Efficacy of Standardized and Quality-Controlled Cord Blood Serum Eye Drop Therapy in the Healing of Severe Corneal Epithelial Damage in Dry Eye

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Purpose: We standardized quality-controlled cord blood serum (CBS)–based eye drops and evaluated the efficacy of 1-month CBS treatment in the healing of diseased corneal epithelium in severe dry eye (DE) patients.

Methods: Seventeen graft-versus-host disease (GVHD) and 13 Sjogren syndrome patients with severe persistent corneal defects were enrolled in the framework of a registered clinical trial (ClinicalTrials.gov NCT01234623). Sterile CBS eye drops were prepared to supply 0.15 ng per eye per day epithelial growth factor and administered for 1 month in a 1-day dose dispensing. The extent of epithelial defect was evaluated in square millimeters area, and subjective symptom score (Ocular Surface Disease Index score), Schirmer test I, break-up time, tear osmolarity, corneal esthesiometry (Cochet–Bonnet esthesiometer), conjunctival scraping, and imprint cytology with goblet cell count were performed at baseline (V0) and after 15 (V1) and 30 (V2, endpoint) days of treatment. Satisfaction and tolerability questionnaires were evaluated at V1 and V2.

Results: A significant reduction was shown at the endpoint versus baseline in corneal epithelial damage (mean ± SD, 16.1 ± 13.7 vs. 40.9 ± 30 mm^2/area, respectively), discomfort symptoms (Ocular Surface Disease Index score, 22.3 ± 10.3 vs. 39.3 ± 16.9), scraping cytology score (3.8 ± 1.2 vs. 6.6 ± 2.1), and tear osmolarity (312.5 ± 7 vs. 322 ± 9.1 mOsm/L), whereas a significant improvement was shown in corneal esthesiometry (48.2 ± 2.1 vs. 49.7 ± 2.1 nylon/mm/length, $P < 0.05$). All patients reported a high degree of satisfaction upon drop instillation.

Conclusions: Heterologous CBS-based eye drops represent a promising therapeutic approach in the healing of severely injured corneal epithelium and in subjective symptom relief. These drops can be obtained as readily available and quality-controlled blood derivative from cord blood banks on a routine basis.

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Failure of the ocular surface epithelia occurs in many clinical conditions with differing pathogeneses; the core of medical management is to stabilize or promote new growth of healthy conjunctival and corneal epithelia. A range of therapeutic approaches has been introduced and considerable advances have been achieved in recent years with the administration of topical products based upon blood preparations. These include eye drops prepared either from the patients' own blood such as autologous serum (AS)[1,2] plasma rich in growth factors[3] and platelet lysate[4] or from donors such as umbilical cord blood serum (CBS).[5] The rationale for the use of all these stands upon their content of biologically active components and in particular of growth factors, shown to be higher than in patients' tears.[6]

CBS, in particular, contains various growth factors at a very high concentration and offers several advantages over the other blood-derived preparations, primarily because a large amount of serum can be obtained from the umbilical vein at one time so that many patients can benefit from this sampling and repeated blood collection from patients are avoided. In addition, the difficulty of obtaining blood from patients with poor general condition or blood dyscrasia and from those with infectious diseases is also avoided. Finally, the unsuitable application of eye drops containing proinflammatory cytokines, as may occur in eye drop preparations from AS in some diseases such as graft-versus-host disease (GVHD)[7] or Sjogren syndrome (SS),[8] may be prevented.

CBS eye drops were found to be more effective than AS in healing epithelial defects,[9] and their efficacy has been successfully demonstrated in various conditions, from severe dry eye (DE) with or without primary Sjogren syndrome (SS-I)[9] to ocular GVHD,[10] persistent epithelial defects,[11] recurrent corneal erosions,[12] chemical burns,[13] and neurotrophic keratitis.[14] Interestingly, the epitheliotropic capacity of CBS in promoting limbal stem cell activity has been recently demonstrated also in corneal cell culture models.[15]

Despite CBS therapeutic potentials, details about the real growth factor content in CBS eye drops used to treat patients were not reported in those previous articles nor were CBS

donor selection, Good Manufacturing Practice preparation procedure, serological and microbiological quality assurance controls, treatment duration, or patient satisfaction.

