UNIVERSITÀ DEL PIEMONTE ORIENTALE

School of medicine

Department of Health Sciences

Master Degree in Medical Biotechnologies

″ Corneal regeneration using dECM enriched with *PACAP – NAP* peptides″

Mentor:
prof.ssa Francesca BOCCAFOSCHI

Firmato digitalmente da Francesca Boccafoschi
Data: 21.02.2026 14:58:02 CET
Organizzazione: UNIVERSITA' DEGLI STUDI DEL PIEMONTE ORIENTALE/01943490027

Candidate:
Asma SOLTANI

Matricular number:20058329

Academic year 2024/2025

INDEX

Summary

The human eye functions are a complex sensory organ, which detects visual data but remains defenseless against physical harm and infectious diseases and age-related deterioration. The cornea functions supports both clear vision and proper refraction. The cornea maintains both optical clarity and mechanical stability through its specific multi-layered structure, which includes the epithelium, Bowman's layer, stroma, Dua's layer, Descemet's membrane and endothelium. The high risk of corneal damage is due to the exposure to environmental factors and physical stress, which leads to abrasions, chemical burns, radiation exposure and microbial keratitis. The cornea fails to heal properly when treatment is delayed, resulting in permanent vision damage through scarring, fibrosis and endothelial dysfunction. Standard treatment methods, which include lubricants, antibiotics or surgical interventions, show limited success in achieving complete transparency and functional tissue regeneration. In this context, the field of regenerative ophthalmology now focuses on biomaterials and bioactive therapies as its main promising approaches. The scientific community shows strong interest in hydrogels made from decellularized extracellular matrix (dECM). In fact, the combination of optical clarity, high-water content and biological activity in these materials makes them suitable for supporting cell adhesion and proliferation. The inadequate mechanical properties and fast degradation rate of these materials necessitate advanced modifications through chemical crosslinking and functionalization techniques. The octapeptide NAP together with Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) have proven themselves as effective therapeutic agents for corneal restoration. The two peptides show protective effects against neuronal damage and inflammation while also preventing cell death. Research on PACAP shows it accelerates epithelial wound recovery while controlling oxidative stress and encouraging neuronal development but NAP protects cells from ultraviolet radiation and oxidative damage through its anti-apoptotic and mitochondrial protective effects. The combination of these peptides with biomimetic hydrogels enables the development of a treatment system, which can fix epithelial damage and protect corneal nerves and minimize inflammation. The research focused on how a hydrogel derived from decellularized extracellular matrix (dECM) and enriched with Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and NAP peptides may support corneal tissue repair. The combination of dECM structure with neuroprotective peptide activity creates a system, which promotes epithelial cell survival and wound recovery and establishes a therapeutic environment for corneal tissue repair. The method represents an advanced therapeutic solution, which establishes a forward-thinking approach for ophthalmic tissue restoration.

1. Introduction

The human eye stands as one of the most vital and intricate sensory organs which exists in our body. The external environment gets recorded by the eye before the optic nerve sends these signals to the brain.

Moreover, the eye faces different diseases, which include bacterial or viral infections together with inflammation. The anterior segment pathologies show effective treatment possibilities through pharmacological interventions. The delivery of therapeutic agents to the posterior segment of the eye presents significant challenges to medical practitioners. The posterior segment contains diseases such as age-related macular degeneration, macular edema, glaucoma, diabetic macular edema, proliferative vitreoretinopathy, cytomegalovirus retinitis, endophthalmitis, and diabetic vitreoretinopathies, which cause severe vision loss when untreated. The eye's special structure creates difficulties for drug delivery but tissue engineering and regenerative medicine work to develop new therapeutic approaches for anterior and posterior eye diseases [1].

The blood-retinal barrier along with the cornea epithelium and tear film functions as natural barriers, which protect the eye but simultaneously block drug delivery to the target area. Simple eye drops fail to deliver their intended effect because the fluid exits rapidly or fails to penetrate properly. Researchers have created innovative solutions to address these problems by developing slow-releasing implants and in-situ gels and small particles [2].

1.1 Anatomy and physiology of the eye

The human eye consists of two main structural parts, which include the anterior segment and the posterior segment (Figure 1). The anterior segment includes the cornea, conjunctiva, iris, ciliary body, aqueous humor and lens which work together to let light enter and perform initial focusing. The posterior segment includes the retina, choroid, optic nerve and vitreous humor functions to detect images and send neuronal signals to the brain [3].

