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Development of Photo-Crosslinked Gelatin methacryloyl –Amniotic Membrane Hydrogel for Ocular Surface Applications

Oküler Yüzey Uygulamaları İçin Foto-Çapraz Bağlanmış Jelatin Metakriloil–Amniyotik Membran Hidrojeli Geliştirilmesi

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Abstract: The amniotic membrane is the innermost layer of the placenta, composed of a thick basal membrane and an avascular stromal matrix. Initially utilized as a skin graft in the early 20th century, it was later introduced to ophthalmology in 1940 when De Rotth proposed its use for ocular surface reconstruction. In this study, clinically used amniotic membrane was converted into a powder form, combined with gelatin methacryloyl (GelMA), and developed as a sustained-release eye drop formulation for ocular surface application. The amniotic membrane–GelMA formulation was prepared at final concentrations of 5% and 10% and photo-crosslinked to support structural integrity. Rheological analysis showed that the elastic component (G′) exceeded the viscous component (G″) across all crosslinking durations, with no marked variation in viscoelastic parameters within the tested range. The formulation exhibited shear-dependent viscosity reduction under increasing shear rates. In vitro cytotoxicity assessment using human limbal stromal mesenchymal stem cells revealed increased mitochondrial activity at 24 hours, with cell viability values of 180% in the 5% amniotic membrane group and 160% in the 10% group relative to control. At 48 and 72 hours, viability values decreased to below 100% while remaining within a non-cytotoxic range. Growth factor analysis demonstrated measurable release of epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β), reaching 313 pg/mL and 350 pg/mL, respectively, at 48 hours in the 5% group. These findings suggest that the amniotic membrane–GelMA formulation exhibits sustained factor release and biocompatible characteristics under in vitro conditions. The system may represent a non-surgical, drop-based approach for delivering amniotic membrane–derived bioactive components to the ocular surface.

Keywords: Amniotic membrane, Transforming growth factor-beta, Epidermal growth factor

Ethics Committee Approval: The study was approved by the Ethics Committee of Eskisehir City Hospital (decision date:15.08.2023, number:9).

Informed Consent: The authors declared that it was not considered necessary to get consent from the patients because the study was a retrospective data analysis.

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Özet: Amniyotik membran (AM), plasentanın en iç tabakası olup kalın bir bazal membran ve avasküler bir stromal matriksten oluşmaktadır. İlk olarak 20. yüzyılın başlarında deri grefti olarak kullanılan AM, 1940 yılında De Rotth tarafından oküler yüzey rekonstrüksiyonu amacıyla oftalmoloji alanına kazandırılmıştır. Bu çalışmada, klinik kullanımda olan amniyotik membran toz formuna dönüştürülmüş, jelatin metakriloil (GelMA) ile birleştirilerek oküler yüzey uygulamasına uygun, sürekli salınımlı bir göz damlası formülasyonu geliştirilmiştir. AM–GelMA formülasyonu %5 ve %10 son konsantrasyonlarda hazırlanmış ve yapısal bütünlüğün sağlanması amacıyla foto-çapraz bağlama uygulanmıştır. Reolojik analizlerde, tüm çapraz bağlama süreleri boyunca elastik davranışın (G′ > G″) baskın olduğu gözlenmiş, viskozitenin ölçüm aralığında belirgin değişiklik göstermediği ve kayma incelmesi (shear-thinning) özelliği gösterdiği belirlenmiştir. İnsan limbal stromal mezenkimal kök hücreleri kullanılarak yapılan in vitro sitotoksisite değerlendirmesinde, 24. saatte mitokondriyal aktivitede artış gözlenmiş; hücre canlılığı %5 AM grubunda %180'e, %10 AM grubunda ise %160'a ulaşmıştır. Buna karşılık, 48 ve 72. saatlerde her iki grupta da hücre canlılığı %100'ün altında ölçülmüştür. Büyüme faktörü analizlerinde, %5 AM grubunda 48. saatte epidermal büyüme faktörü (EGF) düzeyi 313 pg/mL, dönüştürücü büyüme faktörü-beta (TGF-β) düzeyi ise 350 pg/mL olarak ölçülmüştür. Bu bulgular, geliştirilen AM–GelMA formülasyonunun in vitro koşullarda biyolojik olarak aktif faktör salımı sağlayabildiğini ve oküler yüzey uygulamalarında kullanılabilecek bir sürekli salım sistemi olarak değerlendirilebileceğini düşündürmektedir.