The purpose of this study was to standardize the protocol and procedure of CBS eye drop use to evaluate its efficacy in the healing of corneal epithelial defects and amelioration of painful subjective symptoms.

MATERIALS AND METHODS

Patients

The study was conducted under the local Institutional Review Board/Ethics Committee approval (EudraCT: 2008-005757-38; ClinicalTrials.gov NCT01234623) and the protocol followed the guidelines of the Declaration of Helsinki.

Seventeen GVHD (11 men, 6 women, mean age, 38.8 ± 5.8 years, 33 eyes) and 13 SS-I (all women, mean age, 59.6 ± 13.1 years, 26 eyes) patients exhibiting severe corneal involvement at the time of enrollment, graded as grade 4 to 5 according to the Oxford grading level,[16] were included in the study. Eight of 17 GVHD patients and 5 of 13 SS patients had experienced AS therapy not administered by our unit; therefore, details could not be recorded. Informed consent was obtained from each enrolled subject.

The patients were diagnosed as having severe DE according to the Dry Eye Workshop (DEWS) severity level.[17] Exclusion criteria were previous ocular surgery performed in the year leading up to the visit, wearing contact lenses, punctual plug placement or cauterization, and ocular allergy. Subjective symptoms of DE were graded on the basis of the DE discomfort symptoms questionnaire of the Ocular Surface Disease Index (OSDI).[18]

Upon arrival for our evaluation, 24 of 30 patients were using various types of tear substitutes, gels, and ointments, whereas the remaining 6 were using antiinflammatory topical drugs. To normalize the study population to an identical regimen of eye drops/medication use, subjects who met the inclusion criteria of the study were dispensed hyaluronic acid–based eye drops, to be used in both eyes for 4 days (washout period).

Methods

A slit-lamp evaluation and subjective symptom score were performed at baseline (V0) and after 2 (V1) and 4 (V2, endpoint) weeks of treatment. The following tests were performed at V0 and V2.

Corneal sensitivity was measured using a Cochet–Bonnet esthesiometer (Luneau, Chartres, France) and recorded in millimeters per nylon filament length. A measurement of less than 50 mm was considered low corneal sensitivity.[19]

Schirmer test I and break-up time (BUT) were performed according to the DEWS guidelines.[20]

Tear osmolarity was performed with TearLab Osmolarity System as described elsewhere[21]; the cutoff value for severe DE was considered as more than 324 mOsm/L.[21]

To evaluate inflammation, scrapings were performed in both eyes at the lower and upper tarsal conjunctiva, and smears were read following the Scraping Cytology Score System described elsewhere,[22] pathological values of score more than 4 of 12.

Impression cytology samples were obtained from the lower nasal bulbar conjunctiva using strips of polyethersulfone membrane filters, 0.20 μm (Supor-200) as described.[20] The degree of squamous metaplasia was divided into grades (0 to 3) using the grading scheme of Tseng,[23] and periodic acid-Schiff–positive goblet cell (GC) density was calculated as the number of cells per square millimeter (media of 4 ×100 magnification consecutive visual fields).[24]

Corneal epithelial damage was evaluated in square millimeter area at V0, V1, and V2. Briefly, corneal photography was taken after the administration of a 2 μL volume of 1% fluorescein dye, at a magnification of ×16 using a camera attached to a slit-lamp microscope equipped with a 7503 Boston yellow filter kit (equivalent to Kodak Wratten 12) to enhance staining details. Gel Pro Image Analyser Software (Media Cybernetics, Bethesda, MD) was used to estimate the area of damaged corneal epithelium on acquired digital photographs, matched in comparison with a standard calibration area.

Visual Analog Scale[25] (VAS) satisfaction and tolerability questionnaires were provided to patients after 2 (V1) and 4 (V2, endpoint) weeks of CBS treatment. These were compared with a VAS satisfaction questionnaire provided to patients at enrollment, concerning their opinion about the last therapy administered before the study.

