue light. Two solutions were compared: Solution A -CXC-SP diluted 1:50, used as a blank, and Solution B -CXC-SP diluted 1:10. Two absorbance peaks at 365.6 and 464 nm were found in Solution B compared to Solution A (Table I). This result confirms the characteristic broad absorption of SP below 450 nm, indicating that the ophthalmic solution can absorb UV and blue light.

CXC-SP supports wound healing

CXC-SP was able to support wound healing in the tested cell lines. In both A549 and MRC5 cell lines, a nearly constant rate of wound closure was observed from the first hours after scratching. Specifically, in the presence of CXC-SP, both A549 and MRC5 cells showed a significant increase in wound closure compared to the control group (Figure 2). In addition, the average speed of cell edge advancement during the time interval from 1 to 30 hours after scratching was compared. A549 cells treated with the CXC-SP showed a higher edge advancement speed (16.82 ± 0.26 µm/h) compared to the control group (14.8 ± 0.32 µm/h). Similarly, treatment of MRC5 cells with the ophthalmic solution resulted in an increased edge advancement speed (16.6 ± 0.23 µm/h) when compared to the control group (15.84 ± 0.41 µm/h).

DISCUSSION

As DED is a disease with an increasing prevalence worldwide, there is a growing need for the development of novel therapies for its treatment. The present study demonstrated that an innovative

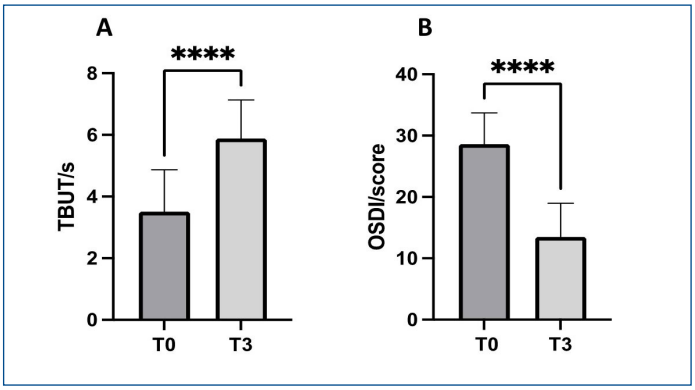


Figure 1. Subgroup one, Tear Breakup Time (TBUT) and Ocular Surface Disease Index (OSDI) score measured before (T0) and after the 3-month (T3) treatment. **A**, The tear film stability, measured as the time in seconds (sec) taken for the first dry spot to appear on the cornea after a complete blink at T0 and T3. **B**, OSDI score at T0 and T3. ****p-value ≤ 0.0001 (Wilcoxon test). Both values clearly demonstrate that there is an improvement in both objective and subjective dry eye disease-related symptoms.

Table I. UV-VIS wavelength and absorbance of CXC-SP solution. CXC-SP showed an absorbance peak at 365.6 and 464 nm, which was absent in solution A used as a blank. This provides clear evidence of the potential photoprotective properties of CXC-SP at the ocular level.

Sample	Wave length (nm)	Absorbance
CXC-SP 1:10	365.6	0.045
	464.0	0.039

CXC-SP: cross-linked carboxymethyl cellulose and Silk Proteins ophthalmic solution.

