

# Time-Dependent Stability of Growth Factors and Endostatin in Human Amniotic Membrane Eye Drops

Ana Boto-de-los-Bueis<sup>1\*</sup>, Almudena Del-Hierro-Zarzuolo<sup>1</sup>, Ignacio Garcia-Gomez<sup>1</sup>, Belen San-Jose Valiente<sup>1</sup>, Mariano Garcia-Arranz<sup>1</sup>, Aquilino Corral-Aragon<sup>2</sup> and Arantxa Acera<sup>3</sup>

<sup>1</sup>La Paz University Hospital, Madrid, Spain

<sup>2</sup>Farmacia Botica de Argensola, Spain

<sup>3</sup>Bioftalmik, Spain

## Abstract

**Purpose:** Amniotic Membrane (AM) is commonly used as a graft for reconstruction in ocular surface surgery. It has been demonstrated that the beneficial effects of the AM graft are due largely to its content in several growth factors. For this reason, we have measured the levels of growth factors in lyophilized AM eye-drops and their variation over time.

**Results:** The levels of bFGF and endostatin in 20% and 30% AM eye drops were higher than those of HGF, NGF and EGF. The concentration of all these growth factors and of total protein remained constant over 6 weeks, independent of the dilution factor.

**Conclusion:** The growth factor and endostatin composition of AM eye drops is stable, at least over 6 weeks, further supporting the viability and potential therapeutic utility of this method for the treatment of ocular surface diseases and warranting its application in clinical trials.

## Introduction

Human Amniotic Membrane (AM) contains a variety of beneficial bioactive factors and has low immunogenicity [1]. Clinical observations have shown that AM transplantation promotes corneal epithelial healing [2], reduces scarring [3], suppresses inflammation [4] and inhibits angiogenesis [5,6]. Many of these properties are attributable to the histological structure of the AM, which has a thick basement membrane that acts as a substrate for epithelial growth, and to its biochemical composition, rich in cytokines and growth factors. Recently, AM eye drops have been reported to have satisfactory clinical effects in the treatment of corneal ulcers, obviating the need for invasive surgical procedures [7-10]. In addition, topical application of an amniotic extract has been shown to have an inhibitory effect on corneal neovascularization *in vivo* [11].

The levels of growth factors in AM preparations depend on a number of factors including: gestational and donor age [12], preservation method applied [13], as well as filtration and centrifugation protocols employed during eye drop preparation. Thus AM eye drop preparation should minimize growth factor depletion, and storage conditions should be designed to minimize protein denaturation. For these reasons, it is important to characterize the composition of AM eye drops in terms of factors which are known to be involved in re-epithelialization, such as bFGF, EGF, HGF, NGF and the endogenous anti-angiogenic factor endostatin, and to determine the best concentration and preservation schedules in order to achieve optimal clinical results.

Consequently, the purpose of this study was (1) to define a protocol for the preparation of AM eye drops, (2) to characterize AM eye drops in terms of levels of a panel of growth factors and endostatin and (3) to examine the stability of AM eye drops over time, thereby evaluating the possibility that this treatment may constitute a new practical and viable alternative to enhance re-epithelialization.

## Materials and Methods

### Preparation of human amniotic membrane

Donors were selected among patients of the Department of Obstetrics of the La Paz University Hospital (Madrid, Spain). The

criteria applied for patient selection were the same criteria required to donate AM for ocular surface surgery. Inclusion criteria were: age between 18 and 42, seronegativity for human immunodeficiency virus, human hepatitis B, C and syphilis; and full-term pregnancies (38-42 weeks). Exclusion criteria were gestational or fetal diseases.

Human placentas were handled according to the tenets of the Declaration of Helsinki. After obtaining proper informed consent, four human placentas were collected after elective caesarean deliveries and were processed immediately after surgery under sterile conditions. First, they were washed with sterile saline solution to remove blood clots. The AM was then carefully detached from the chorion by blunt dissection and rinsed several times with a saline solution containing antibiotics and antimycotics (penicillin 10,000 U/ml, streptomycin 50 mg/ml and amphotericin B 2.5 mg/ml). Each AM was then introduced into a sterile container with a 1:1 solution of Dulbecco's Modified Eagle's Medium: glycerol (Gibco® DMEM, USA) and stored at -80°C until use.