Anahtar Kelimeler: Amniyotik membran, Transforme edici büyüme faktör-beta, Epidermal büyüme faktörü

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1. Introduction

The amniotic membrane (AM), the innermost layer of the placenta, consists of a thick basal membrane and an avascular stromal matrix rich in pluripotent cells, organized collagen, growth factors, antifibrotic and anti-inflammatory cytokines, immunomodulators, and matrix proteins. This unique composition accelerates wound healing, stimulates cell proliferation, and prevents the perforation of atrophic tissues (1, 2). Since its first use in ophthalmology by De Rotth in 1940, AM has become a preferred material for ocular surface reconstruction because it does not elicit immune responses and provides an optimal substrate for the growth, migration, and adhesion of corneal and conjunctival epithelial cells (3, 4). In addition to its antifibrotic activity, AM exhibits potent anti-inflammatory, antimicrobial, and anti-angiogenic properties, which make it an effective therapeutic option for chemical and thermal ocular surface injuries (OSIs), severe dry eye disease, and neurotrophic keratopathy (5-8).

Despite these advantages, AM transplantation has several disadvantages, including the need for a surgical procedure and the non-transparent nature of the tissue. To overcome these limitations, the AM has been processed into extract form to provide a non-surgical, patient-friendly alternative. Conventional preservation techniques such as lyophilization and cryopreservation extend shelf life but require controlled cold storage. In contrast, AM extraction enables sterilization by filtration and facilitates the preparation of formulations that can be applied topically without surgery (9). The development of AM extract–based eye drops, which can be rehydrated prior to use and easily administered, represents a promising alternative to transplantation (10).

However, even with correct administration, eye drops deliver only about 10 % of the administered dose to ocular tissues (11). Under normal physiological conditions, the tear volume in the conjunctival cul-de-sac ranges between 7 and 9 µL, with a turnover rate of 0.5 to 2.2 µL/min, leading to rapid dilution and drainage. Because growth factors must remain bound to their receptors for at least 6–8 hours to influence cell-cycle signaling, rapidly cleared topical drops fail to maintain sufficient concentrations for therapeutic efficacy (12-14). In severe OSIs, the lacrimal gland, the primary endogenous source of growth factors, and its ducts may also be damaged, leaving exogenous preparations such as AM extracts, autologous serum, or platelet rich plasma **(**PRP) as the sole regenerative sources.(13) Although these formulations are widely used for persistent epithelial defects, severe dry eye, and neurotrophic keratopathy, their short ocular residence time remains the major limitation to clinical success.

To address these pharmacokinetic and bioavailability challenges, recent research has focused on hydrogel-based controlled-release systems. Hydrogels are hydrophilic, three-dimensional polymer networks capable of absorbing large amounts of water and enabling controlled drug delivery. Their porous structure, flexibility, and biocompatibility make them particularly suited to ocular use (15). These systems resist mechanical stress from blinking and tear flow and allowing drugs to remain longer on the ocular surface. Hydrogel carriers can encapsulate high concentrations of therapeutic agents and release them gradually through diffusion, swelling, or external stimuli. Among hydrogel candidates, gelatin methacryloyl (GelMA) has attracted attention because of its structural similarity to the extracellular matrix (ECM) and its photo-crosslinkable properties, which permit precise control of stiffness, degradation rate, and release kinetics (16-18).

In this study, the aim was to develop and characterize a GelMA-based amniotic membrane eye drop formulation by converting clinically used AM into a powdered form and incorporating it into a hydrogel system suitable for ocular surface application. The formulation was evaluated in terms of its rheological properties, in vitro cytocompatibility using limbal stromal mesenchymal stem cells, and short-term growth factor release behavior. This approach enables the utilization of the AM's regenerative potential in a non-surgical, topical drop-based format, while providing a proof-of-concept evaluation of a GelMA-based carrier system for ocular surface delivery.

