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Autologous versus allogeneic versus umbilical cord sera for the treatment of severe dry eye disease: a double-blind randomized clinical trial

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ABSTRACT.

Purpose: **To measure the effects of Autologous serum (AS), Allogeneic Serum (HS) and Umbilical Cord serum (CS) eye drops in severe dry eye disease (DES), as well as to characterize and quantify several molecules in the three sera (albumin, fibronectin; Vitamin A and E; IgG, IgA and IgM; Transforming growth factor β; Epithelial growth factor).**

Methods: **Randomized, double-blind, single-centre, three-arm (AS, HS and CS) clinical trial. Sixty-three subjects were included with severe DES, 21 in each arm of the study. Visual acuity, Schirmer test, Breakup time (BUT), lissamine green, fluorescein staining measurements and a questionnaire were performed prior to treatment, and after one-month and three-month follow-up.**

Results: **There was a significant main effect of time on visual acuities, Schirmer and BUT tests and fluorescein and lissamine green staining measurements and questionnaire scores ($p = 0.015$, $p = 0.002$, $p < 0.001$, $p < 0.001$, $p = 0.031$ and $p < 0.001$, respectively), although there was no significant interaction between time and serum type, nor between serum type and the test performed. Regarding the concentration of molecules, in our study AS contained significantly higher concentrations of IgA, IgG and fibronectin whereas HS contained significantly higher concentration of IgM, vitamins A and E, TGF and albumin. Contrary to previous reports, CS did not show higher concentration of any of the molecules analysed.**

Conclusions and relevance: **The three sera were effective in the treatment of severe DES. CS did not contain a higher proportion of molecules compared to AS/HS. More research is needed to assess the effect of AS in patients with DES and autoimmune diseases.**

Key words: allogeneic serum eye drops – autoimmune disease – autologous serum eye drops – dry eye – growth factor – haemoderivatives eye drops – ocular surface – Sjögren's syndrome – umbilical cord serum eye drops

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Introduction

Dry eye syndrome (DES) is a prevalent health condition that affects patients' quality of life (Miljanovic et al. 2007; Gayton 2009) and means an important economic burden in developed countries (Pflugfelder 2008). Much has progressed over the last few years within the field of DES physiopathology and treatment. However, current therapeutic outcomes may be frustrating due to the chronic nature of the condition without a specific etiological treatment.

Conventional treatments include environmental modifications, tear film substitutes, topical and/or systemic corticosteroids, tetracyclines and immunomodulators such as cyclosporine A. In severe cases, surgical techniques (Murube 2003) as punctal occlusion (Yu 2004), submandibular gland transfer (Borrelli et al. 2010; Qin et al. 2013) and several eyelid procedures (Murube 2003) may be employed. An encouraging alternative is the use of blood-derived sera that not only provide hydration but also nutrition and growth factors.

Therapeutic effects of autologous serum (AS) are known since the 1980s (Fox et al., 1984), but widespread use did not begin until the 1990s (Tsubota et al. 1999). AS presents similar pH and osmolarity than the tear film. Its mechanism of action is based both on the lubricant properties and the concentration of vitamins A and E, growth factors and fibronectin (Nelson & Gordon 1992; Bosch-Valero et al. 2008) that promote cellular trophism within the epithelium thus improving regeneration. Besides, AS indirectly enhances epithelial viability by binding and neutralizing inflammatory cytokines (Yoshino et al. 1996; Tsubota et al. 1999). AS also holds bactericide components – lysozyme, lactoferrin and immunoglobulins, thus reducing the risk of contamination and infection (Geerling et al. 2004).

Over the last few years, the applications of AS in the ocular surface have expanded due to the increase of conditions in which it might be indicated besides DES, such as chemical burns, Stevens–Johnson disease or Sjögren's syndrome (SS) (Tsubota 1999), persistent epithelial defects (Young 2004), recurrent erosions (Benítez del Castillo 2002), neurotrophic ulcers (Matsumoto 2004), graft-host disease (Ogawa 2003), aniridic keratopathy (López-García 2008) and superior limbic keratoconjunctivitis (Goto 2001). However, there are concerns regarding the use of AS in some situations, such as active systemic inflammation or infection and when venepuncture is contraindicated or inconvenient (Chiang et al. 2007). Therefore, allogeneic serum (HS) may be an alternative for these patients.

Umbilical cord serum (CS) has recently been applied in DES (Yoon et al. 2006) and EICH (Yoon et al. 2007b) with good outcomes. Presumably, its effects are superior to those of AS and HS (Campos et al. 2020; Yoon et al. 2007a). Moreover, CS, as well as HS, can be prepared in large quantities from a single donor, be used for many patients, as well as being useful in patients who may not be ideal candidates for AS drops (Marks & van der Meer 2017).

The aims of the present study were to measure the effects of AS, HS and CS on the tear film and ocular surface in patients with severe DES over three months of treatment and compare them, as well as to characterize and quantify the three serum modalities by means of: protein levels of albumin, fibronectin; vitamin A and E; IgG, IgA and IgM; transforming growth factor β (TGF-β); epithelial growth factor (EGF).

This is the first study comparing the effects of these three types of sera for the treatment of DES to date, to the authors' knowledge.

Material and methods

This was a randomized, double-blind, single-centre, three-arm (AS, HS and CS) clinical trial for the comparison of the effects of three types of sera for the treatment of severe DES. Objective and subjective criteria were assessed prior to treatment, one month into treatment and after a three-month follow-up. Patients were recruited among those who attended the Hospital Regional Universitario de Málaga from September 2011 through May 2015.

The study protocol was approved by the Ethics Committee of the research institution and was performed according to the tenets of the Declaration of Helsinki. Characteristics of the clinical trial and potential risks were explained to the patients prior to enrolment. Upon patient agreement, two copies of informed consent forms were signed and then the clinical history was completed, and a comprehensive ophthalmologic assessment was carried out, quantifying the degree of dry eye and ocular surface alteration, as well as any other concomitant ophthalmological pathologies.

Inclusion criteria implied two or more of the following: symptoms of DES for more than 3 months that did not improve with conventional treatment (i.e. artificial tears and/or ointments) and/ or Type I Schirmer test ≤5 mm and/or Tear breakup time (BUT) ≤5 s and/or Oxford test ≥ grade III. Exclusion criteria included less than 18 years of age, pregnancy, contact lens use, history of ocular allergy within the three months prior to enrolment, intraocular or refractive surgery within the six months prior to enrolment, eyelid and/or eyelash anatomical alterations, or infectious contagious disease (HIV, HCV and HBV). DES patients complying with two or more of the inclusion criteria and none of the exclusion criteria previously mentioned were selected. Subjects with ocular pathologies different from dry eye disease or cataract diagnosis were also excluded.