### Evaluation of total protein, growth factor and endostatin content in native amniotic membrane

Three cryopreserved AM samples obtained from three donors were sent for analysis to Bioftalmik (Vizcaya, Spain). The AM were cut into small segments in ice-cold saline solution. Then, they were

**\*Corresponding author:** Ana Boto-de-los-Bueis, Medical Assistant Cornea Unit, Department of Ophthalmology, La Paz University Hospital, Paseo de la Castellana 261, 28046 Madrid, Spain, Tel: +34 91 7277259, E-mail: [mail@anaboto.es](mailto:mail@anaboto.es)

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homogenized with phenylmethanesulfonyl fluoride (1 ml) and phosphate buffered saline (10 ml) followed by centrifugation at 3,000 rpm, 5 min at 4°C. The collected supernatants were aliquoted, passed through a 0.22 µm filter, and stored in a new container.

The Nanodrop and the EZQ methods were used to assess total protein content and data were expressed as µg/ml. bFGF, HGF, NGF, EGF and endostatin were analyzed by ELISA according to the manufacturer's instructions. Data were expressed as pg/µg of total protein.

### Lyophilization of amniotic membrane

The four AMs were defrosted. The AMs were pooled together, mechanically disaggregated by grinding and sonication and then subjected to centrifugation (3,000 rpm, 15 min; see Figure 1). The supernatant was extracted and centrifuged again (3,000 rpm, 15 min). The resulting supernatant was dosed (2 ml each) into 40 bottles by a Watson-Marlow pump and lyophilized in three phases as follow: 4 hours prefreeze at -80°C, followed by 8 hours at -22°C, and finally a three stage secondary dryness with 0.020 mBar high vacuum, 4 h at -10°C, 2 h at 0°C and finally 2 h at 25°C. The bottles were closed and injected with sterile nitrogen gas. A quality test was performed to check residual humidity (<0.5%). Because this work was performed under sterile and high biological risk conditions (in an ISO 5 room), at a temperature lower than 8°C, no filtration was required. Three microbiological tests showed negative results and the forty bottles were sent to Biofalmik (Bizkaia, Spain) to proceed with the quantification of proteins and growth factors (Figure 1).

### Evaluation of total protein, growth factor and endostatin content in amniotic membrane eye drops

The light protected bottles with lyophilized AM were divided in two groups: 1) AM rehydrated with 2 ml sterile water (30% AM eye drops; n=20) stored at 4°C for different times: time 0 (n=2), day 1 (n=2), day 2 (n=2), day 3 (n=2), day 7 (n=2), day 14 (n=2), day 21 (n=2), day 28 (n=2), day 35 (n=2) and day 42 (n=2) and 2) AM rehydrated with 3 ml sterile water (20% AM eye drops; n=20) conserved at 4°C for different times: Time 0 (n=2), day 1 (n=2), day 2 (n=2), day 3 (n=2), day 7 (n=2), day 14 (n=2), day 21 (n=2), day 28 (n=2), day 35 (n=2) and day 42 (n=2). After each re-hydration, total protein content in 30% and 20% eye drops at the different time points was measured using an EZQ

protein quantification kit. Data was expressed in µg/ml. Using ELISA, we similarly measured the levels of Basic Fibroblast Growth Factor (bFGF), endostatin, Hepatocyte Growth Factor (HGF), Nerve Growth Factor (NGF) and Epithelial Growth Factor (EGF). These results were expressed as pg/µg of total protein.

### Statistical analysis

All experiments and chemical determinations were performed in duplicate. Data was expressed as mean + SD. To evaluate growth factor stability over time, we performed a statistical analysis using the regression of mixed models. P <0.05 was considered to be statistically significant.

### Results

#### Comparative analysis of peptide levels in intact amniotic membrane and eye drops

Peptide levels in three different non-lyophilized human amniotic membranes were measured by ELISA and these levels were compared with those measured in AM eye drops (two 20% AM eye drops and two 30% AM eye drops) after 6 weeks of re-hydration (Figure 2).

Endostatin, HGF and NGF levels (pg/µg) were higher in the cryopreserved AM (n=3) than in the 20% (n=2) and 30% (n=2) lyophilized AM eye drops. NGF levels were significantly lower in both eye drop preparations, perhaps due to its lability. Peptide levels were higher in 30% vs 20% eye drops, suggesting that the peptides may be more stable at higher concentrations (Figure 2).