Treatment—Preparation of CBS Eye Drops

Total quality system and good manufacturing practice facilities were used. The umbilical cord blood was obtained from mothers with vaginal or cesarean section delivery after informed consent. Tests to detect the presence of infection (hepatitis B and C virus, human immunodeficiency virus, TPHA, TOXO, CMV, HIV/HBV/HCV-NAT, HTLVI/II) were performed at delivery and after the "window period" of 6 months (quarantine) during which infections cannot be detected but the donor may be infectious, according to European Medicines Agency regulations. In Food and Drug Administration regulations, NAT tests are recommended and not mandatory.

A mean volume of 80 mL of the umbilical cord blood was collected from the umbilical vein, and blood was clotted for 2 hours at room temperature. After centrifugation at 3000 rpm for 15 minutes, the serum was carefully isolated under a laminar flow hood (BacT/Alert; Biomerieux; sterility tests was performed in each batch). Serum was frozen at −80°C for 6 months (quarantine period).

To assess whether the procedure and storage could reduce growth factor content, levels of epithelial growth factor (EGF) and transforming growth factor (TGF)-β1 were tested in 7 CBS preparations, in the different procedure step points (Elisa Kit Quantikine; R&D Systems, Abingdon, UK). Screening of CBS to be used as the eye drop preparation

source was achieved by EGF content analysis before CBS storage (EGF content >1.5 ng/mL was selected as threshold).

After the quarantine, the preselected sera were thawed and pooled to obtain the needed amount of serum to treat all patients, diluted to 20% with refrigerated sterile phosphate-buffered saline with aseptic technique, and filtered (Millex HV 0.4 μm). The preparation was then aliquoted into luer-lock cap 1-mL sterile syringes. Filled syringes were sealed and packed in single labeled envelopes for delivery to patients. A single syringe was used for the daily supply of CBS preparation. In 5 of 30 patients, the COL-20 medical device (Biomed, Modena, Italy) was used to prepare mono-dose vials, packed, frozen, and stored as above. Patients were instructed to administer one drop per eye for 8 times a day, after having thawed 1 syringe or vial the evening before the day of use.

Statistical Evaluations

Statistical evaluation was performed by using MedCalc and SPSS 14 software and applying the Wilcoxon signed-rank test to determine the significance of changes after CBS eye drop therapy ($P < 0.05$ was considered to be statistically significant). Pearson (r) or Spearman (ρ) correlation coefficients were applied when appropriate; correlations were considered statistically significant at $P < 0.05$ (small correlation strength, 0.10–0.29; medium 0.30–0.49; large, 0.50–1.00). Descriptive statistics for tests and variables analyzed in subjects are reported as mean ± SD.

RESULTS

The preparation of CBS eye drops is explained in Figure 1. Data from EGF and TGF-β1 determinations in CBS are shown in Figure 2; the content of these factors did not show any significant variation throughout the procedure. EGF content was found to be well correlated to CD34+ cell content (Pearson r = 0.54, $P < 0.001$). EGF supply was estimated as 0.15 ng per eye per day in the final preparation.

All patients administered CBS eye drops for the entire study period and were fully compliant with the treatment regimen. Neither patient dropout nor any adverse event was registered. Patients successfully and homogeneously responded to CBS treatment, with no appreciable difference between those affected by GVHD and those suffering from SS-I. All parameters evaluated were found to have changed to a statistically significant extent from baseline to endpoint in all patients. Subjective symptoms of ocular surface discomfort significantly decreased after 2 weeks of treatment, and further at endpoint (Table 1). Corneal sensitivity, Schirmer test I, and BUT recovered with respect to baseline and all significantly increased at endpoint (Table 1). Tear osmolarity, inflammatory score evaluated by scraping cytology score, and conjunctival epithelium squamous metaplasia evaluated by imprint cytology score significantly decreased at endpoint versus baseline (Table 1). Periodic acid-Schiff–positive GC density did not show any increase in 14 of 30 patients; in these patients GC absence had been found in imprint samples at baseline. In the remaining 16 patients, GC density significantly increased from baseline to endpoint (number of GCs per square millimeter, mean ± SD; 57.6 ± 51.9 vs. 126.8 ± 79.8/mm^2, respectively, $P < 0.001$).