Size sample calculation

To determine the minimum sample size, advanced statistical methods were used due to the complexity of the study, considering three treatment groups to be compared with each other. The starting point (pilot sample) was the weighted means and standard deviations of Yoon's study (2007a) in which he compared AS with UC in the treatment of dry eye. The millimetre (mm) has been taken as the unit of measurement. For the Schirmer test, an average of $\mu = 3.63$ mm and a weighted standard deviation of $\sigma = 1.45$ mm (corresponding to the aforementioned work) have been considered. If we take 25% as the estimation error of the measurement, we obtain in expression (1) a $\delta = 0.90$ (0.9 being 25% of 3.63). It is a moderately high value since the coefficient of variation (CV) in our case is:

$$CV = \frac{\sigma}{\pi} = \frac{1.45}{3.63} = 0.399 (\approx 39.9.\%).$$

To apply the analysis, we consider a significance $\alpha = 0.05$, the sample size (n) of each of the groups and the minimum difference $\delta = 0.9$ between the means, which can be detected with a power of 1 - β, are related by equality: (Douglas, 2001, n.d.)

$$= \sqrt{\frac{n\delta^2}{2kS^2}} = \sqrt{\frac{n0.9^2}{2\,3\,1.45^2}}.$$

The power 1 – β and φ are related by means of a family of curves that depends on the degrees of freedom $\nu 1 = k - 1 = 2$ and on $\nu 2 = k\,(n–1)$. It must be taken into account that the power used must meet $\nu_2 > 81$ ($n–1 > \frac{81}{3} \Rightarrow n - 1 > 27$). For a power for the test of 0.7, $1.6 \Rightarrow \varphi = \sqrt{\frac{n0.9^2}{2\,3\,1.45^2}} \Rightarrow 41$ (N total = $3 \times 41 = 123$).

Randomization

After enrolment, patients were attended in the Regional Blood Transfusion Centre of Málaga, where blood extraction was carried out, independent of the type of serum to be applied. The laboratory technician carried out elaboration of collyria, as well as early

detection tests for infectious disease. Once infectious diseases were ruled out, the haematologist randomized the treatment allocation of patients' eyes. Such randomization was stratified by means of block minimization to ensure balance between groups. The patient then received a closed envelope with the number of the assigned container, so that masking was effective for both the patient and the ophthalmologist. The eye drops were stored in the Regional Blood Transfusion Centre of Málaga. Patients collected the eye drops on days 1, 19, 38 and 57.

The patients were instructed on the collyrium instillation technique and the importance of therapeutic compliance. Previous to serum use, a washout period of two weeks with only carmellose eye drops five times was required. Once finished, serum was used five times daily (instillation was recommended every three–four hours during daytime, with nocturnal rest). The ophthalmologist, following the same protocol as previously described, re-evaluated the patients one month and three months after the beginning of the treatment period.

Serum preparations

The manufactured drug consisted of a 20% dilution in a solution of Sterile Irrigating Solution, BSS® of AS from patients, allogeneic serum from AB-blood donors or umbilical cord samples that were not valid for the transplant of haemopoietic progenitors. Currently, there is no consensus on the concentration at which to prepare serum eye drops (Meer et al. 2016; Marks & van der Meer 2017), but the 20% concentration seems (Geerling et al. 2004) sufficient and avoids the discomfort derived from the higher viscosity of more concentrated preparations and considerably reduces the number of blood draws. The time elapsed between the cord blood collection and the preparation of the CS eye drops was less than 24 hrs, but this period was certainly higher compared to the preparation time of AS and HS eye drops (around three hours from the flebotomy to the introduction of the aliquot in sterile bottles) (Geerling et al. 2004).

Serum determinations

The three serum-based treatments used in this study were analysed measuring the following chemical and biological determinations: IgA, IgG and IgM immunoglobulins (measured in mg/dl), A and E vitamins (μg/dl), EGF and TGF (ng/ml), serum albumin (g/dl) and fibronectin glycoprotein (μg/ml).

Subjective and objective patient measurements

All the patients in the three groups were evaluated three different times. At baseline, patients were given the serum treatment according to their analysis group, as well as the instructions to be followed during the whole duration of the study. All the measurements were carried out in both eyes, although in this study, only the results from the right eye were included (Karakosta et al. 2012).

Objective measurements for the analysis of the disease evolution included, following the guidelines from the International Dry Eye Workshop Clinical Trial Design Subcommittee (International Dry Eye Workshop (DEWS), 2007), the following parameters were included in the clinical trial: Tear film volume and production determination by type I Schirmer test without topical anaesthesia (Alcon Tear Test Schirmer, Alcon Labs, Forth Worth, Texas). Schirmer test was performed first to avoid bias in the BUT test; Assessment of corneo-conjunctival epithelium status using lisamine green strips (Dina Strip Lissaver-Plus V, Gecis labs, Villemorant, Neung-sur-Beuvron, France) and using van Bijsterveld classification (Bron et al. 2003), Tear film stability by fluorescein tear film breakup time (BUT) (Sodium Fluorescein 20 mg/10 ml, Xalabarder Farma, Spain); Ocular surface integrity assessment by fluorescein staining (Colircusí Fluotest, Alcon Labs, Forth Worth, Texas) using Oxford scale (Bron et al. 2003), Subjective patient symptoms assessment by a validated dry eye questionnaire (Donate et al. 2002). The second and third visits were scheduled 1 and 3 months after the baseline visit, respectively, and the same objective and subjective measurements were taken in each one.

Statistical analysis

Measurements were evaluated using SPSS v.24 (IBM Corp., New York, NY, USA). Normality was evaluated by the Kolmogorov–Smirnov test, while homogeneity of variances was evaluated by the Levene's test. The statistical significance limit was set at $p < 0.05$ in all cases.

Chemical and biological determinations of the three sera types

To test the differences between the three groups for each dependent variable, and in case that homogeneity of variances between groups can be assumed, a one-way ANOVA or a Kruskal–Wallis test was applied depending on whether the distributions for the dependent variables were or were not normally distributed, respectively. And in case of significance, *post hoc* Tukey or Mann–Whitney tests were applied to test for pair group differences. In case that homogeneity of variances cannot be assumed, a Welch ANOVA or a median test was used depending on whether the distributions were or were not normally distributed, respectively. And in case of significance, post hoc Tamhane or median tests with Bonferroni correction were applied pairwise.

Subjective and objective patient measurements

A two-way 3×3 mixed ANOVA test (between-subjects independent factor: serum type; within-subjects independent factor: time) with repeated measures on time variable was used for each dependent variable to study the effect of serum type, time and the possible interaction between them on the dependent variable. Prior to the mixed ANOVA, the homogeneity of variance–covariance matrices assumption was checked using the Box's M-test, while the sphericity assumption was checked using the Mauchly's test. The Greenhouse–Geisser correction was applied in those cases in which sphericity could not be assumed. Multiple post hoc group comparisons with Bonferroni correction were performed when the ANOVA procedure revealed statistically significant differences between groups.