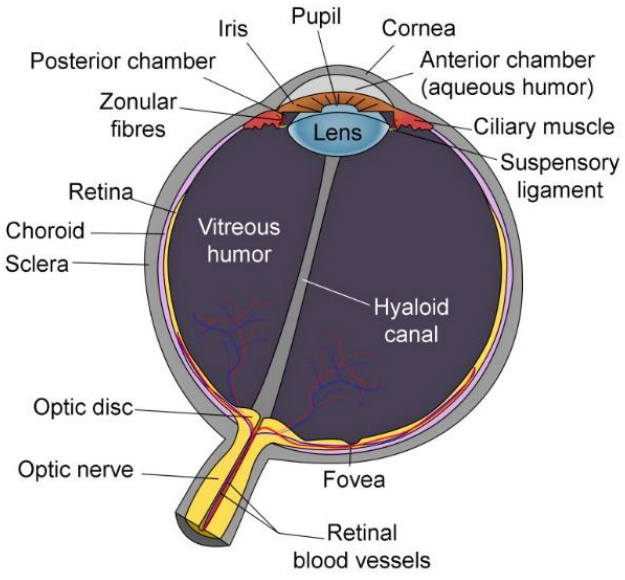


Figure 1: Anatomy of the Human Eye Schematic of Eye Anatomy. Contributed by R.H. Castilhos and Jordi March i Nogué (CC by SA-3.0) https://creativecommons.org/licenses/by-sa/3.0/deed.en

1.1.1 Cornea

The cornea is represented by a transparent curved structure which serves as the front section of the eye. It has two essential roles by upholding the inner eye structure and through its curvature it directs about two-thirds of incoming light for optical power [1].

The cornea exists as a thin smooth structure which lacks of blood vessels and stands as one of the most sensitive body tissues while receiving dense nerve supply. The cornea extends into the transparent conjunctiva and the white-eye structure called sclera. A pluripotent stem cell responsible for corneal surface regeneration exists at the limbus, which marks the boundary between the cornea and sclera (Figure 2) [4].

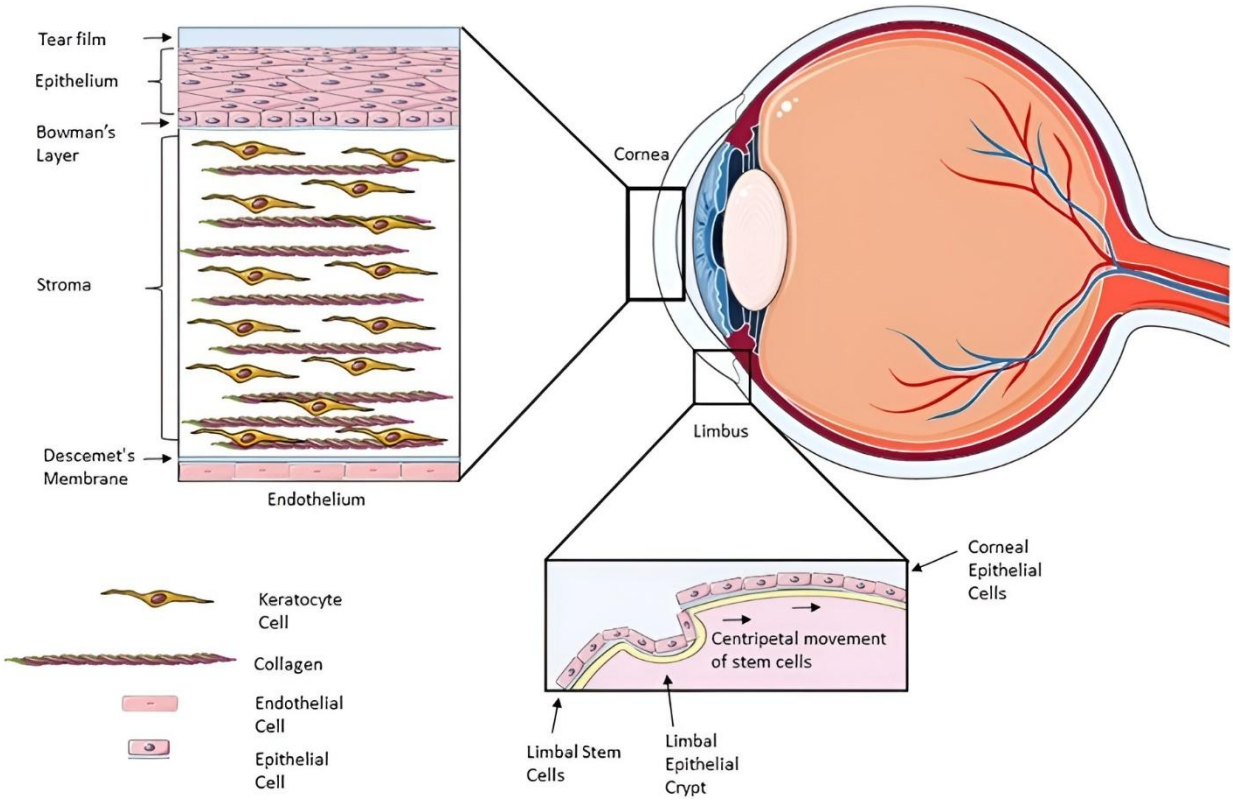


Figure 2: Corneal Architecture and Limbal Stem Cell Niche [5].The cornea consists of multiple layers which include epithelium followed by Bowman layer then stroma and Descemet membrane and finally endothelium. The limbal region at the corneo-scleral junction contains limbus epithelial cells that drive cornea epithelial regeneration and wound healing through their centripetal migration process [6].