2. Materials and Methods

2.1. Preparation, lyophilization, and grinding of amniotic membrane extract

The AM extract was obtained through sequential lyophilization, freezing, and grinding steps. Amniotic membranes were collected immediately after delivery from a placenta obtained during a cesarean section from a donor who had tested negative for infectious disease markers prior to childbirth. The chorion layer was separated from the placenta by blunt dissection to isolate the AM. The AM was then washed separately with phosphate-buffered saline (PBS) solutions containing 1000 U/mL penicillin, 20 mg/mL streptomycin, and 2.5 µg/mL amphotericin B. Following washing, the AM was cut into 3 × 3 cm fragments and placed on nitrocellulose membranes with the epithelial surface facing upward. The prepared AMs were immersed in Dulbecco's Modified Eagle Medium (DMEM) and stored at –80 °C until use.

For extract preparation and hydrogel production, the AM samples were spread on Petri dishes, rapidly frozen using liquid nitrogen, and subsequently lyophilized for 48 hours at –55 °C under a pressure of <0.133 mBar. The lyophilized tissues were stored at –80 °C and then ground into a fine powder using a cryogenic mill (CryoMill, Retsch GmbH, Haan, Germany). The powdered form of the AM obtained after the grinding process is presented in Figure 1.

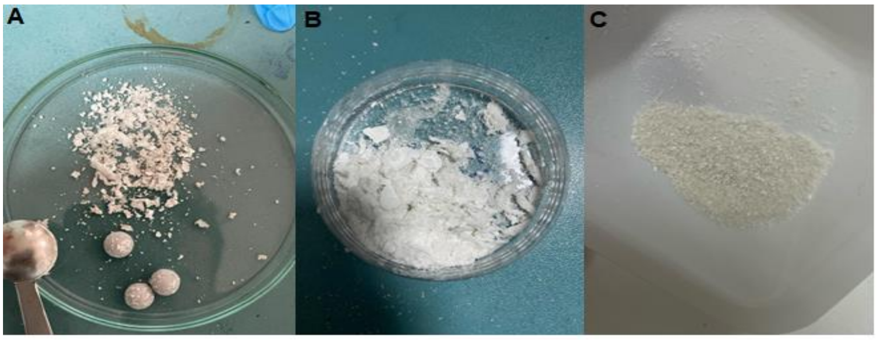

Figure 1. *Images of the frozen and lyophilized amniotic membrane (A, and B) and the appearance of the cryoground amniotic membrane granules obtained after lyophilization using a cryogenic mill (C).*

2.2. Preparation of amniotic eye drop by combining amniotic membrane extract with GelMA and rheological characterization

Following the grinding procedure, powdered AM was suspended in PBS to obtain a final concentration of 20%. GelMA was similarly prepared at a 20% concentration. The prepared AM and GelMA solutions were then mixed at a 1:1 ratio, followed by the addition of a photoinitiator to obtain the AM-based eye drop formulation. The appearance of the photo-crosslinked amniotic eye drop formulation after 8 minutes of exposure on both hydrophilic and hydrophobic surfaces is shown in Figure 2.

To examine the photo-induced gelation kinetics of the hydrogel-containing system, small-amplitude dynamic oscillatory time and frequency sweep tests (0.1–100 rad/s) were performed, and the shear storage modulus (G') and the loss modulus (G") were recorded (19). All measurements were carried out in situ at 25 °C using a plate rheometer. Photopolymerization was induced using a Xenon light source (Smith & Nephew, 500XL light source, Watford, UK, filter range: 450–550 nm) with an irradiance of 100 mW/cm² delivered through a light guide. Each experiment was performed in triplicate (n = 3), representing technical replicates derived from the same formulation batch. The same samples were also analyzed for density (DMA 4500 Density Meter, Anton Paar GmbH, Graz, Austria), kinematic viscosity, and dynamic viscosity (AMVn Automated Micro Viscometer, Anton Paar GmbH, Graz, Austria) at 25 °C and 37 °C, each measured in ten replicates.