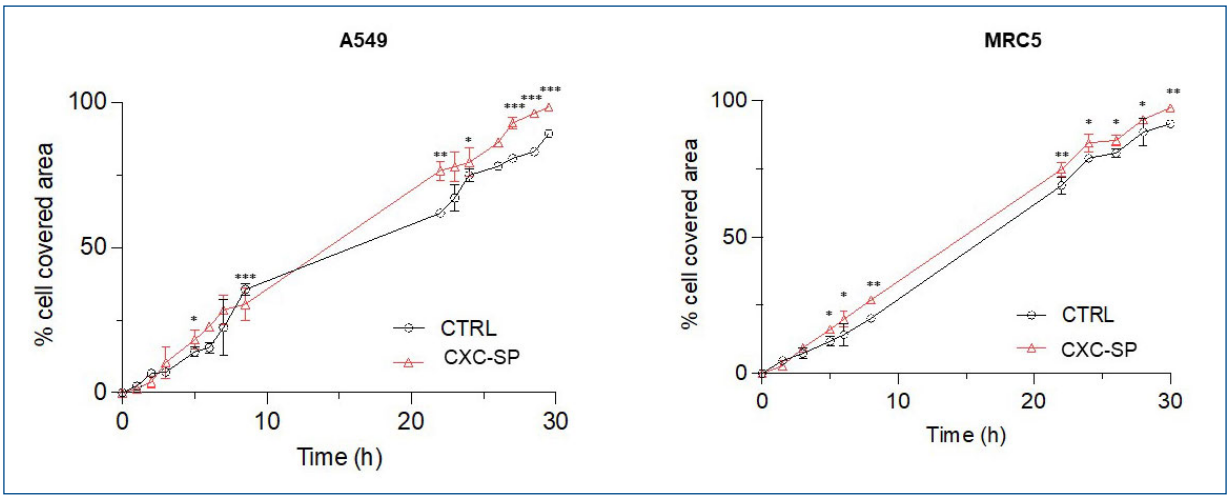


Figure 2. Effect of the ophthalmic solution on wound closure (% of covered area) in A549 and MRC5 cell lines. Mean values of wound closure were calculated from at least four repeated experiments and are presented as the mean ± SD. Student's *t*-test: **p*-value ≤ 0.05, ***p*-value ≤ 0.01, and ****p*-value ≤ 0.001, when CXC-SP treatment was compared to the control group. CXC-SP: cross-linked carboxymethyl cellulose and Silk Proteins ophthalmic solution.

ophthalmic solution containing cross-linked CMC and silk protein was able to improve both the objective and subjective symptoms of DED after 3 months of treatment. Furthermore, this solution exhibited the capacity to absorb UV and blue light, and the preliminary wound healing data collected suggested that the solution is able to facilitate physiological corneal healing processes.

While the use of linear CMC is well-established in several clinical settings, including the treatment of DED[19,31,33], in this research, CMC has been cross-linked to create a three-dimensional polymeric hydrophilic network capable of absorbing higher amounts of water compared to its linear version. Indeed, cross-linked CMC can reach an equilibrium between the thermodynamic swelling forces and cross-linking elastic retraction forces, thus forming a hydrogel[22]. The advantage of cross-linked CMC lies in its stability obtained through the cross-linking process and the lack of specific enzymes in the body that can degrade the molecule, making it extremely resistant. Additionally, cross-linked CMC is a hydrogel with unique properties for visco-supplementation, hydration, and drug delivery in its natural state[34].

In contrast to linear CMC, which is typically used in concentrations ranging from 0.5 to 1% in artificial tears[19,31,33], the results collected indicated that cross-linked CMC can alleviate DED symptoms even at a concentration as low as 0.1%.

In a recent systematic review[14], evidence about CMC efficacy was inferred from 64 different clinical trials. The study supports the effectiveness of CMC in improving the signs and symptoms of dry eyes, pointing to CMC as an adjuvant treatment for various conditions, including anterior eye trauma, infection, inflammation, and discomfort associated with contact lens use. Additionally, the study emphasized that artificial tears containing CMC can enhance epithelial healing; this is attributed to the presence of hydrogel-like components, which are known to activate the epidermal growth factor receptor. This activation promotes the healing of corneal epithelial wounds[35].

Silk fibers consist of two main components: fibroin and sericin[23,36]. Silk fibroin has a complex molecular structure, including a heavy and a light chain, which gives silk better mechanical properties than other natural and synthetic materials[37]. Fibroin is biocompatible, biodegradable, non-toxic, non-immunogenic, and has hemostatic properties[25,37,38]. Indeed, silk fibroin is used as a scaffold for the transplantation of tissue constructs, as a surgical reabsorbable suture material, as well as a wound dressing, and ophthalmic devices[39-41]. Moreover, the use of fibroin in DED restores tear volume, improves corneal smoothness, and results in the recovery of the corneal epithelial cells and conjunctival goblet cells[25].

Sericin is a globular protein consisting of random coil and β-sheets. Its biochemical features give sericin important biological properties such as biocompatibility, antioxidant, and moisturizing properties[42]. The amino acid composition of sericin is identical to the human Natural Moisturizing Factor, a natural component of the human skin structure[43]. The human Natural Moisturizing Factor is a mixture of various water-soluble and hygroscopic substances capable of absorbing and retaining water. This factor is important for maintaining the hydration of the outermost layer of the epidermis[44]. Due to its high serine content (30-33% of the total amino acids) and its amino acid composition very similar to human Natural Moisturizing Factor, sericin has moisturizing and

anti-ageing effects on the skin[45]. An *in vivo* study on the moisturizing effect of sericin on human skin highlighted its action in increasing the level of hydroxyproline and the hydration of epidermal cells, due to its occlusive effect, which prevented transepidermal water loss, a factor responsible for skin dryness[43]. In an animal model[46] of type 2 diabetes mellitus, corneal lesions healed faster when treated with sericin diluted in saline, and when added to the medium of a corneal cell line, sericin induced increased adhesion. Moreover, sericin possesses photoprotective properties, absorbing UV rays in the range of 223-300, inhibiting tyrosinase, reducing oxidative stress, COX-2, and cell proliferation, and retaining water[36,45,47].