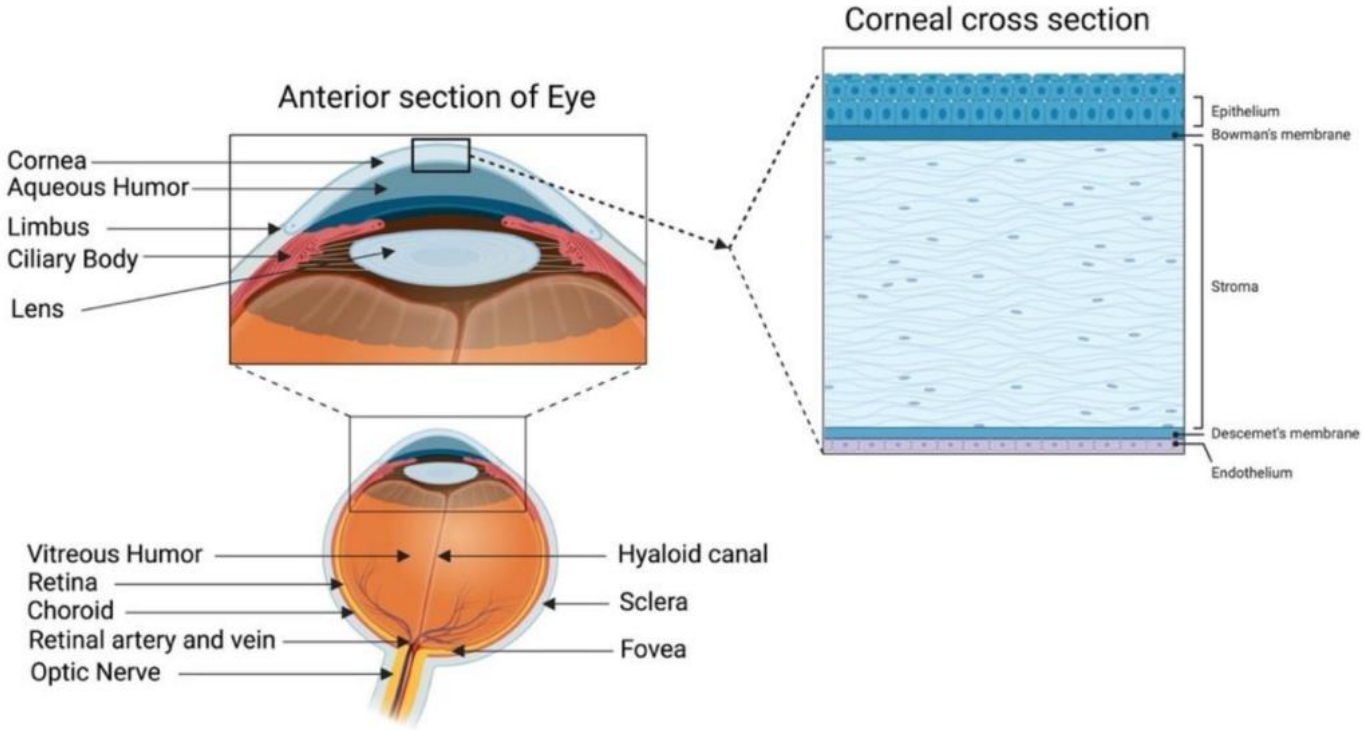
The cornea has two layers of protection: the tear film at the outer surface and aqueous humor at the inner surface. The cornea becomes thinner and more curved moving from the centre to the periphery [7]. The collagen fibers in the stroma are highly organized to allow efficient transmission and refraction of light with uniform diameters and spacing [8]. This structure reduces light scattering helping to preserve corneal transparency. As it does not have a direct blood supply, the cornea receives oxygen and nutrients from surrounding fluids [7].

Moreover, the cornea contains dense innervation, reaching 300–400 times higher than those found in the epidermis [9]. The corneal nerves create a subepithelial plexus through sensory fibers which stem from the ophthalmic branch of the trigeminal nerve. The fibers penetrate the cornea through the limbal region before they shed their myelin sheath and split into branches that penetrate the stromal layer [10]. The exposure of corneal nerve endings through epithelial damage leads to intense ocular pain and significant discomfort [11].

Furthermore, the cornea contains five distinct specialized layers which include the epithelium followed by Bowman's layer then stroma and Descemet's membrane and, finally, the endothelium. The maintenance of the transparency, mechanical strength and optical performance are made through the five distinct layers which differ in structure and cellular composition and function. The appropriate treatment selection depends on how each corneal layer responds to injury or disease [12].

1.1.2 Corneal Epithelium

The corneal epithelium represents the first protective layer on the eye surface which defends against environmental threats and physical injuries, functioning as a protective barrier through tightly bound non-keratinized, stratified, squamous epithelial cells. The tear film interaction



with this layer determines the cornea's hydration level and its smoothness and optical transparency (Figure 3). The corneal epithelium consists of five to seven flat cell layers that function mainly as an external barrier [5]. The epithelium receives its support from

limbal epithelial stem cells which reside in crypts at the corneoscleral junction also known as the limbus [13], [14].

Figure 3: Human cornea: anatomy and structure. In the anterior segment, the cornea is highlighted in relation to the rest of the eye. A schematic representation of the structure and composition of the cornea is presented in the corneal cross-section. It consists of 3 cellular layers (epithelium, stroma, and endothelium) and 2 basement membranes (Bowman's layer and Descemet's membrane) (figure created using BioRender.com, BioRender, Toronto, ON, Canada) [15].