#### Growth factor and endostatin levels 6 weeks after reconstitution

Six weeks after reconstitution of lyophilized amniotic membrane, mean bFGF and endostatin levels in AM eye drops presented highest values. The rank order of concentration of the other growth factors was HGF>NGF>EGF. Results are expressed as pg/µg and also as pg/ml in Table 1.

#### Temporal stability of growth factors in human amniotic membrane eye drops

Growth factor levels were expressed relative to total protein

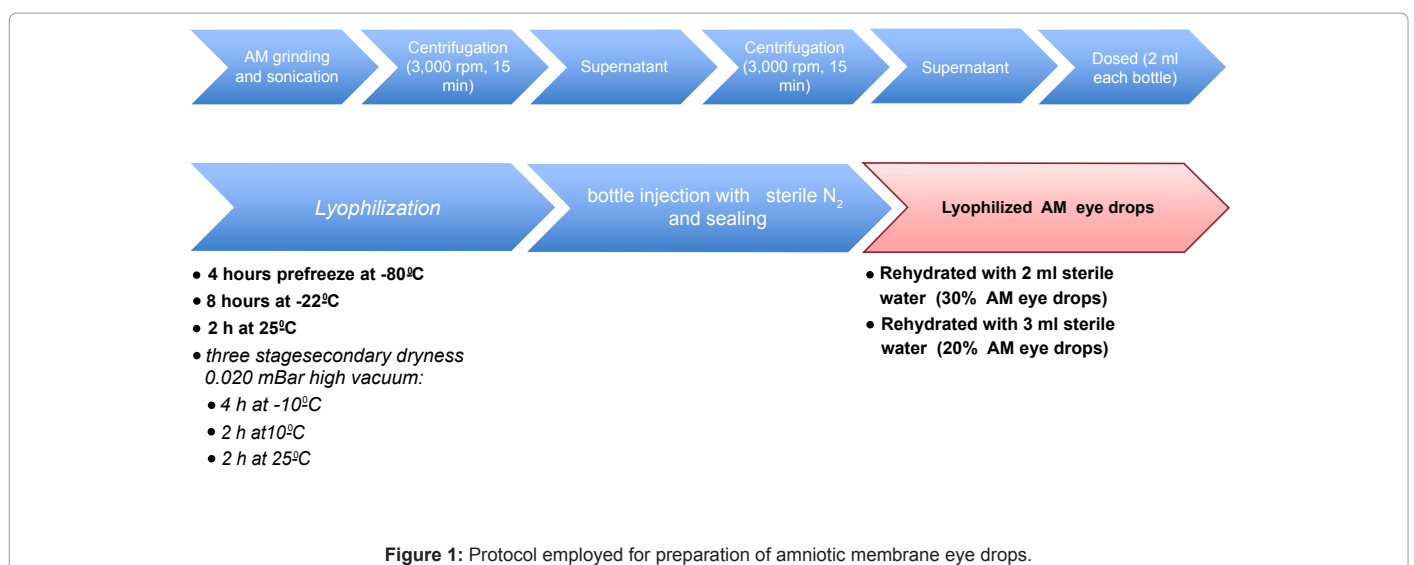
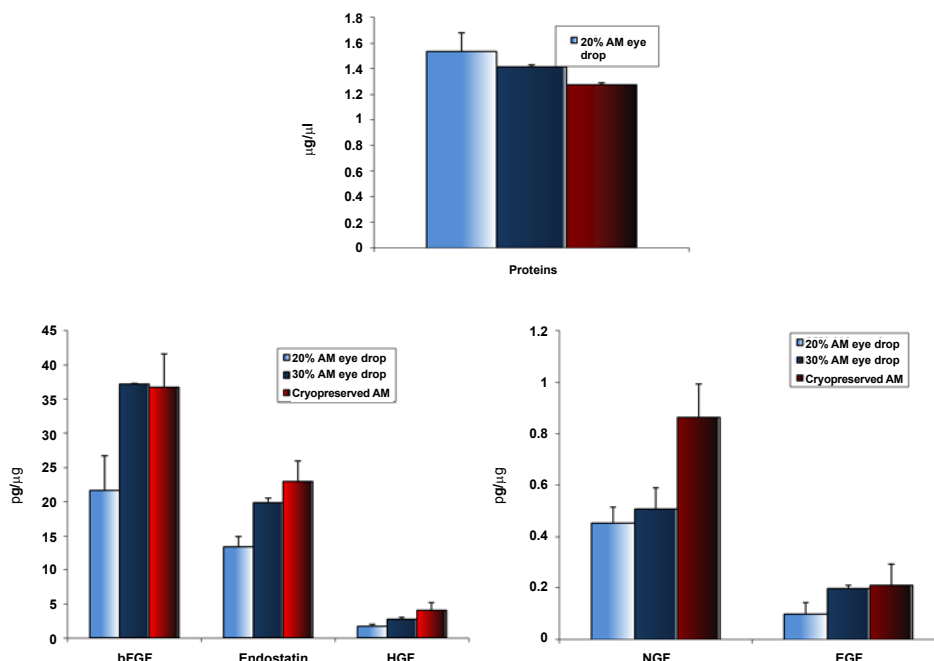
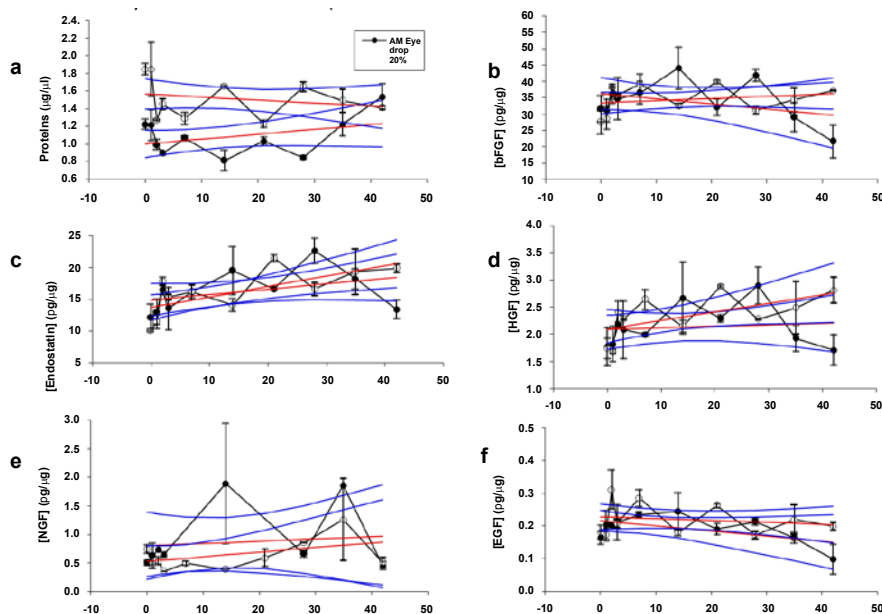