Results

Demographics

A total of 125 eyes of 63 patients, 55 women and eight men, with mean age

of 61.2 ± 12.2 years old (range 26–88), were randomly included in three different groups. Each one of these groups received three different serum-based eye drops for the treatment of DES: 21 patients received AS (58.7 ± 11.9 years, 20 women), another group of 21 subjects (61.1 ± 16.7 years, 17 women) received HS and 21 patients included in the third group (63.8 ± 7.8 years, 18 women) received CS. The groups were comparable and there was no statistically significant difference between the basal characteristics of the groups ($p = 0.065$, Kruskal–Wallis test).

Serum determinations

The chemical and biological analysis of the three serum-based treatments revealed statistically significant differences for all of the determinations performed. The IgA, IgM, IgG immunoglobulins, A and E vitamins, TGF, serum albumin and fibronectin for the AS and HS concentrations were significantly different from the values obtained in the CS (all values were $p < 0.001$, except for fibronectin $p < 0.02$). When comparing AS and HS, AS contained significantly higher concentrations of IgA ($p = 0.048$), IgG ($p < 0.001$), and fibronectin ($p < 0.001$), whereas HS contained significantly higher concentration of EGF ($p < 0.001$). There were no significant differences between AS and HS in TGF values ($p = 0.28$), IgM ($p = 0.89$), vitamin A ($p = 0.50$), vitamin E ($p = 0.45$), or albumin ($p = 0.6$) (Figs 1-3).

Subjective and objective patient measurements

There was a significant main effect of time overall on all the measured parameters, that is, all parameters improved over time: visual acuities ($p = 0.015$), lissamine green dye ($p = 0.031$), Schirmer test ($p = 0.002$) and $p < 0.001$ in the questionnaire scores, fluorescein staining and BUT test. However, there was no significant interaction between time and serum type in any of the parameter measured; that is, the differences between serum types were statistically the same in the three scheduled measurements in terms of visual acuity scores ($p = 0.811$), questionnaire scores ($p = 0.133$), fluorescein staining scores ($p = 0.973$), lissamine green dye ($p = 0.647$), Schirmer test ($p = 0.634$) and BUT ($p = 0.941$). There was no significant main effect of serum type on any of the parameters measured overall; that is, there were not statistically significant differences between the three sera for the measured parameters overall: visual acuity scores ($p = 0.942$), questionnaire scores (0.421), fluorescein staining scores ($p = 0.662$), lissamine green dye ($p = 0.322$), Schirmer test ($p = 0.349$) and BUT ($p = 0.921$).

Pairwise comparisons results were the following: significant reduction in questionnaire scores from baseline to 1 month and from baseline to 3 months ($p < 0.001$ in both cases, mean differences of 9.0 and 9.2, respectively), although not between 1 month and 3 months ($p = 1.000$) (Fig. 4); significant improvement on visual acuity scores from baseline to 3-month measurement ($p = 0.042$, mean difference of 0.05), although not between baseline and 1 month ($p = 0.085$), nor between 1 month and 3 months ($p = 1.000$); significant reduction on fluorescein staining score between baseline and 1 month ($p < 0.001$, mean difference of 0.9), and between baseline and 3 months ($p < 0.001$, mean difference of 1.2), although not between 1 month and 3 months ($p = 0.337$); significant reduction on lissamine green staining score between baseline and 1 month ($p = 0.045$, mean difference of 1.0), although not between baseline and 3 months ($p = 0.144$), nor between 1 month and 3 months ($p = 1.000$) (Fig. 5); significant improvement from baseline to 1 month ($p = 0.042$, mean difference of 2.8 mm), and from baseline to 3 months ($p = 0.001$, mean difference of 3.4 mm), although not between 1 month and 3 months ($p = 1.000$) for Schirmer test. As to BUT test, pairwise comparisons revealed a significant improvement from baseline to 1 month ($p < 0.001$, mean difference of 2.0 s), and from baseline to 3 months ($p < 0.001$, mean difference of 2.8 s), although not between 1 month and 3 months ($p = 0.072$) (Fig. 6).

Patients with autoimmune disease

Previous autoimmune disease was found in 101 eyes of 51 patients (80.15%, 45 women and 6 men). Mean age was 57.69 years, range 26-79. The mean age was 55.33 (±10.28) years in the group treated with AS, 54.87 (±19.47) years for the HS and 64.81 years (±8.79) in the group treated with CS. This difference was statistically significant ($p = 0.016$).

The autoimmune diseases present were as follows: primary SS (46 eyes of 23 patients), rheumatoid arthritis (26 eyes of 13 patients), Raynaud´s disease (eight eyes of four patients), systemic lupus erythematosus (six eyes of three patients), fibromyalgia (six eyes, three patients), spondyloarthrosis (four eyes, two patients), thrombocytopenic purpura (two eyes, one patient), scleroderma (two eyes, one patient) and Hashimoto thyroiditis (one eye, one patient). Secondary SS had already been diagnosed in 51 of the 55 eyes that were not primary SS patients. Thus, 80% of the patients included in this study had been previously diagnosed with various autoimmune diseases, from whom 96% had previous SS (45.45% with primary SS and 50.49% secondary SS).

AS was received by 17 of the patients with autoimmune diseases, 16 were treated with HS and 18 with CS. When considering the effect of the sera comparing the three treatments between them (AS versus HS versus CS), we did not obtain statistically significant values for any of the test performed: Schirmer ($p = 0.28$), the fluorescein staining ($p = 0.14$), lissamine green staining ($p = 0.11$) nor the questionnaire score ($p = 0.97$). Almost significant differences were found in the BUT test ($p = 0.005664$), with a tendency for AS to be superior.

Complications

No complications related to the sera use were reported during the study.