1.1.3 Bowman's Layer

The first layer of the corneal stroma is Bowman's layer which exists below the epithelial basement membrane [16]. The layer measures between 15–18 µm in thickness and contains collagen fibrils that lack any lamellar arrangement [17], [18], [19]. The acellular layer provides mechanical strength to the cornea which maintains its smooth curved shape [20]. The layer shows age-related changes and does not heal after laser surgery injuries [21], [22]. The Bowman's layer connects to the epithelium through anchoring structures which include collagen types IV and VII [23], [24]. The layer exists independently from other stromal components because it possesses unique collagen structures and compositions

(Figure 4) [25], [26].

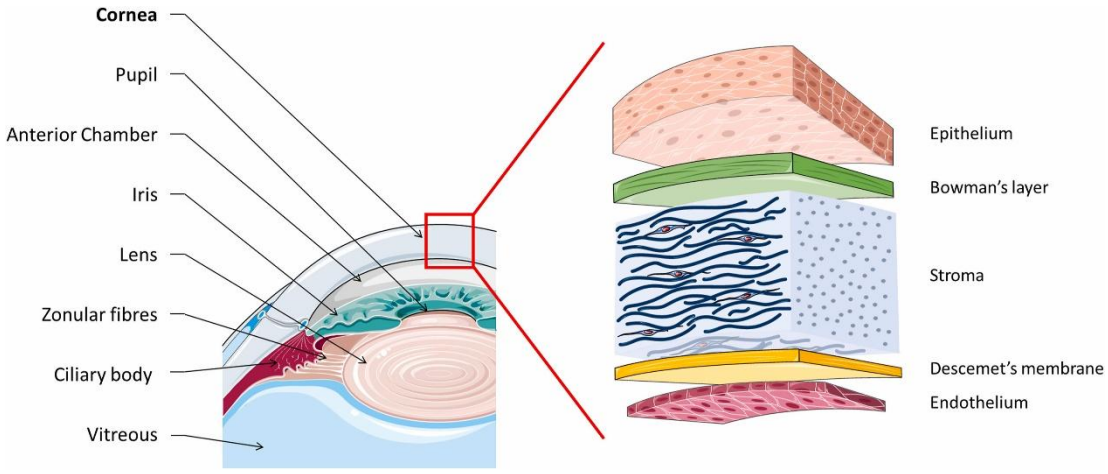


Figure 4: Cornea's layer. The cornea is composed by five distinguishable layers, three of these are cellular (epithelium, stroma and endothelium), and two acellular (Bowman's and Descemet's membranes) [27].

1.1.4 Stroma of the cornea, Dua, and Descemet membrane

The corneal stroma represents the thickest part of the cornea which makes up 80–90% of its total thickness [28], [29]. It consists of a structured collagen fibril network which contains type I collagen as the main component, together with types III, V and VI and a proteoglycan (PG)-rich ground substance that includes keratan sulfate (KSPG) and dermatan sulfate (DSPG). The

collagen and extracellular matrix (ECM) structure between Bowman's membrane and Descemet's membrane maintains both optical transparency and mechanical strength which are vital for maintaining a proper vision (Figure 5) [30], [31].

The anterior stroma lamellae are tightly packed creating a rigid structure that helps shaping the cornea. The lamellae in the posterior stroma show a more relaxed arrangement. The posterior stroma's loose structure makes it more prone to hydration imbalances that could result in corneal edema or clouding if not properly managed. The main cellular component of the corneal stroma consists of keratocytes which derive from neural crest fibroblast-like cells. The maintenance of stromal homeostasis depends on keratocytes which produce extracellular matrix components and control matrix remodeling through matrix metalloproteinases (MMPs) enzymes [28].

Moreover, the newly discovered anatomical structure Dua's layer exists directly below the stroma according to recent findings [32]. The layer consists of 58 lamellae made of type I collagen and is approximately 15 μm thick, acellular and collagenous [28], [32]. Right below the Dua's layer, there is the Descemet membrane, which represents the basement membrane of the corneal endothelium. The Descemet membrane starts its development in fetal development at 8 weeks gestation and continues to be produced by endothelial cells throughout human life [28]. The membrane consists mainly of type IV collagen and laminin. The membrane thickness is about 3 μm at birth but it increases to 10 μm in adulthood, presenting nine smooth layers (amorphous) with a uniform structure that lacks organized bands [29], [33]. The Descemet membrane serves as a communication link between the stromal and aqueous humor compartments, while providing essential structural support to the corneal endothelium. The three layers together (the stroma, Dua's layer, and Descemet's membrane) form the basic structural and functional backbone of the cornea, contributing to transparency, mechanical stability, and fluid homeostasis [34].