A highly significant reduction of corneal epithelial damage was shown in all eyes from baseline as early as 2 weeks of treatment, which was further confirmed at endpoint (Fig. 3A). Only 2 of 59 eyes healed completely after 4 weeks of CBS treatment; in these eyes, the corneal epithelial damage at baseline had been estimated below 10 mm^2. The extent of initial corneal damage correlated with the magnitude of the corneal responses, measured by the area of injured epithelium at endpoint (Pearson r = 0.7, $P < 0.0001$). Baseline epithelial damage less than 25 mm^2 healed almost completely after 1 month of CBS treatment (Fig. 3B). No correlation was found between corneal responses and duration of epithelial defects or of systemic disease diagnosis nor between SS and GVHD patients.

Pre–post variations (ie, endpoint minus baseline value) for all parameters were calculated and correlated with each other. A significant correlation was found (r = 0.41, $P = 0.02$) between amelioration of subjective symptoms and inflammation score reduction in the whole population; the extent of initial corneal damage greater than 25 mm^2 increased the correlation strength (r = 0.62, $P < 0.01$).

A significant difference was observed with the overall satisfaction questions estimated by a VAS score, already after 2 weeks of CBS treatment and at the endpoint visit (Fig. 4). The E point columns in the figure show the answers given by patients at enrollment, which refer to their feeling about the last topical therapy administered.

DISCUSSION

The central core of DE is now thought of as a downward spiral in which cascading inflammatory processes are initiated by tear hyperosmolarity, and the final stage comprises loss of compensatory mechanisms[26]; the process can lead to ocular surface tissue damage with effects varying from mild to severe.

Current treatment options are targeted to the disease severity level.[27] At severity levels 3 and 4 (the most severe), AS therapy is recommended because of its supplement in growth factors and other nutrients needed for proliferation, migration, and differentiation of the corneal and conjunctival epithelium. These natural components may support the healing of injured ocular surface epithelia, in cases of diminished tear growth factor content, as may occur in DE disease.[28] The use of AS in the ophthalmic setting dates back at least 3 decades, but despite its proved efficacy there is no standard procedure of preparation, quality control, storage, and administration.[29,30]

In more recent years, the platelet lysate obtained from nongelified autologous platelet-rich plasma has attracted increasing interest[31] because platelet-rich plasma is also a source of a variety of growth factors with an important role in the wound-healing process in many tissues.

To overcome the need for peripheral blood sampling from each single patient, heterologous blood sources have been proposed, with the advantage that they can be obtained as readily available and quality-controlled blood derivatives