Discussion

The scope of the present work was to assess the effect of AS versus CS versus HS eye drops at one- and three-month follow-up, measuring different ocular surface metrics (Donate et al. 2002). This is, to the authors' knowledge, the first randomized, double-blind clinical trial comparing the outcomes and composition of the three types of sera for the treatment of severe DES. We found that the three sera were effective in the management of severe DES. We also performed determination of essential

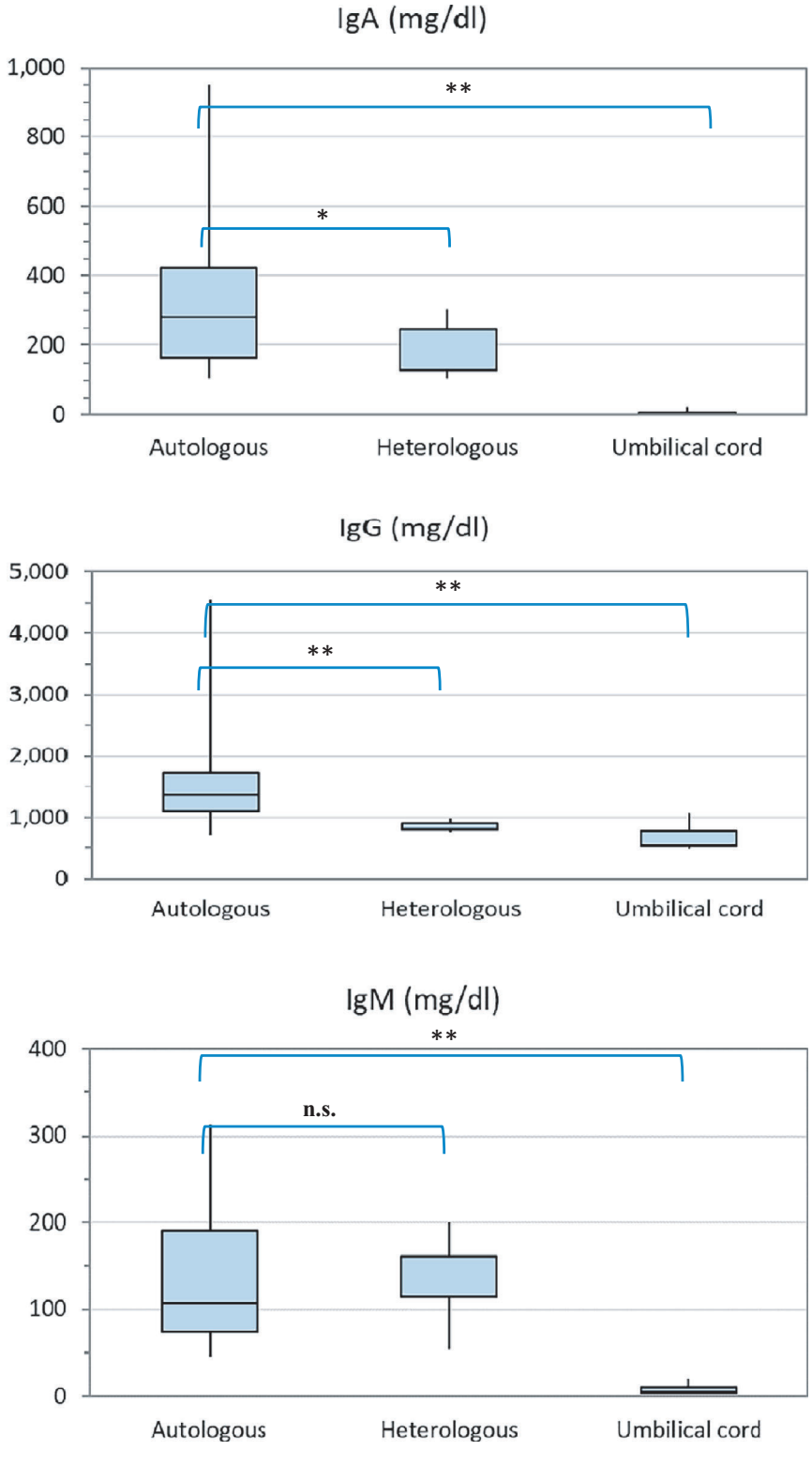

Fig. 1. Box-plots of distributions obtained for the different immunoglobulin measurements. Statistically significant comparisons with $p < 0.05$ are marked with * and those with $p < 0.001$ are marked with **. Non-statistically significant comparisons with are marked with 'n.s.'.

molecules for the homeostasis of the corneal surface: albumin, fibronectin, IgA, IgG, IgM, vitamin A, EGF and TGF. All of them have been described in the literature and referenced for AS (Tsubota 1999) with the exception of vitamin E, which has been determined at the initiative of this study. Vitamin E is a fat-soluble vitamin that acts as an antioxidant at the level of cell membranes.

The effects of AS eye drop for DES are well known. Almost all authors found a subjective improvement of the ocular surface (Tsubota 1999; Poon et al. 2001; Noble et al. 2004). As summarized in the latest DEWS II report (Jones et al. 2017), AS can be effective in the management of ocular