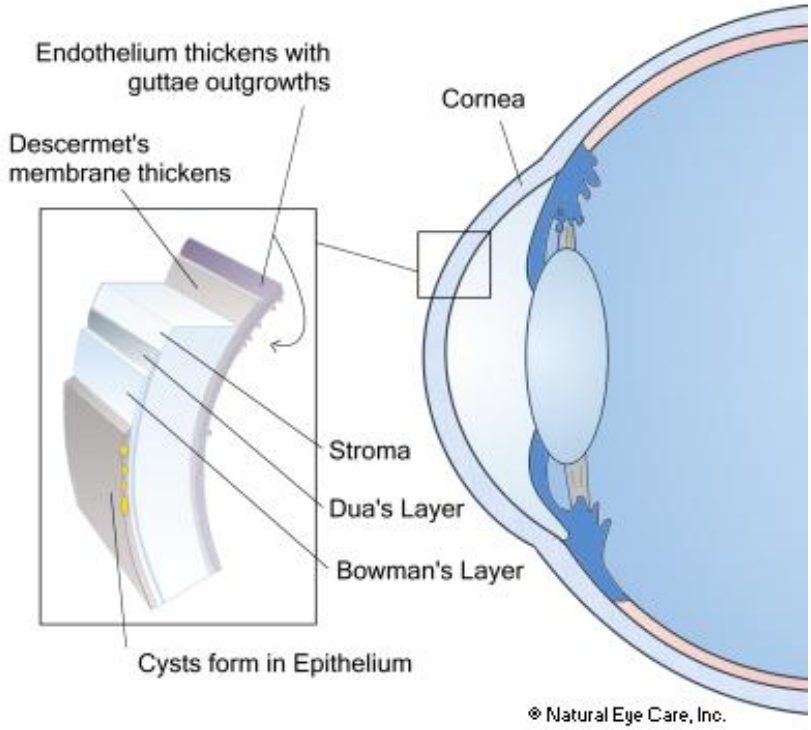


Figure 5: The cornea contains multiple structural layers which include the epithelium and Bowman's layer and stroma and Dua's layer and Descemet's membrane and endothelium. The figure shows age-related and pathological changes which include epithelial cysts and Descemet's membrane thickening and guttae outgrowths in the endothelium. The cornea achieves transparency and maintains curvature and mechanical strength through the combined function of these layers.

1.1.5 Corneal Endothelium

The corneal endothelium consists of a single hexagonal cell layer that covers the posterior surface of the cornea (Figure 6). The endothelium exists as a single cell layer which contacts the aqueous humor directly while Descemet's membrane provides structural support to this layer [35]. The corneal endothelial cells stay in the G1 phase of the cell cycle and have limited proliferation potential in vivo compared to other corneal layers [36]. The layer enables waste and nutrient exchange through active transport and diffusion processes [35]. Endothelial cell loss from disease or trauma leads to corneal edema and opacity which eventually causes vision loss [37].

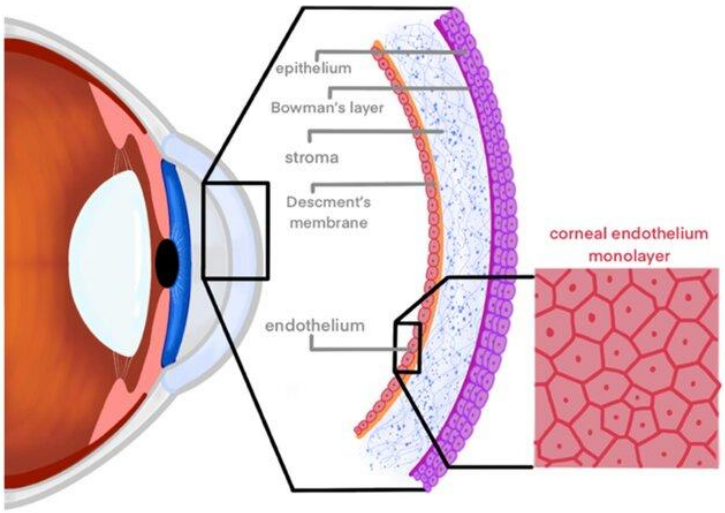


Figure 6: Cornea anatomy. Layers of the cornea depicted from inner to outer: endothelium, Descemet's membrane, stroma, Bowman's layer, and epithelium. The endothelium is highlighted to show its hexagonal monolayer arrangement of cells, crucial for maintaining corneal transparency and hydration balance [38].

1.2 Corneal Damage

Vision loss primarily related to corneal opacities which affect mainly low–income regions of Africa and Asia where timely diagnosis and treatment are not readily available [39]. The global programs have reduced blindness caused by infectious conditions like trachoma, but non–trachomatous corneal diseases continue to pose a significant and growing challenge. These trends highlight the urgent need for effective, accessible, and biotechnology–based solution that can be scaled across diverse healthcare settings [39]. The cornea suffers damage through multiple causes including mechanical trauma from fingernail or paper abrasions and improper

contact lens use and chemical injuries (Figure 7). Other notable examples include thermal burns, infections such as herpes simplex keratitis or Acanthamoeba keratitis, degenerative disorders like Fuchs' endothelial dystrophy, and autoimmune conditions such as Stevens–Johnson syndrome [31] [40], [41], [42], [43]. Among chemical agents, alkali substances are particularly hazardous due to their ability to deeply penetrate ocular tissues [32], [44]. The cornea becomes structurally weakened by radiation damage which includes UV keratitis that affects skiers and welders [45].