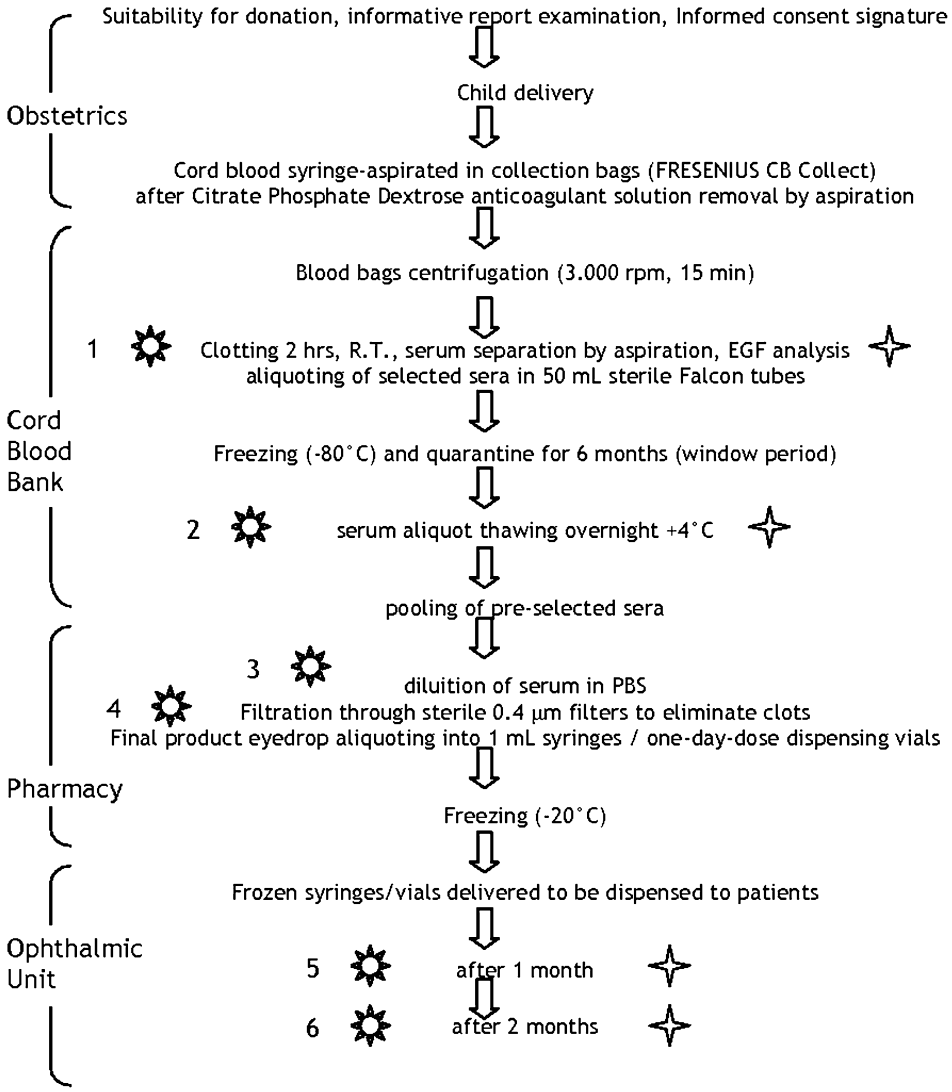

FIGURE 1. CBS preparation procedure is summarized from donor suitability to delivery. All steps were conducted in UNI EN ISO 9001:2008 certified laboratories. ✹ indicates the points of the procedure where determination of EGF and TGF-β1 were carried out, numbered 1 to 6 to match data summarized in Figure 2. ✦ indicates the points where sterility controls were performed.

from blood banks on a routine basis. In this perspective, CBS eye drops may represent a powerful nonimmunogenic biological supplement source because the preparation contains an elevated growth factor content, higher than in peripheral blood serum[6,9,10] and natural antiinflammatory cytokines.[32]

In the present article, we showed that there is a certain biological variability in mother donors with respect to the presence of growth factor in CBS, and optimizing donor selection and collection strategies is a matter of ongoing research. In the present study, we showed a strong correlation between EGF and CD34+ cell content, but not with the obstetric factors including gestational age, parity of the mother, sex and weight at birth of the newborn, weight of the placenta, and mode of delivery. Because CD34+ cell content is routinely estimated by blood banks, particular efforts should be made to collect CBS from donors with a high CD34+ cell content to preselect the serum and reduce preparation time and costs.

The efficacy of CBS in the healing of persistent corneal epithelial defects has been reported in the past literature[5,9–14] and evaluated by the clinical evidence of injured area reduction after treatment. The results had been related mainly to growth factor supplementation, but recent in vitro data demonstrated that CBS-supplemented culture medium supports not only the proliferation but also the differentiation of human conjunctival and limbal epithelial cells.[15]

However, previous articles did not report any details in terms of the selection of the donor, standardized protocol for microbiological quality assurance, or growth factor content in the final preparation. The present study examined these issues and the data obtained showed that EGF and TGF-β1 content did not change significantly through the various preparation steps, quarantine and storage periods. The eye drop solution used in the present study was prepared to achieve a standardized EGF content estimated as 0.15 ng per eye per day in the final preparation, and this allowed us to avoid bias of response related to composition variability.