Figure 1: Protocol employed for preparation of amniotic membrane eye drops.



**Figure 2:** Mean peptide levels (pg/μg total protein) in cryopreserved, non-denatured AM and in AM eye drops 6 weeks after re-hydration (20 and 30%). Whereas bFGF and EGF levels were similar in native AM and 30% AM eye drops, endostatin, HGF and NGF showed somewhat higher levels in intact AM tissue.



**Figure 3:** Normalized values of the concentration of total protein (μg/μl) and of growth factors and endostatin (pg/μg of total protein) at different re-hydration times over a 6 week period, with the minimum / maximum values of both samples and their lines of regression. a) Mean total protein levels in AM eye drops did not show significant variation over 6 weeks for 20% AM eye drops (p=0.3894) and 30% AM eye drops (p=0.5364). b-f) The mean peptide levels in 20% and 30% eye drops for b) bFGF, c) endostatin, d) HGF, e) NGF and f) EGF, were similar throughout the 6 weeks.

content. Protein content was found to be stable over time for both eye drop dilution regimes (20% AM eye drop, p=0.3894 and 30% AM eye drop, p=0.5364). Moreover, there was no significant difference in the temporal stability of total protein in the two different eye drop dilutions (p=0.0968) (Figure 3A).

The concentrations of growth factors and endostatin in both 20% and 30% AM eye drops were invariant over time (Figure 3) (Table 2). There were no differences in the stability of bFGF, EGF, NGF, HGF and endostatin in the 20% and the 30% AM eye drops, over the 6 weeks of duration of these experiments.

	bFGF pg/ml	bFGF (pg/μg)	Endostatin pg/ml	Endostatin (pg/μg)	HGF pg/ml	HGF (pg/μg)	NGF pg/ml	NGF (pg/μg)	EGF pg/ml	EGF (pg/μg)
20% AM eye drops	32,467.52	21.62	20,376.68	13.40	2,585.97	1.70	682.95	0.44	144.42	0.09
30% AM eye drops	52,371.45	37.13	28,024.34	19.86	3,966.57	2.80	714.76	0.50	303.5	0.19

**Table 1:** Mean growth factor levels (pg/μg total protein and pg/ml) in amniotic membrane (AM) eye drops, measured 6 weeks after re-hydration at 20% and 30% dilutions.