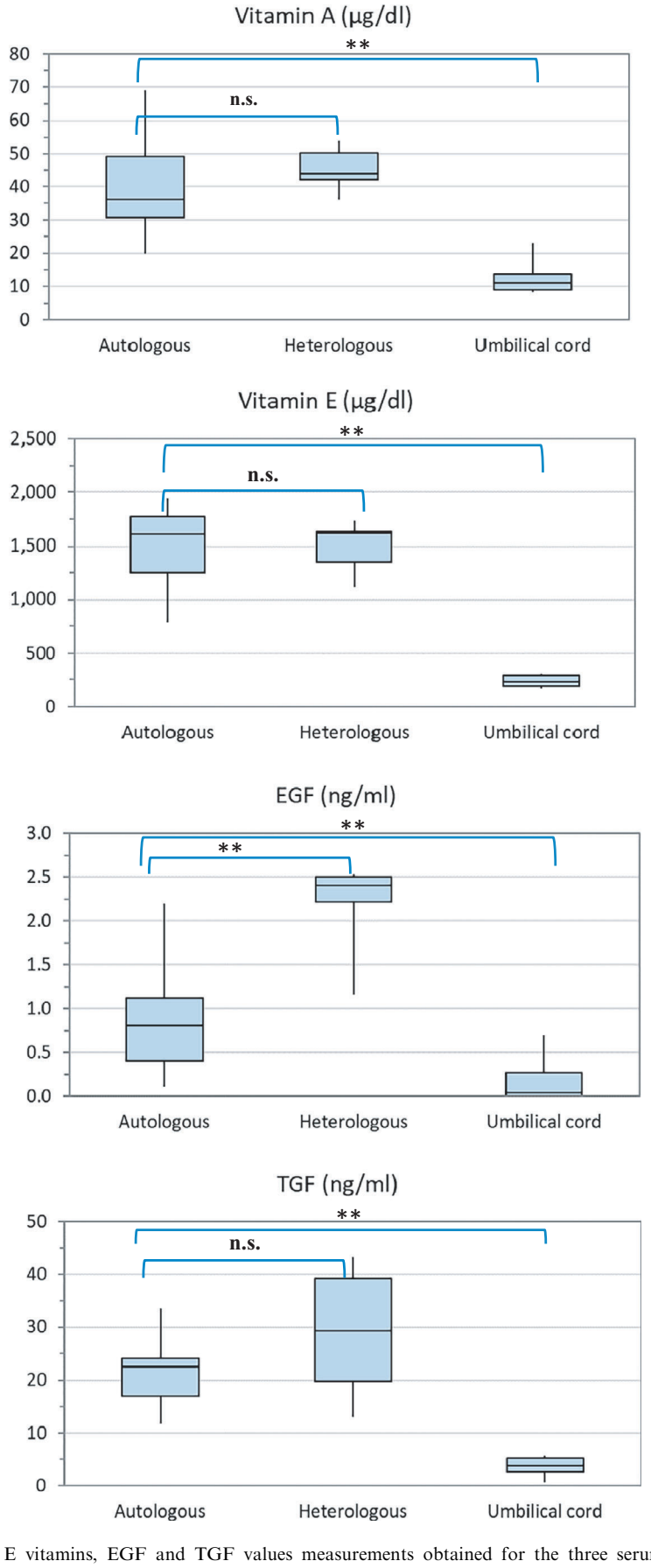

Fig. 2. Box-plots of the A and E vitamins, EGF and TGF values measurements obtained for the three serum-based treatments. Statistically significant comparisons with $p < 0.05$ are marked with * and those with $p < 0.001$ are marked with **. Non-statistically significant comparisons with are marked with 'n.s.'.

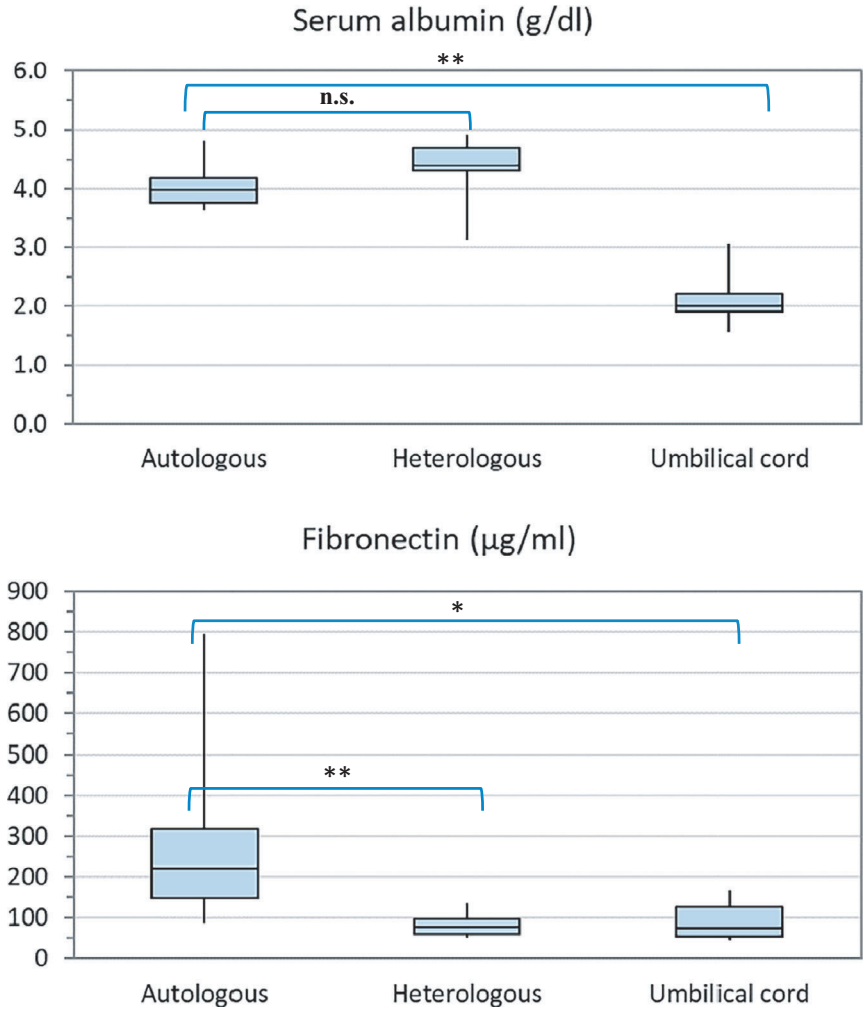

Fig. 3. Box-plots of the distributions obtained for both the serum albumin and the fibronectin measurements in the three serum types. Statistically significant comparisons with $p < 0.05$ are marked with * and those with $p < 0.001$ are marked with **. Non-statistically significant comparisons with are marked with 'n.s.'.

surface disease (OSD) secondary to DES. Several authors have reported superiority further improvement in patients treated with AS compared to conventional artificial tears (Kojima et al. 2005).

The advantage of blood-derived sera is that many of its biochemical characteristics, including pH, nutrient content, vitamins, fibronectin, growth factors such as EGF or NGF, are similar to that of human tears, probably enhancing corneal epithelial wound healing, as shown in different *in vitro* and *in vivo* studies (Geerling et al. 2001; Freire et al. 2014), inhibiting the release of inflammatory cytokines and increasing the number of goblet cells and mucin expression in the conjunctiva (Lopez-García et al. 2016a,2016b).

With regards to the role of HS, there are limited but positive clinical data on its use for the treatment of DES (Na & Kim 2012; Harritshøj et al. 2014; Meer et al. 2015). CS, on the other side (Giannaccare et al. 2020), seems to contain a higher concentration of tear components compared to other sera (Campos et al. 2020; Yoon et al. 2007a) and it has demonstrated to significantly improve OSD markers after its application in patients with DES resistant to conventional treatment (Yoon et al. 2006; Yoon et al. 2007b). Yoon et al. (2007a) found an improvement in the subjective questionnaire, BUT and fluorescein staining, both with AS and CS eye drops after one and 2 months of treatment. Versura et al. (2013) reported positive changes in Schirmer test, BUT, osmolarity, squamous metaplasia and questionnaire as soon as one month after the treatment. Although previous comparisons between outcomes after treatment with CS with AS showed improvement with both sera, the outcomes in those treated with CS were better in severe DES (Yoon et al. 2007a), possibly due to a higher concentration of growth factors and cytokines. A recent clinical trial comparing the effect of HS and CS showed that CS eye drops were more effective in epithelial healing and symptoms relief (Campos et al. 2020).