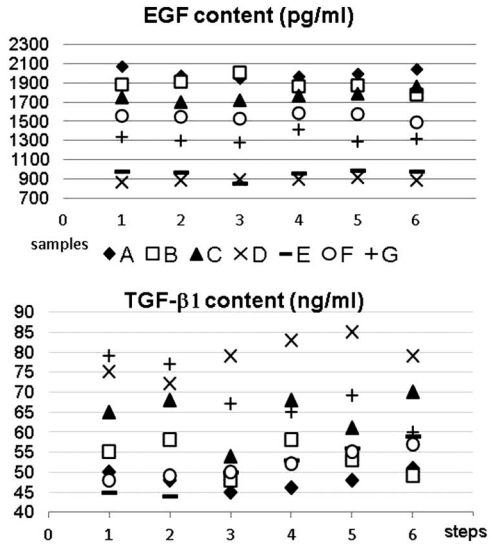

FIGURE 2. Evaluation of EGF and TGF-β1 content in different step points of the preparation procedure of CBS eye drops, preliminarily carried out on 7 (A through G) samples. Steps were numbered 1 to 6, as detailed in Figure 1 and specifically as follows: 1: freshly collected CBS; 2: after the quarantine period; 3: after dilution; 4: after filtration; 5: after 1 month of freezing; 6: after 2 months of freezing. A considerable biological variability was shown in both EGF (700–2100 pg/mL) and TGF-β1 (800–1900 ng/mL) over the samples analyzed, but no significant variation was observed throughout the whole process, suggesting that the procedure does not affect or reduce the content of both growth factors.

Because of cost limitations, growth factor content control was restricted to EGF only, although we recognize that a wider range would be recommended. EGF, a cytokine secreted by the lacrimal gland, was arbitrarily selected as the biomarker to standardize the final product for its mitogenic effects and role in ocular surface homeostasis.[28,33] However, the introduction of pooling collection could be a reasonable compromise in the standardization of the final product, in contrast to the one donor–one patient basis used in previous works.

In this study, we demonstrated that 1 month of CBS treatment significantly reduced injured corneal epithelium area and improved painful disease sensation in all enrolled patients. Complete epithelial healing was achieved only in 2 of 59 eyes, whereas in another 23 eyes a nearly complete resolution was achieved; the extent of initial corneal damage but not duration of epithelial defects correlated with response, suggesting that CBS treatment should be initiated at the earliest and attempted also in those cases with a long history of corneal epithelium damages. Systemic diseases did not apparently affect the response to the treatment, although the relatively small number of patients did not make it possible to statistically analyze data for stratification.

The ocular surface is considered as a functional unit and several parameters were shown to be improved as a consequence of epithelial healing in this study. BUT and Schirmer test results significantly increased at endpoint, in agreement with previous reports,[10,11] although a normal value was never detected in any eye after 1 month of CBS treatment.

Conjunctival epithelial metaplasia was also shown to be significantly improved, confirming the in vitro data[15] on the role of CBS in promoting human conjunctival epithelial cell proliferation. Inhomogeneous results were obtained with respect to conjunctival GC recovery after CBS treatment; an increase in GC density was demonstrated in agreement with previous studies,[11] but no treatment response was observed in eyes showing GC loss at baseline. Perhaps this is related to the duration of the CBS therapy, and we could speculate that a longer period of treatment is needed to promote GC differentiation. More likely, CBS eye drops for this study were prepared by checking EGF content only, and it is feasible that other suppliers more specific for GC promotion[34] were insufficient in the final product. Preselection of CBS from donors based on more growth factor content (in addition to EGF) is planned for future studies.