In vitro and *in vivo* data on the efficacy of silk protein on corneal epithelium wound healing, UV light absorption, and DED treatment were already documented[25,27,28,45,47-49]. In particular, in the manuscript of Abdel-Naby et al[28], the authors used an alkaline burn animal model of corneal wound healing to test the ability of SP to promote re-epithelialization. It is worth mentioning that, for both concentrations used in their study, a significant improvement in the percentage of wound closure occurred after 6 h. Similarly, both cell lines used in the present study showed similar wound closure kinetics when treated with CXC-SP.

In line with this scenario, our results show that the inclusion of silk protein in an ophthalmic formulation containing cross-linked CMC was effective in improving tear film stability (TBUT value), confirming what was previously observed in a mouse model of dry eye[25]. In addition to the objective measurement of DED symptoms, patients also reported a subjective improvement of their symptoms as assessed by the OSDI score. The observed clinical efficacy of the ophthalmic solution could be related to the synergic action of both molecules.

CXC-SP was successful in the UV and blue light absorption test *in vitro*, and this result was consistent with previous work demonstrating the ability of SP to absorb UV light wavelengths[45,47,49]. To further evaluate the potential use of the ophthalmic solution in supporting corneal wound healing, two different cell lines were tested: an epithelial-derived (i.e., A549, resembling corneal epithelium) and a fibroblastic-derived (i.e., MRC5). As observed in other studies[46,50-53] for corneal epithelial cells treated with silk protein, the cell lines used showed a better wound healing property compared to untreated cells, both in terms of wound closure rate and speed of cell edge progression. These results confirm that CXC-SP, with its composition comprising cross-linked CMC and silk proteins in a single formulation, can reproduce the well-known properties of its individual components, including restoring the tear film stability and DED symptoms, UV and blue light absorption, enhanced wound healing capacity, and thus becomes a promising tool in the treatment of DED symptoms.

Recently, a clinical trial[54] on about 500 subjects investigating the clinically beneficial effects of silk proteins in dry eye has been published. In particular, researchers have been able to demonstrate that silk-proteins can significantly increase TBUT *vs.* control at days 28 and 56, and patient symptomatology measured *via* "Symptom Assessment in Dry Eye" score from baseline. Our findings are well in line with these results and further expand them by highlighting the ability of CXC-SP to promote wound healing and absorb UV and blue light.

Based on the reported results, we hypothesize that the mechanism of action through which CXC-SP achieves its therapeutic effect is linked to a silk-enhanced scaffold as previously described[55]. While the results presented in this manuscript are intriguing, there are limitations to the study, including a small participant pool, which highlights an opportunity for further exploration of the topic. In particular, a powered enough randomized controlled trial, eventually with multiple control groups (i.e., CMC alone, silk protein alone), is auspicious to fully elucidate and define the potential superiority of the CXC-SP treatment compared to routinely used lubricant eye drops or some other pharmacological or non-pharmacological therapeutic options. At the same time, a corneal re-epithelization study in an animal model is recommended to further support the *in vitro* evidence on the re-epithelization properties of the CXC-SP ophthalmic solution.

CONCLUSIONS

In conclusion, the present study provides valuable insights into the potential efficacy of a novel ophthalmic solution containing cross-linked CMC and silk protein in improving the objective and subjective symptoms of DED disease by absorbing UV and blue light to support epithelial wound healing.

Conflict of Interest

The authors declare no financial or personal relationships that could have influenced the work reported in this paper. D&V FARMA Srl had no role in the study design, data collection, statistical analysis, data interpretation, or manuscript preparation. The study was independently designed and conducted by the investigators within hospital and university settings.

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Authors' Contributions
Conceptualization, FS and ADM.; investigation, FS, CC, and FB; biochemical experiments and formal analysis, GDS; writing and reviewing, FS and ADM.; editing, GC, and GP. All authors have read and approved the final manuscript.

Data Availability
The data supporting this article will be made available by the authors upon request.

Ethics Approval
The study adhered to the Declaration of Helsinki and its later amendments. The study was approved by the Ethics Committee "(CET) Lazio Area 3" of the Fondazione Policlinico Universitario A. Gemelli IRCCS with protocol number ID: 7509 and date 27/05/2025. The authorization covered the retrospective analysis of fully anonymized clinical data collected between April 2023 and September 2024. No interventions beyond standard clinical care were performed.

Informed Consent
Informed consent was obtained from all subjects involved in the study.

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