Yoon et al. (2007a) compared 92 eyes of 48 patients with moderate to severe DES. Versura et al. (2013) applied CS in 33 patients with GVHD and 26 with SS with a grade 4 or more in the Oxford scale. Campos et al. (2020) included 24 females and seven males with moderate to severe symptomatic dry eye. In our study, we included 125 eyes of 63 patients. Most of the patients in the present study were females (55 versus 8 males) with a mean age of 61.2 ± 12.2 years old (range 26–88). Yoon et al. (2007b) provides a

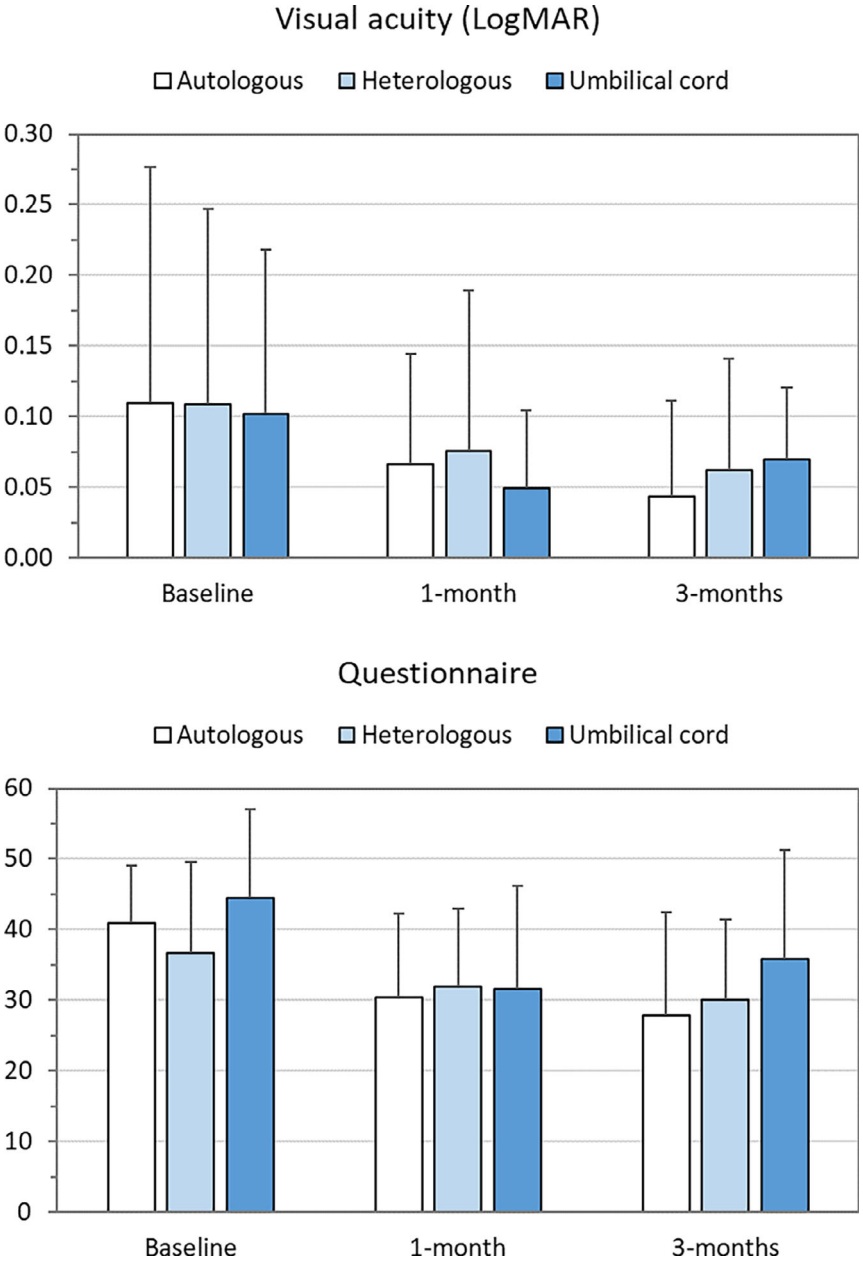

Fig. 4. Improvement achieved in visual acuity scores (LogMAR) and Questionnaire scores as a function of time for the three serum types during the whole follow-up period.

more homogeneous group, with a female/male ratio of 11:10 for the AS group and a 15:12 ratio for the CS Group. Besides, the population in their work was also younger compared to ours. On the other side Versura et al. (2013), reported 59 eyes, 19 were female patients and 11 male patients. Lastly, Campos et al. (2020) included 24 females aged 65.5 (61.5–69.0) and seven males aged 52.0 (45.0–71.5). DES affects mainly post-menopausal woman, elderly patients and patients with autoimmune diseases (Gayton 2009). While the subgroup with autoimmune diseases of Versura et al. (2013), Campos et al. (2020) and the patients of the present study are coherent with the described epidemiology of DES, the demographic sample of Yoon's article (2007a) is not, even when considering a Korean population, as was the case (Han et al. 2011).

Both Yoon et al. (2007a), Versura et al. (2013) and Campos et al. (2020) found improvement in the performed tests with CS eye drops treatment. In our study, all measurements improved from baseline to the following visits for all the tests performed. However, there was no significant interaction between time and serum type in any of the tests, nor there was significant main effect of serum type in any of the measurements.

Unlike others (Yoon et al. 2006; Yoon et al. 2007a, 2007b; Versura et al. 2013; Campos et al. 2020), we did not find CS to be superior to AS/HS, nor did it contain a higher proportion of any of the molecules measured. In our study, AS contained significantly higher concentrations of IgA, IgG and fibronectin whereas HS contained significantly higher concentration of IgM, vitamins A and E, TGF and