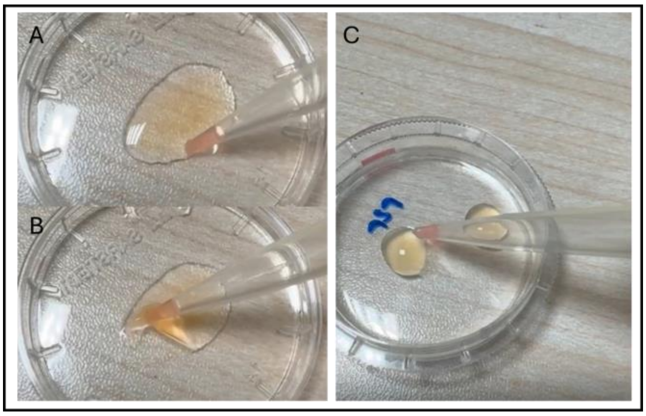

Figure 2. Appearance of the amniotic eye drop formulation crosslinked with GelMA on a hydrophilic surface after 8 minutes of photo-crosslinking (A). The formulation gained structural integrity and increased stiffness after gelation (B). Appearance of the amniotic eye drop crosslinked with GelMA on a hydrophobic surface after 8 minutes of photo-crosslinking (C).

2.3. Evaluation of in vitro cytotoxicity of the hydrogel

Corneolimbal tissues that were not suitable for patient transplantation were procured from the Eye Bank of the Eskisehir Osmangazi University Hospital (IRB approval ESOGU Non-Interventional Clinical Research Ethics Committee no: 55-15.02.2022). Limbal stromal mesenchymal stem cells (LSMSCs) were selected as the model for in vitro cytotoxicity testing, as they are considered to yield results most closely resembling in vivo conditions for chemical substances intended for application to sensitive tissues such as the eye (20-22).

2.3.1. Thawing, culture, and characterization of limbal stromal stem cells

Previously isolated and characterized LSMSCs were thawed and expanded for experiments. The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2.5 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1.25 µg/mL amphotericin B. Culture medium was replaced every three days, and the cells were maintained at 37 °C in a 5% CO_2 incubator until reaching 80–90% confluence. Once sufficient cell density was achieved, the cells were detached using 0.25% trypsin-EDTA.

Immunophenotypic characteristics were determined by flow cytometry to demonstrate that cells retained their stemness properties after freezing. LSMSCs were labeled with MSC surface marker antibodies at passage 3 to examine their stemness. CD90, CD73, HLA-B-C, CD44, CD69, CD105, CD34, CD25, CD29, HLA-DR, CD19, CD14, and CD45 conjugated antibodies (BioLegend, San Diego, USA) were used for hLSMSC characterization. Briefly, cells (1 106 viable cells/mL) were incubated with antibodies diluted 1:100 at 4C in the dark. Flow cytometry was performed using a NovoCyte D3005 flow cytometry (Agilent, Santa Clara, USA). The data were analyzed using NovoExpress flow cytometry software (version 1.5.0, Agilent). All experimental groups were co-cultured with LSMSCs, and the effects on cell viability were evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay.

2.3.2. Mitochondrial activity-based MTT assay

After the LSMSCs reached a sufficient number for the MTT assay, they were counted and seeded into plates containing the amniotic eye drop formulation at 5×10^3 cells per well. To evaluate the effects of the formulation via direct contact, the cells were treated with the amniotic eye drop formulation at concentrations of 5% and 10% (v/v), diluted in culture medium. After 24 hours of incubation, the culture medium was replaced with serum-free medium, and the cells were incubated for 24, 48, and 72 hours. At the end of these periods, to assess the effects of the 5% and 10% AM concentrations on cell viability, the cells were treated with a 5 mg/mL MTT stock solution and incubated in a CO_2 incubator for 3 hours. At the end of the incubation period, 100 µl dimethylsulfoxide **(**DMSO) was added to each well and the optical densities of the cells in the plates were read at 570 nm wavelength in a microplate reader (ELx808IU, BioTek Instruments, Winooski, Vermont, USA)(23). All experiments were performed in triplicate with eight technical replicates.