Thus, the development of corneal damage results from multiple causes which trigger intricate pathological and molecular processes [33], [44]. The corneal epithelium functions as a vital protective layer because it represents the outermost defensive barrier which usually sustains the initial damage. The cornea requires immediate and effective epithelial wound healing because this process safeguards both optical clarity and long-term ocular surface stability [46], [47], [48]. The regulatory mechanisms function in the most active ECM environment of the human body which exists in the anterior part of Bowman's layer that continuously remodels to enable cell adhesion and migration and tissue regeneration [49], [50], [51], [52], [53], [54].

The cornea demonstrates strong self-healing properties when it experiences superficial epithelial injuries. The healing process of epithelial tissues depends on cell migration which enables fast wound closure and normal function recovery. Moreover, chronic injuries become more complex because it involves stromal keratocyte inflammatory activation and fibrotic remodelling and permanent scarring risks [47]. In addition, corneal healing depends also on the synchronized work of growth factors, cytokines and ECM components which direct the tissue regeneration [55].

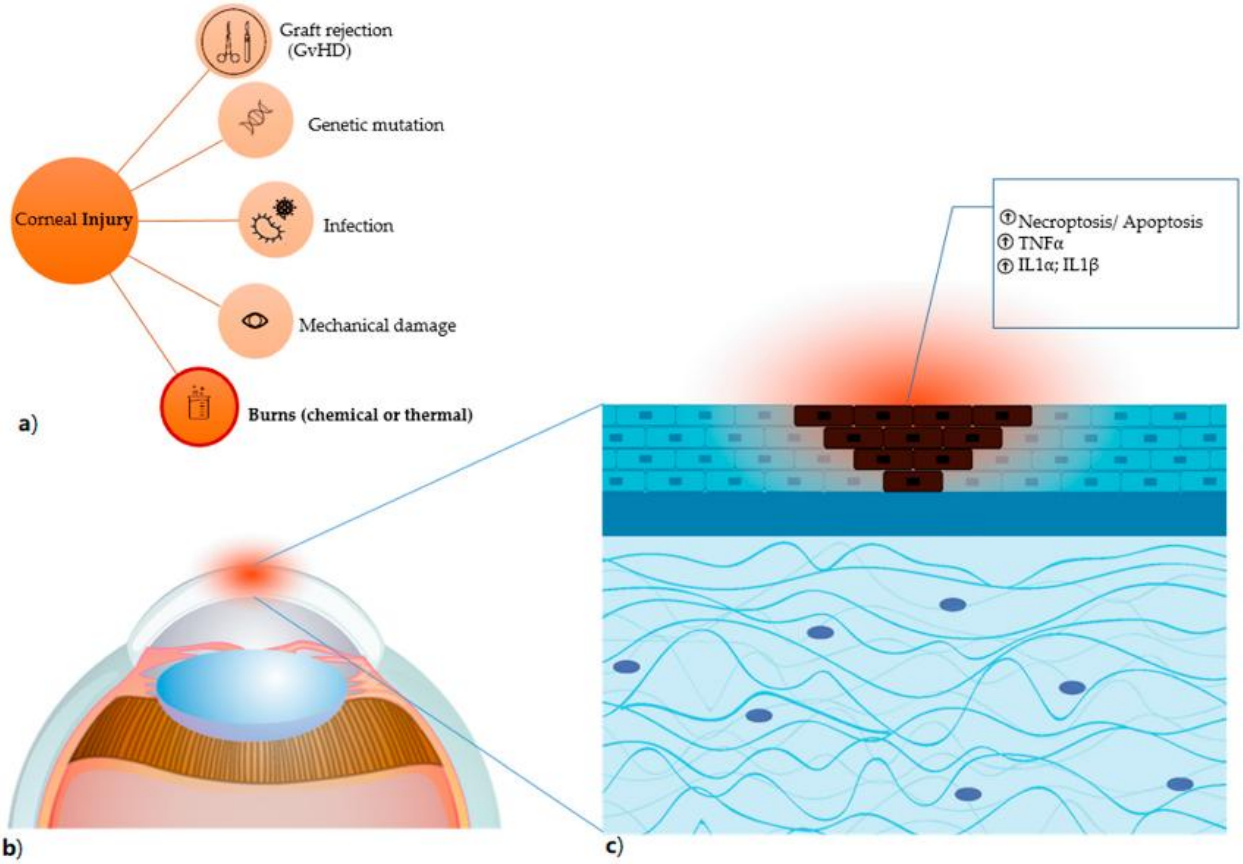


Figure 7: Corneal injury (**a**) the different types of corneal damages are listed, in particular burns, which comprise both chemical (for instance, induced by alkali exposure) and thermal burns. (**b**) Anatomical detail of the damaged corneal area, highlighted in red. (**c**) the damage caused by alkali burn induces necroptosis and apoptosis (shown as dark cells) with result in the release of factors that trigger the inflammatory process and have TNFα, IL1α and IL1β as primary drivers [56].