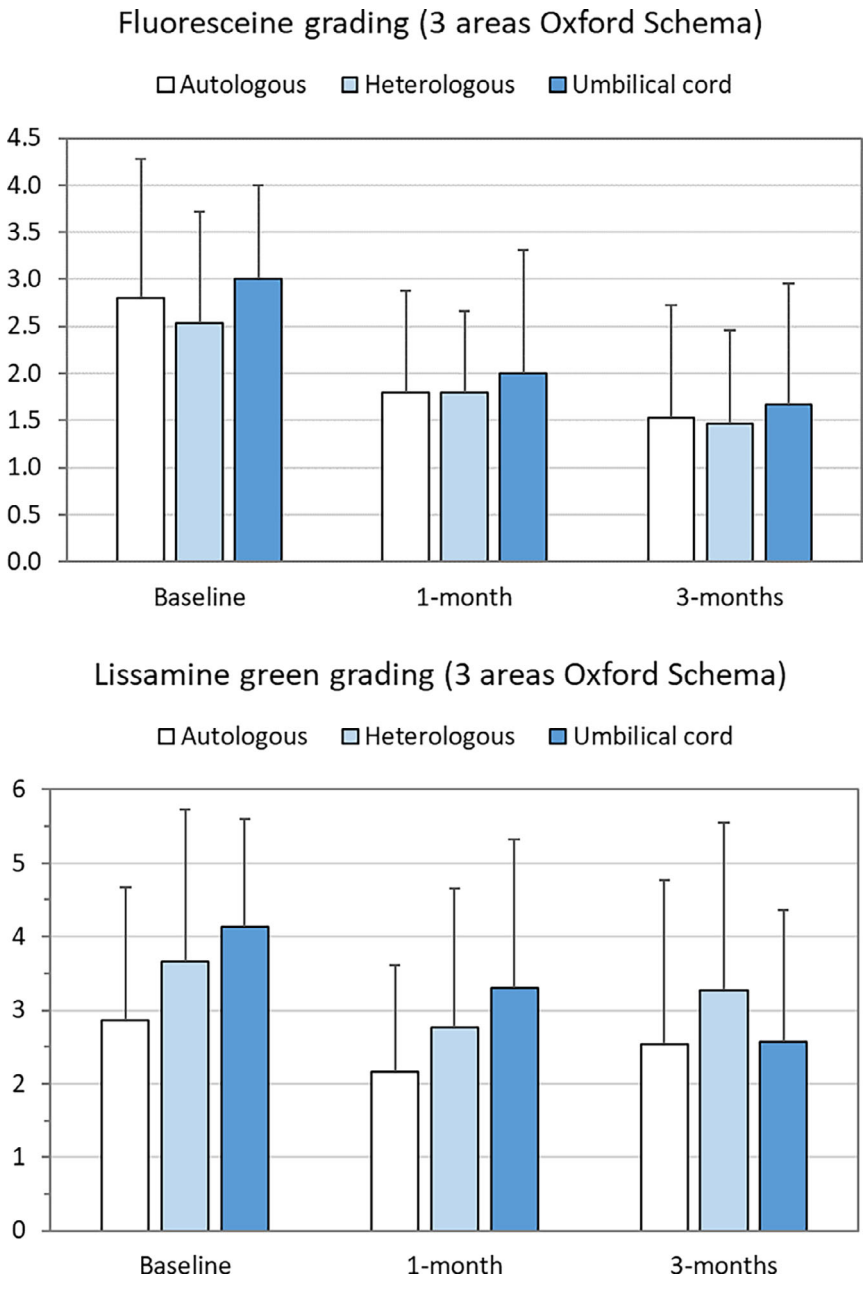

Fig. 5. Mean fluorescein and lissamine green measurements as a function of time.

albumin. The higher concentrations of fibronectin (a protein related to inflammatory processes and cell migration in corneal repair processes), IgA and IgG in AS may be due to the inflammatory process associated with the autoimmune diseases that most of the patients suffered from.

The Centro Regional de Transfusión Sanguínea of Málaga, Spain, owns the bigger Cord Blood Bank in Europe in terms of stored units. We do not believe that there were critical mistakes in the technique of preparation of the serum eye drops: the concentrations of molecules of AS eye drops were similar to those reported by other authors (Geerling et al. 2004; Yoon et al. 2007a; Versura et al. 2013; Lopez-García et al. 2016a,2016b).

The lower concentration of the molecules in the CS eye drops samples was compared to AS and HS, in contradiction to the results published by other authors (Yoon et al. 2006; Yoon et al. 2007a; Versura et al. 2013), may be caused by various reasons. Firstly, it is important to take into account that the determinations previously reported (Yoon et al. 2006; Yoon et al. 2007a; Versura et al. 2013; Yoon 2014; Versura et al. 2015) were calculated directly in serum samples. We have used the determinations in plasm that is diluted in anticoagulant (20 ml of anticoagulant solution for every 60 ml of plasm, approximately).

Secondly, other authors (Yoon et al. 2006; Yoon et al. 2007a; Yoon et al. 2007b; Versura et al. 2013; Yoon 2014; Versura et al. 2015) prepare the CS eye drops in a similar fashion to AS eye drops, centrifuging the sample after two hours of storing it at room temperature. Our hospital is a reference centre of haematopoietic transplantation; therefore, the CS eye drops for the present study were prepared

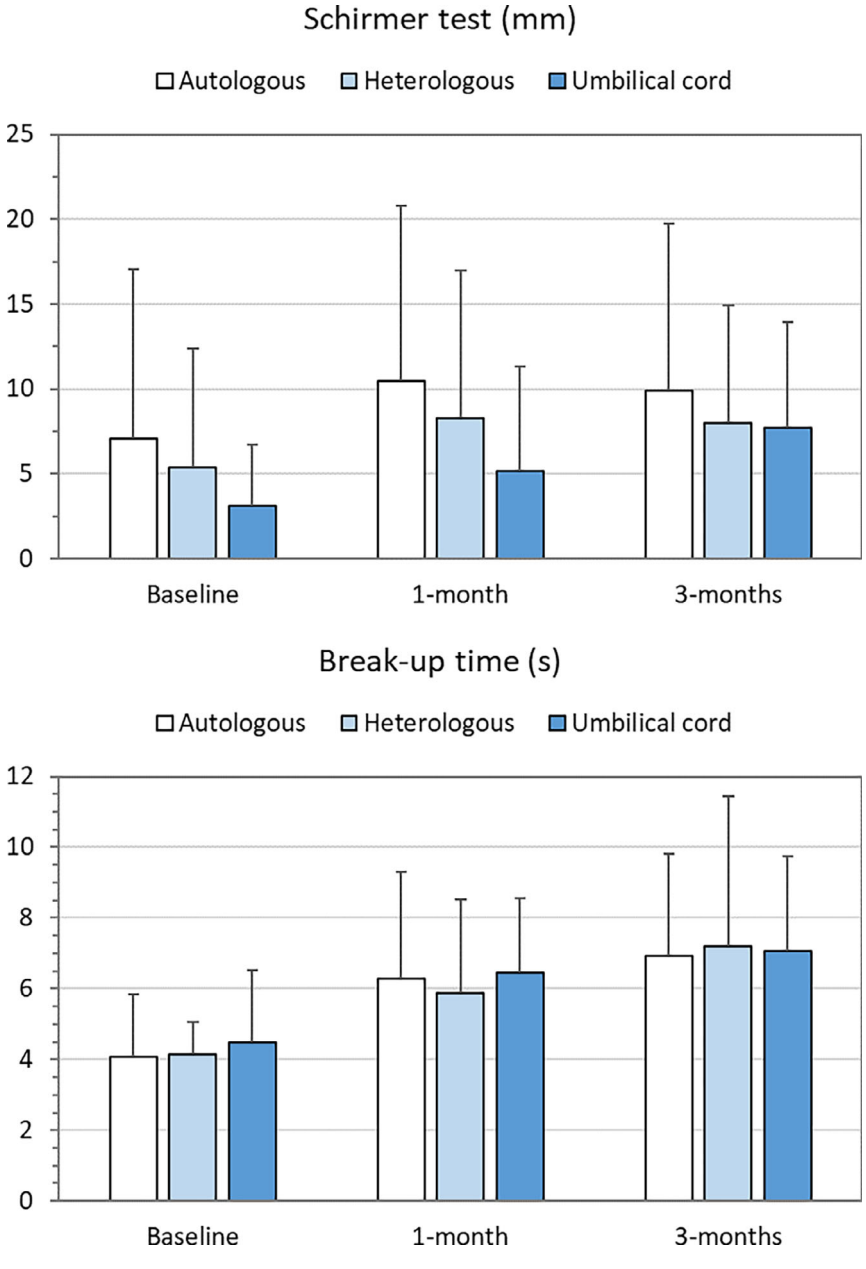

Fig. 6. Mean improvement in tear tests scores during the whole follow-up period.

from those samples that were no valid for the transplant of haemopoietic progenitors. The time elapsed between the cord blood collection and the preparation of the CS eye drops was less than 24 hours, but it was certainly higher compared to the preparation time of AS and HS eye drops. This larger amount of time between extraction of the umbilical cord blood and preparation of CS eye drops may have caused the decrease in molecules in CS eye drops we found in our study compared to others (Yoon et al. 2006; Yoon et al. 2007a; Yoon et al. 2007b; Versura et al. 2013). The work of Liu et al. (2005) seems to support our hypothesis.