2.4. Evaluation of TGF-B and EGF release by ELISA

The samples were cut into equal pieces measuring 1x1cm². Prepared samples were placed in 15 mL conical tubes containing 10 mL of Dulbecco's

Phosphate Buffered Saline Solution (DPBS) (pH 7.4) and incubated at 37 °C for 24 and 48 hours. At each time point, 5 mL supernatant was collected, and 5 mL fresh buffer was added. The concentrations of transforming growth factor-β1 (TGF-β1) and epidermal growth factor (EGF) in the supernatants were determined using human TGF-β1 and EGF ELISA kits (ABclonal Technology, Massachusetts, USA). Briefly, after washing the 96-well plates provided in the kit, 100 µL of sample was added to each well and incubated for 2 hours at room temperature. Following washing, 100 µL of biotin-labeled working solution was added and incubated for 1 hour, followed by another wash and the addition of 100 µL streptavidin-HRP solution for 30 minutes. After a final wash, 100 µL of substrate solution was added and incubated for 20 minutes. The reaction was stopped with 100 µL stop solution, and absorbance was measured at 450 nm using a monochromator microplate reader (ELx808IU, BioTek Instruments, Winooski, Vermont, USA)(24-27).

3. Results

3.1. Evaluation of rheological moduli in amniotic eye drops photo-crosslinked for different durations

Rheological analysis of the amniotic eye drops showed that as the photo-crosslinking time increased (0 second [black], 30 seconds [red], and 60 seconds [gray]), both the storage modulus (G') and the loss modulus (G") increased (**Figure 3A**). For all groups, the G' values were higher than the G" values, suggesting that the elastic properties of the material were dominant over its viscous behavior.

Furthermore, no marked variation in G′ and G″ values was observed with increasing photo-crosslinking time, with the material exhibiting a relatively stable viscoelastic profile within the measurement range.

3.2. Evaluation of viscosity changes at constant shear rate in amniotic eye drops photo-crosslinked for different durations

In the non-crosslinked liquid form of the amniotic eye drop, viscosity values fluctuated over time (**Figure 3B**), reflecting incomplete structural stabilization during the initial phase. The sample photo-crosslinked for 30 seconds exhibited a comparatively more stable viscosity profile with reduced fluctuations compared to the liquid state. The 60-second photo-crosslinked sample showed the least time-dependent variation among the tested groups. The rheological profile showed reversible, time-dependent changes in viscosity during the measurement period.

3.3. Evaluation of viscosity changes at variable shear rates in amniotic eye drops photo-crosslinked for different durations

Viscosity measurements of the material under different shear rates ($\dot{\gamma}$) and over time showed a shear-dependent decrease in viscosity, where viscosity decreased as shear rate increased (**Figure 3C**). Descriptive comparison between groups suggested that as the photo-crosslinking duration increased (0, 30, and 60 seconds), overall viscosity also increased. The difference in viscosity between groups was more pronounced at low shear rates, while at higher shear rates the differences diminished, and viscosity values converged. This observation may reflect time-dependent structural rearrangements, although no statistical inference was performed.

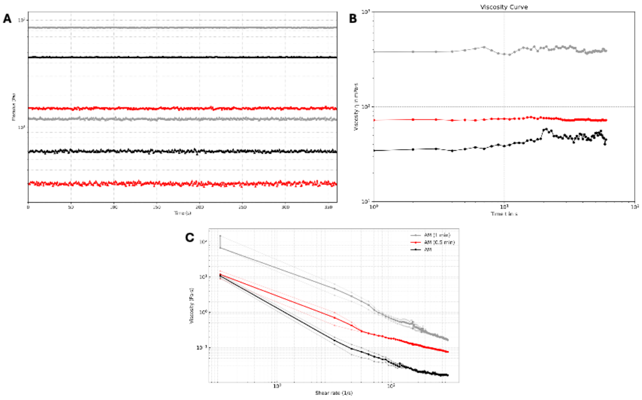

Figure 3. *Evaluation of the rheological moduli (Panel A, ■ represents G' (storage modulus), ▲ represents G" (loss modulus)), viscosity at constant shear rate (Panel B), and viscosity at variable shear rates (Panel C) of the amniotic eye drop formulations photo-crosslinked for different durations (0 second [black], 30 seconds [red], and 60 seconds [gray]).*