1.3 Treatments

The appropriate treatment for corneal epithelial injuries depends on the source of the injury and the degree of epithelial damage. The initial treatment approach includes the use of artificial tears, together with topical antibiotic drops to prevent infection and topical analgesics to control pain [57]. The treatment of severe or unresponsive cases requires additional therapeutic approaches, including autologous serum eye drops, amniotic membrane transplantation and therapeutic contact lenses [58], [59], [60].

The main goal of new treatments focuses on enhancing corneal epithelial regeneration and neurotrophic support to achieve complete healing and functional recovery [61]. The list includes ReGeneraTing Agents (RGTAs) and recombinant human nerve growth factor (rhNGF which is sold as Oxervate®) and corneal neurotization techniques [62], [63], [64].

However, the complex nature of corneal regeneration has led to the development of advanced therapeutic methods which treat both tissue damage at the macroscopic level and cellular dysfunction. The therapeutic strategies for corneal regeneration include stem cell-based therapies and emerging molecular interventions such as exosome-mediated delivery systems gene therapy, gene therapy, and regulatory RNA technologies [65].

In this context, the field of regenerative ophthalmology has seen recent advancements through biodegradable scaffolds made from dECM which create biochemical and mechanical gradients that enhance epithelial surface regeneration through better cell adhesion and structural similarity [66].

1.4 Hydrogels in Corneal Repair

The unique properties of hydrogels as highly hydrated polymers make them similar to native ECM which has led to significant interest in corneal tissue engineering applications. The soft elastic nature and high- water content of hydrogels matches the natural corneal stroma composition very closely, which makes them suitable for ocular surface applications. Interestinlgy, the natural optical transparency of hydrogels makes them suitable for use without vision impairment which remains a critical requirement for any corneal implant or regenerative scaffold [67].

The biomimetic scaffolds of hydrogels enable epithelial cells to adhere, proliferate and migrate, which are vital processes for corneal repair during wound healing. The use of natural hydrogels (e.g., collagen, gelatin, hyaluronic acid and alginate) and synthetic ones, such as polyethylene glycol (PEG) and polyvinyl alcohol (PVA), have been explored for this purpose [68], [69], [70]. However, the preference for natural hydrogels results from their built-in bioactivity because they possess biological signals which enable cell adhesion and signalling. The natural

materials, in fact, contain collagen and hyaluronic acid which support epithelialization, minimize inflammation and fibrosis to establish an ideal healing environment [71].

Thus, the development of injectable or in situ-forming hydrogels represents a future breakthrough because they can gel at physiological temperatures or respond to physiological conditions. These minimally invasive systems can be directly applied to the corneal wound area and they can fit irregular geometries of defects and provide a supportive and protective microenvironment for new tissue formation [67].

The native cornea achieves its transparency because of the ordered lamellar arrangement of collagen fibrils in the stroma and minimal light scattering. The hydrogels used for corneal applications must be optically clear while supporting the regeneration of structured ECMs to restore and maintain visual clarity [72]. The structural organization of the cornea will be severely affected if any disturbance occurs from scarring or fibrosis or when implanted materials have incompatible refractive indices [72].The development of smart or stimuli-responsive hydrogels has become possible through recent advances which allow these materials to detect local microenvironment changes including temperature and pH and enzyme activity fluctuations to trigger structural modifications or therapeutic release. The control of drug delivery is crucial for ocular applications because it enhances treatment outcomes and minimizes adverse effects. Advanced hydrogel systems demonstrate effective results in supporting epithelial and stromal regeneration and nerve repair and scar reduction [73], [74].

The hydrogels derived from decellularized extracellular matrix (dECM) hold great promises in corneal tissue engineering. In fact, the preserved matrix contains essential components including collagens, glycosaminoglycans (GAGs), laminin and fibronectin which create a highly bioactive environment that closely resembles the native corneal microenvironment and tissue-specific regeneration [75], [76]. These hydrogels can be engineered to match the shape of the injured ocular surface, providing both mechanical compliance and optical transparency while creating a bioactive biochemical environment that enables epithelial cells to adhere, migrate and proliferate [68], [74].

The main limitation of using dECM in hydrogels is its insufficient mechanical strength and rapid degradation when used *in vivo*. The stability and durability of dECM-based hydrogels have been improved through several crosslinking methods, such as glutaraldehyde and polyethylene glycol diacrylate (PEGDA) as well as natural crosslinkers like genipin and enzymatic systems (e.g., EDC/NHS). These approaches have contributed, to some extent, to the preservation of biocompatibility and optical transparency [75], [77]. The modifications enable hydrogels to stay on the ocular surface for longer durations which extends their therapeutic impact.

However, the ECM-mimicking structure, combined with slow degradation properties of these hydrogels, makes them suitable for delivering bioactive compounds including growth factors and anti-inflammatory agents and stem cells and therapeutic peptides [78], [79].

1.5 Bioactive Peptides: PACAP and NAP in Corneal Regeneration

Short peptide chains consisting of 2–50 amino acids join through peptide bonds formed through condensation reactions between adjacent residues' amino and carboxyl groups [80]. The basic units of protein structure are peptides, but their limited length creates distinct biological and

chemical and pharmacological characteristics that differ from proteins [81]. Thus, functional bioactive peptides integrated into biomaterial scaffolds represent a groundbreaking approach for tissue engineering applications.