	20%	30%
bFGF	<i>p</i> =0.4609	<i>p</i> =0.5286
Endostatin	<i>p</i> =0.3792	<i>p</i> =0.1982
HGF	<i>p</i> =0.7926	<i>p</i> =0.2738
NGF	<i>p</i> =0.8079	<i>p</i> =0.5387
EGF	<i>p</i> =0.3817	<i>p</i> =0.6318

**Table 2:** The concentrations of growth factors and endostatin in both 20% and 30% AM eye drops were invariant over time. P values were derived from the regression of mixed models to determine differences which were statistically significant. All p values > 0.05, which was the limit considered to be statistically significant.

## Discussion

### Growth factor levels in native AM and AM eye drops

We initially evaluated if AM prepared as eye drops contains mitogenic growth factors. Here, we have demonstrated that AM eye drops contain high levels of bFGF, endostatin and HGF, and lower levels of NGF and EGF. The levels of some growth factors, such as HGF, NGF and endostatin, were lower in the lyophilized AM eye drops than in the native AM tissue, whereas other growth factors such as bFGF and EGF maintain similar levels in both native tissue and 30% AM eye drops.

The values which we reported differ in some respects from those reported in previous studies of growth factor concentrations. For example, HGF has been reported to have the highest levels in AM [12] and also in AM suspension [8]. These differences are likely due to differences in AM preparation methods such as the use of fresh vs cryopreserved AM, the use of Dulbecco's Modified Eagle Medium vs sterile water for the dissolution of powdered AM, and whether centrifugation is performed after reconstitution, or before lyophilization [8].

The growth factor levels which we reported here are also different to those reported in other biological eye drops used to treat corneal epithelial diseases. Thus, similar levels of EGF have been reported in autologous serum (410 pg/ml) [14] and plasma rich in growth factors (PRGF) (468.9 +/- 97.6 pg/ml) [15], whereas EGF levels were somewhat lower (303.5 pg/ml, 30% AM eye drops) in our study. In contrast, the levels of other growth factors were higher in our eye drops than in PRGF [15]; (our study vs 82.6 pg/ml), HGF (3,966.57 pg/ml vs 149.5 pg/ml) and for example, FGF (52,371.45 pg/ml NGF (714.76 vs 37.7 pg/ml)). Although the autologous PRGF eye drops also have a valuable effect on corneal wound healing, the AM eye drops provide some additional advantages. Since the AM has anti-inflammatory, anti-angiogenic and anti-scarring properties, it may well be the treatment of choice for epithelial defects accompanied by inflammation.

The biochemical factors derived from the AM epithelium which are involved in re-epithelialization are known to be bFGF, HGF, NGF, EGF and KGF. However, most useful clinical information has been obtained with NGF which has been applied alone to diseased human corneal tissue. In clinical studies [16-20], topical treatment with purified murine NGF was found to induce complete healing of corneal ulcers, as well as a marked improvement in both corneal sensitivity and visual acuity. This purified murine NGF solution was composed of 100

or 200 μg/ml NGF, which is much higher than that contained in our AM eye drops (0.00071 μg/ml).

### Stability of growth factors in AM eye drops

Next, we evaluated if growth factor levels were stable in the AM eye drops. The stability of growth factors in eye drops is important for its clinical application because growth factor denaturation has been reported to occur in other eye drop preparations, such as autologous serum eye drops [21]. The levels of all the growth factors which we studied (bFGF, HGF, NGF and EGF), and of endostatin, were found to be nicely maintained over a period of at least 6 weeks. This biochemical stability over time is an added advantage for the clinical usefulness of AM eye drops, since it can be stored without freezing at 4°C for at least for 6 weeks, and it can be applied immediately if required or if any contraindication exists for autologous serum. Further biological tests will be required to evaluate if the factors present at 6 weeks post de-freezing continue to be functionally efficacious in a clinical context.

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