3.4. Evaluation of in vitro cytotoxicity of the hydrogel

3.4.1. Thawing, culture, and characterization of human limbal stromal mesenchymal stem cells (hLSMSCs)

Immunophenotypic characterization of hLSMSCs cultured up to passage 3 was performed using antibodies specific to surface antigens defined as the minimal phenotypic criteria by the International Society for Stem Cell Research (ISSCR) (Figure 4). Flow cytometric analysis revealed that hLSMSCs expressed CD90 (100%), CD73 (100%), HLA-B-C (100%), CD44 (100%), CD105 (99.60%), and CD29 (99.81%) positively, while showing low or negative expression for CD69 (5.50%), CD34 (27.78%), CD14 (15.26%), CD25 (0.02%), CD19 (0.00%), CD45 (0.02%), and HLA-DR (0.03%) (Figure 4).

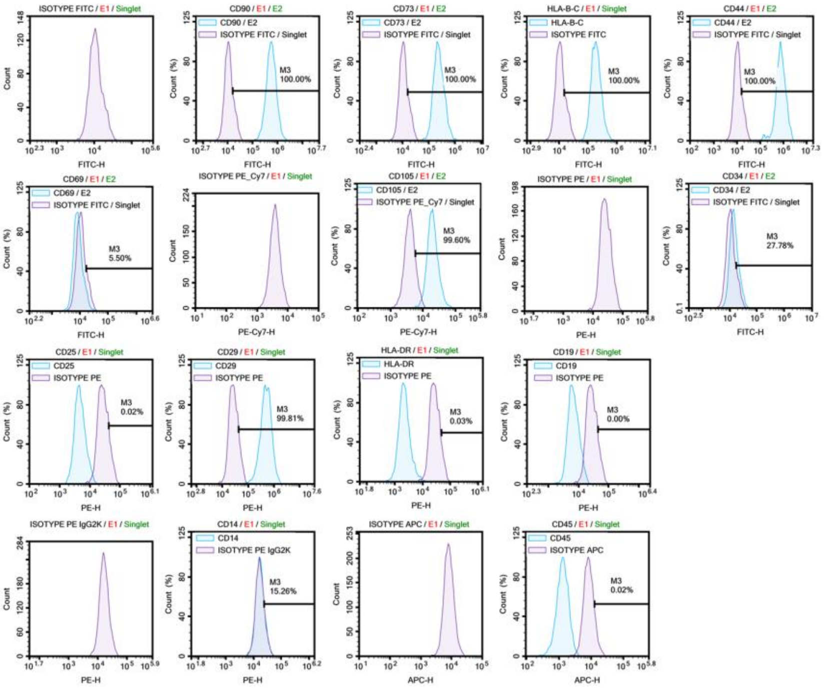

Figure 4. Flow cytometric immunophenotypic analysis of third-passage hLSMSCs. Target antibodies are shown in blue, while isotype antibodies (negative controls) are shown in purple.

3.4.2. Mitochondrial Activity-Based MTT assay

Amniotic membrane combined with GelMA was applied to hLSMSCs at final concentrations of 5% and 10% for 24, 48, and 72 hours. At 24 hours, cell viability was found to be 180% in the 5% AM concentration group and 160% in the 10% concentration group compared to the control. At 48 and 72 hours, cell viability in both groups was below 100% (Figure 5).

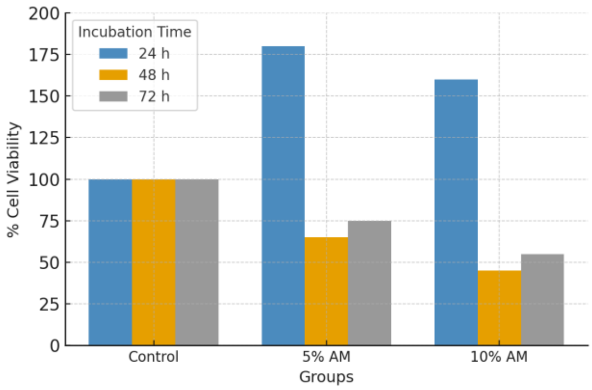

Figure 5. Evaluation of the effects of amniotic membrane application on hLSMSCs cell viability at 24, 48, and 72 hours using the MTT assay.