Regarding the efficiency of sera, various vials HS and CS eye drops can be prepared from one single donor and kept in storage for urgent cases and/or those patients that are not candidates for blood extraction. For the latter group of patients, a finger-prick autologous blood may also be a viable option (Than et al. 2017). Neither HS nor CS contains pro-inflammatory molecules present in the sera of patients with autoimmune diseases (Giannaccare et al. 2020). Besides, CS eye drops are prepared from the discarded blood samples from haemopoietic progenitors' transplantation, instead of using a valuable blood donor. Moreover, CS is not antigenic because it does not contain agglutinins.

Eighty per cent of the patients included in this study had been diagnosed with various autoimmune diseases, from whom 96% suffered from SS. DES in SS is often severe and refractory to treatment (Hyon et al. 2007). The high proportion of patients with SS included in our study may have influenced the outcomes, as the ocular surface in these patients shows further inflammation. Before starting this

study, we had a concern about using AS in patients with autoimmune diseases, given that the concentration of growing factors is lower in the serum of these patients (Yoon et al. 2006; Yoon et al. 2007a; Yoon et al. 2007b; Harloff et al. 2008; Baturone et al. 2009; Coghill, et al. 2011; Versura et al. 2013). Tsubota (1999) argues that AS is the preferred therapy in patients with SS, due to the similar biochemical characteristics of AS compared to the tear fluid, for being hypoallergenic and for containing growth factors and other necessary molecules for the ocular surface homeostasis. Noble et al. (2004) and Kojima et al. (2005) in their prospective and randomized studies treating SS patients with AS only found improvement in the conjunctival cytology and symptom scoring, whereas Ali et al. (2018) found that AS was effective in the resolution of punctate epithelial erosions and improving the subjective response. Hwang et al. (2014) described that AS treatment was effective only in primary SS, but not in secondary SS, due to the higher content of inflammatory molecules in the serum of the latter group. Moreover, patients with primary SS show higher levels of IL-6 in serum (McDonnell et al. 1988; Baturone et al. 2009). In our study, we found higher concentrations of inflammatory molecules in AS, that is, fibronectin, IgA and IgG, which agrees with the reported evidence. For those patients with autoimmune diseases treated with AS, it has been recently published that is possible to perform an inactivation of immune components (immunoglobulins and fibronectin) while maintaining the rest of molecules, but it was published after the finalization of our study (Sanchez-Avila et al. 2017).

Nonetheless, the statistical analysis performed in the present work did not show differences between patients with autoimmune diseases assigned to SA group and those that were treated with HS or CS eye drops. Various questions arise in the treatment of patients with autoimmune diseases with AS: can systemic treatments influence the quality of the serum? To what extent will immunosuppressive drugs be present in the serum and the AS eye drops? Would it increase or decrease the efficiency of the eye drops? In patients without current systemic treatment when blood-derived eye drops are prescribed, is own patient´s serum eye drops the most suitable for the preparation of eye drops or would it be more advisable to use donor eye drops? Further work needs to be done to establish the role of AS and other blood-derived sera in the treatment of DES in patients with autoimmune diseases.

Among limitations of the study is the number of molecules determined. We would have liked to include other growth factors as Platelet-Derived Growth Factor (PDGF), substance P and Insulin-type Growth Factor 1 (IGF-1), but the availability of resources was limited.

The larger amount of time between extraction of the umbilical cord blood and preparation of CS eye drops compared top the AS and HS eye drops may have caused the lower concentration of molecules in CS eye drops we found in our study. To assess this issue, we will perform a study regarding the stability of the molecules in the CS eye drops depending on the time elapsed between extraction of the umbilical cord blood and preparation of the eye drops.

For the present work, we chose as ophthalmologic tests: Schirmer test, BUT, fluorescein staining, lissamine green staining and a questionnaire. We performed conjunctival impression cytology of every patient in all the visits, but unfortunately due to logistic reasons, most of them were damaged and could not be analysed. More advanced techniques and devices as an osmometer, meibometry, meniscography, rate of tear clearance, esthesiometry, thermography, confocal microscopy and the objective assessment of the blinking time are not available in our centre. On the other hand, it could have been interesting to analyse the concentration of the molecules in the tear fluid. However, various authors (Foulks 2003; Savini et al. 2008) and specially the Report of the Diagnostic Methodology Subcommittee of the International Dry Eye Workshops (2007), both in 'Methodologies to Diagnose and Monitor Dry Eye Disease' and in 'Design and Conduct of Clinical Trials' sections, emphasize that no test can be chosen as a Gold Standard for the DES diagnosis and no variable may be taken as definitive (International Dry Eye Workshops (DEWS), 2007).

Lastly, we did not consider including a control group treated with conventional therapy in our study. Firstly, because the patients included in the present work have severe DES refractory to conventional treatment. Besides, AS has already proved to be superior compared to artificial tears in several works (Noble et al. 2004; Urzua et al. 2012; Celebi et al. 2014; Yılmaz et al. 2017).

The present study shows that all DES indicators measured improved over time with the three types of sera, but the difference between the different types of sera found by other authors was not found in the sample analysed in the present report. This fact may be caused because mainly patients with autoimmune diseases, specifically those with SS, fulfilled the inclusion criteria for the study (80% of the included patients had a diagnosed autoimmune disease, from whom 96% suffered from SS). In fact, in our sample AS eye drops contained higher proportion of inflammatory molecules that may be associated with the autoimmune diseases that most of our patients suffered from. Unlike others, we did not find CS to be superior to AS/HS. The time elapsed between obtaining the cord blood sample and processing it to prepare the eye drops could have influenced the concentration of molecules and, therefore, its effect on the ocular surface.

In conclusion, the three sera were effective in the treatment of severe DES. CS contained a lower proportion of molecules as EFG and TGF compared to AS/HS. More research is needed to assess the effect of AS in patients with autoimmune diseases.

Data availablity statements

Data of this study are available on request.

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