TABLE 1. Descriptive Statistics for Tests and Variables Analyzed in Patients From Baseline (V0) to Endpoint (V2) After CBS Treatment

Test	Measure	V0	V1	V2	*P*
OSDI	Score	39.3 ± 16.9	24.4 ± 10.3	22.3 ± 10.3	V0 vs. V1, $P < 0.0001$ V0 vs. V2, $P < 0.0001$ V1 vs. V2, $P < 0.004$
Corneal esthesiometry	mm/nylon filament length	48.22 ± 2.85		49.66 ± 2.42	<0.0001
Schirmer test	mm/wet strip after 5 inch	2.64 ± 2.31		3.38 ± 2.05	0.0001
BUT	Seconds	5.1 ± 3.2		5.7 ± 3	<0.0001
Tear osmolarity	mOsm/L	322 ± 9.1		312.5 ± 7	<0.0001
Scraping cytology	Score	6.6 ± 2.1		3.8 ± 1.2	<0.0001
Imprint cytology	Score	1.89 ± 0.45		1.64 ± 0.51	0.0002

Results are summarized (mean ± SD) from baseline to endpoint for subjective symptom reduction (OSDI score), corneal esthesiometry recovery, Schirmer test increase, BUT increase, tear osmolarity decrease, ocular surface inflammation reduction (scraping cytology), and conjunctival epithelium metaplasia decrease (imprint cytology). All data were shown to be statistically significant.

$P < 0.05$.

OSDI, Ocular Surface Disease Index; V1, intermediate step point after 15 days of CBS treatment.

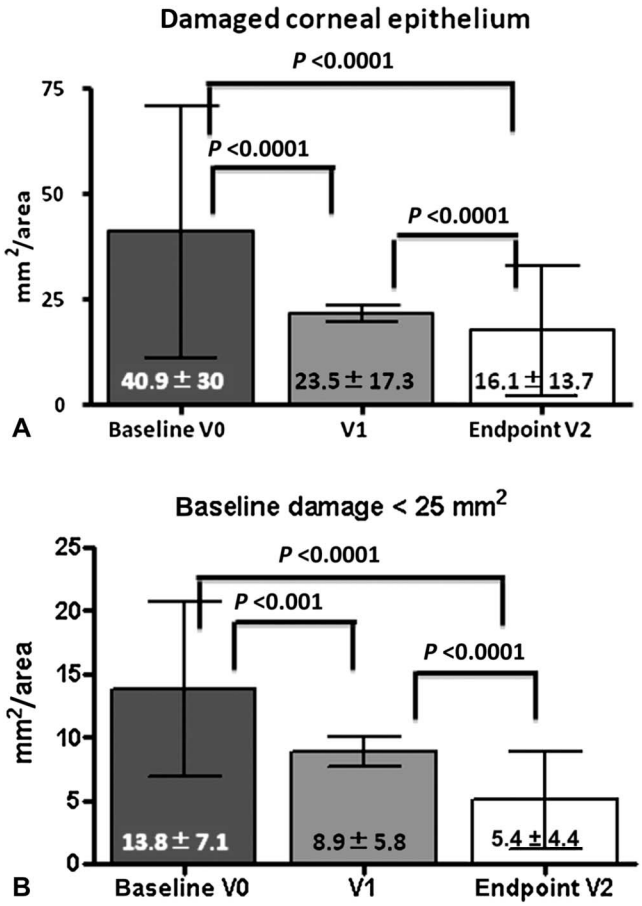

FIGURE 3. Corneal epithelial damage estimated as square millimeter per area (mean ± SD) at baseline and after 2 weeks (V1) and 1 month (endpoint, V2) of CBS treatment. Data from the whole population showed that epithelial damage was significantly reduced as early as 2 weeks of treatment and further at endpoint (A). Data from the population stratified by initial epithelial damage showed that baseline damage less than 25 mm^2 healed nearly completely after 1 month of CBS treatment (B).