3.5. Evaluation of TGF-β and EGF Release by ELISA

Prepared samples were applied to hLSMSCs cultures for 24 and 48 hours, after which ELISA analyses were performed. The results showed that EGF concentrations reached 313 pg/mL in the 5% AM group at 48 hours. For TGF-β, concentration measured 350 pg/mL in the 5% AM group at 48 hours. At 72 hours, EGF levels were undetectable, while TGF-β concentrations decreased to 100.5 pg/mL.

3. Discussion

This study suggested that the AM can be successfully incorporated into a GelMA-based hydrogel system to develop a slow-release amniotic eye drop with favorable physicochemical and biological properties. Rheological analyses showed that the formulation exhibited stable viscoelastic behavior with shear-thinning characteristics, which may be compatible with ocular surface application. In vitro studies using hLSMSCs showed no evidence of cytotoxicity under the tested conditions, and increased metabolic activity was observed at lower concentrations at early time points. Furthermore, ELISA results showed measurable release of EGF and TGF-β, particularly at 48 hours.

The combination of AM extract with GelMA yielded a photo-crosslinkable hydrogel capable of forming a stable, uniform, and elastic film on both hydrophilic and hydrophobic surfaces. Rheological characterization showed that the storage modulus (G') exceeded the loss modulus (G") across all crosslinking durations, suggesting predominant elastic behavior within the tested range. Increasing photo-crosslinking time (0, 30, and 60 seconds) did not result in marked differences in G' and G" values within the measurement range.

Cytocompatibility analysis showed no detectable cytotoxic effect under the experimental conditions. Human LSMSCs cultured with 5 % and 10 % AM concentrations exhibited cell viabilities of 180 % and 160 %, respectively, after 24 hours relative to control. At 48 and 72 hours, viability declined below 100 %, remaining within a non-cytotoxic range. These findings suggest that the formulation may be compatible with ocular application, although further biological validation is required.

When compared with previous studies, this GelMA-based system represents a substantial advancement in AM-derived ocular formulations. Boto-de-los-Bueis et al. quantified the time-dependent stability of growth factors and endostatin in AM eye drops, showing that proteins such as EGF, HGF, and endostatin remained stable for up to six weeks under cryopreserved storage (28). Their study confirmed biochemical integrity of AM extracts but also highlighted a critical pharmacokinetic limitation; solution-based eye drops exhibited short ocular retention and limited corneal penetration, consistent with the general estimate that only approxiametly 10 % of topically applied drugs reach ocular tissues (11, 28). In contrast, the present GelMA–AM hydrogel may increase contact time and modulate diffusion of bioacvite factors, thereby potentially addressing this limitation.

Similarly, Basasaro et al. developed two AM-containing hydrogels; one based on thiolated hyaluronic acid (HA-SH) and gold-mediated dynamic crosslinking, and another using polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), Eudragit S100, and hyaluronic acid (29). These hydrogels encapsulated AM proteins at 1 mg/mL and were fabricated as solid conjunctival films requiring surgical or manual placement (29). While Basasaro’s system achieved improved ocular residence and demonstrated compatibility in rabbit conjunctiva, its application remained invasive and lacked on-surface formability (29). In comparison, the GelMA–AM system developed here offers a potentially less invasive alternative, as its photo-crosslinkable design allows gelation on the ocular surface. This capability eliminates the need for implantation or sutures while maintaining bioactive release control.

Kilian et al. in their recent review provided a comprehensive synthesis of AM-extract therapy mechanisms (30). It reported that AM extracts contain a broad array of bioactive components—including EGF, KGF, HGF, TGF-β, TIMP-1/2, IL-10, and endostatin—that persist after lyophilization or mild extraction (30). These molecules collectively promote epithelial migration, suppress inflammation, inhibit neovascularization, and modulate fibroblast activity. Clinical findings summarized in the review showed accelerated corneal epithelial closure and reduced discomfort in alkali-burn and persistent epithelial defect models, with efficacy comparable to or exceeding that of autologous serum drops (30). However, the review emphasized key translational barriers: rapid tear-film clearance, inconsistent growth-factor dosing, and short residence time on the ocular surface (30). The current GelMA–AM formulation was designed to address these limitations by incorporating a photo-crosslinkable delivery matrix intended to prolong surface residence time (12).

In alignment with these findings, the sustained release of EGF and TGF-β observed in this study mirrors the temporal profile of physiological wound-healing cascades. ELISA analysis showed that EGF and TGF-β levels reached 313 pg/mL and 350 pg/mL, respectively, at 48 hours in the 5% AM group. EGF predominantly acts during the early proliferative phase by promoting epithelial proliferation and migration, whereas TGF-β regulates extracellular matrix synthesis and fibroblast differentiation, orchestrating stromal remodeling and stabilization in subsequent phases (31, 32). These concentrations are consistent with a sustained-release pattern within the evaluated time frame.

At 24 hours, hLSMSCs treated with amniotic membrane–GelMA constructs demonstrated markedly elevated MTT viability values, reaching 180% in the 5% AM group and 160% in the 10% AM group compared with controls. This finding reflects increased metabolic activity as measured by mitochondrial dehydrogenase function (23). Values exceeding 100% in MTT assays have been well documented in the literature, particularly at early time points following exposure to bioactive matrices or growth factor–rich environments, and are interpreted as indicators of metabolic stimulation and proliferative activation rather than methodological artifacts (23, 33). The subsequent decline of viability values to below 100% at 48 and 72 hours in both groups suggests attenuation of this early hypermetabolic response and progression toward cellular adaptation, a temporal pattern consistent with previous reports involving hydrogel-based and amniotic membrane–derived biomaterial systems (18).

Furthermore, the rheological characteristics of the formulation, where G' exceeded G" and shear-thinning behavior was maintained, ensured film integrity during blinking and tear flow. Rheological studies confirm that when the storage modulus (G') dominates the loss modulus (G"), the network behaves more elastically and resists deformation under dynamic loading (34). Moreover, shear-thinning behavior, where viscosity decreases with increasing shear rate, has been shown to facilitate spreading and conformal contact on curved ocular surfaces while still providing mechanical stability once the shear is removed (35). In ocular-specific hydrogel formulations, these controlled viscoelastic properties correlate with improved residence time and comfort on the eye surface, as the gel maintains its structure under blinking yet flows during instillation (15, 36, 37). By exhibiting these viscoelastic traits, the GelMA–AM drop may support surface retention and mechanical conformity.

The lyophilization–cryomilling–photo-crosslinking sequence was designed to preserve growth-factor bioactivity while facilitating handling stability. Kilian et al. highlighted in their recent review that maintaining protein integrity through appropriate drying and sterilization methods is essential for preserving the therapeutic efficacy of AM-derived formulations, and the current protocol aligns with those recommendations (30). This design therefore integrates biochemical efficacy, mechanical adaptability, and logistical practicality into a single system suitable for widespread clinical translation.

However, this study has important limitations. All experiments were conducted in triplicate (n = 3) as technical replicates derived from the same formulation batch, without independent biological replicates. Accordingly, inferential statistical testing was not performed, as such analyses would not be methodologically appropriate and could produce unreliable or non-generalizable results.

Collectively, these findings suggest that the developed photo-crosslinked GelMA–AM hydrogel eye drop shows biocompatibility and sustained-release characteristics under in vitro conditions. This formulation may represent a non-surgical, sustained-release approach addressing limitations of conventional AM therapies. By combining the biological potency of the AM with the functional advantages of a smart polymeric carrier, the system provides a potential translational platform integrating biochemistry and materials engineering in ophthalmic therapy.

Future investigations should include in-vivo evaluations in chemical and thermal ocular injury models to assess epithelial regeneration, stromal remodeling, and potential inflammatory modulation. Long-term storage stability, mechanical degradation kinetics, and receptor-level bioactivity of released factors should also be characterized. With such validation, the GelMA–AM formulation could evolve into a clinically feasible, patient-friendly alternative to both AM transplantation and solution-based AM eye drops.

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