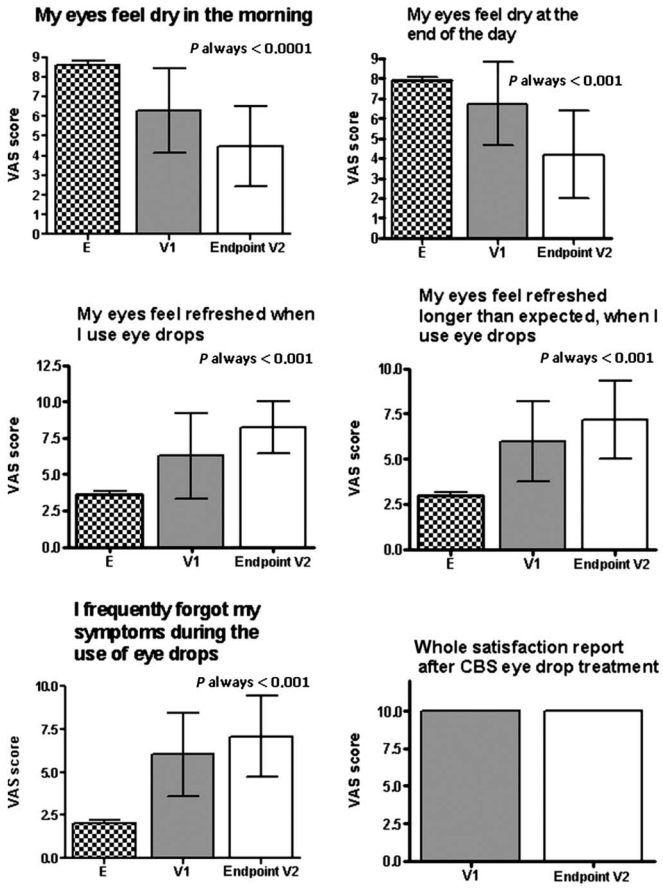

FIGURE 4. Subjects' satisfaction analyzed with a VAS score system, with the improvement of subjective symptoms at 2 weeks (V1) and at the endpoint (V2) visit after CBS treatment. The E point columns in the figure show the answers given by patients at enrollment, which refer to their feeling about the last topical therapy administered.

Subjective discomfort symptoms as measured by Ocular Surface Disease Index were demonstrated to be significantly reduced already after 2 weeks of CBS treatment. This amelioration did not occur as a consequence of a decrease of the sensitivity threshold as occurs in injured eyes. On the contrary, corneal sensitivity was shown to be significantly increased after CBS treatment, which suggests the occurrence of neural fiber promotion during epithelial healing, but we were not able to confirm this result in vivo by confocal analysis.

Discomfort symptom reduction was demonstrated to be in correlation to a significant decrease of inflammatory score after CBS treatment in all subjects. The relationship between subjective symptoms and ocular surface inflammation has been debated in the literature[22,35] and correlated to the increase of interleukin (IL)-6 in tears of DE patients. In addition, the presence of natural antiinflammatory cytokines such as IL-10 has been documented in cord blood[36]; the plasma concentrations of this protective cytokine reaches far higher levels in infants than adults. We did not evaluate the IL-10 content in the final CBS eye drop solution in the present study; the appropriateness of estimating its content in the serum preselection phase is now strongly recommended for future preparations.

The role of tear hyperosmolarity in determining pain sensation has recently been hypothesized and demonstrated by our group in DE patients and in an in vitro human conjunctival primary cell culture model,[37] and in analogy with these results, we suggest that the subjective symptom decrease after CBS treatment could also benefit from the reduction in tear osmolarity shown in this study.

The level of satisfaction with the treatment was very high for all patients, who homogeneously reported a significant improvement of their discomfort as early as 2 weeks, reaching full satisfaction at endpoint. DE associated with epithelial corneal impairment considerably affects patients' quality of life and involves an increasing burden to the patient as the disease becomes more severe. We are currently evaluating whether discomfort symptom relief lasts longer and how long in the patients' follow-up after CBS treatment.

In conclusion, in this work we confirmed that CBS eye drops are a promising therapy for the healing of persistent epithelial defects in severe cases of DE (DEWS severity levels 3 and 4). BUT, Schirmer test, tear osmolarity, corneal esthesiometry, and conjunctival epithelial metaplasia significantly improved after 1 month of treatment. Subjective symptoms of pain and discomfort were satisfactorily relieved after 1 month as a possible consequence of inflammation decrease. The extent of area of corneal injury rather than its duration is the main factor determining the early response and repair process.

CBS eye drops need to be prepared by well-equipped and trained laboratory staff. Preparation steps include efficacy and microbiology controls for a reproducible quality of the final product that can be prepared in advance in 1-day doses and stored at −20°C ready to be dispensed.

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