In the field of corneal regeneration, the neuropeptides NAP and PACAP have been identified as promising therapeutic agents, due to their neuroprotective, anti-inflammatory, and epithelial-healing properties. Preclinical studies have shown that PACAP and NAP peptides possess significant regenerative and cytoprotective effects for this purpose. In fact, the peptides have been proven to boost epithelial healing while promoting corneal nerve growth and maintaining tissue structure after damage according to preclinical research [78], [79].

1.5.1 PACAP: A Multifunctional Regenerative Neuropeptide

The two biologically active isoforms of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) are PACAP38 and PACAP27. The signaling peptide functions to protect neurons, prevent cell death, regulate immune responses and promote epithelial tissue repair [82] [83]. The properties of PACAP make it a promising candidate for corneal wound healing by supporting epithelial restoration and inflammation control. Recent animal studies demonstrate that topical PACAP27 accelerates corneal epithelial healing and promotes tissue recovery [78].

Particularly, PACAP controls the expression of genes that regulate inflammation (e.g., TNF-α) and oxidative stress and apoptosis which demonstrates its extensive role in maintaining. The development of biocompatible hydrogels and decellularized ECM scaffolds in advanced bioengineering serves as delivery systems for PACAP through controlled release mechanisms that protect the molecule from enzymatic breakdown [84], [85].

1.5.2 NAP (Davunetide): Cytoprotective Modulator of Stress and Apoptosis

The octapeptide NAP originates from activity-dependent neuroprotective protein (ADNP) which was first recognized for its functions in brain development including synaptic plasticity regulation and circadian rhythm control [86], [87]. The biologically active domain of ADNP is NAP, which shows high therapeutic potential because of its small size and favourable pharmacokinetics and efficient permeability across the blood-brain barrier and nasal bioavailability. The characteristics of NAP make it a promising therapeutic agent for treating neurodegenerative and neuromuscular and ocular diseases [88]. Interestingly, NAP shows strong antioxidant and anti-apoptotic effects in experimental models of corneal injury caused by UV-B irradiation. In particular, the cytoprotective effects of NAP include blocking mitochondrial reactive oxygen species (ROS) production and maintaining mitochondrial membrane stability and blocking c-Jun N-terminal kinase (JNK) signalling, which is a major pro-apoptotic pathway that activates during cellular stress [79].

2. AIM

The proper functioning of vision depends on both corneal transparency and effective epithelial regeneration. The extracellular matrix (ECM) provides essential mechanical and biochemical signals which direct corneal cell activities and support wound healing and tissue homeostasis. The current research investigates how a decellularized extracellular matrix (dECM)-based hydrogel enriched with PACAP and NAP peptides affects tissue regeneration. In particular, in this study we evaluated a dECM peptideenriched hydrogel in order to promote corneal regeneration, improving epithelial cell viability.

3. Materials and Methods

3.1 dECM Hydrogel Preparation and Characterization

Hydrogel was derived from bovine pericardium extracellular matrix (dECM) through a decellularization process developed by Tissuegraft Srl (Alessandria, Italy) (Italian Patent: 102020000007567, International Patent: PCT/IB2021/052779). The decellularization method, validated by Tissuegraft, effectively removed cellular components while preserving the ECM essential protein content. The dECM liquid hydrogel alone or in combination with 10 nM NAP (NAPVSIPQ) or 100 nM PACAP (HSDGIFTDSYSRYRKQMAVKKYLAAVLGK RYKQRVKNK) (New England Peptide, MA, USA) at a final concentration of 2 mg/ml was finally used for cytocompatibility and regenerative potential testing. Hydrogels at different concentrations (9 mg/ml, 4 mg/ml 2 mg/ml and 2 mg/ml with NAP or PACAP) were prepared in 96 well plate and transmittance to visible light was assessed using Victor4X Multilabel Plate Reader (Perkin Elmer, Milan, Italy), PBS was used as blank. The obtained absorbance values were converted to transmittance by Beer Lambert's law. Mean transmittance was derived by averaging the values obtained for the visible light (595 nm). Moreover, in order to assess the transparency over time, transmittance was observed up to two weeks at different hydrogel concentrations.

3.2 Cell culture:

The ATCC PCS-700-010 Normal Human Primary Corneal Epithelial Cells (ATCC, LGC Standards, Milan, Italy) received standard physiological medium incubation at 37°C inside a 5% CO2 humidified incubator. The cells were maintained using the Corneal Epithelial Cell Basal Medium (ATCC, LGC Standards, Milan, Italy) with Corneal Epithelial Cell Growth Kit (ATCC, LGC Standards, Milan, Italy) supplemented with penicillin (100U/mL), streptomycin (0.1 mg/mL), amphotericin (0.25 µg/mL) (Euroclone, Milan, Italy). The cells underwent subculturing at 70-80% confluence through standard sub-culturing procedures.

3.3 Cell viability

Cell viability was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide salt (MTT) (Sigma-Aldrich). Briefly cells were seeded at a density of 1 × 104 cells/well for 3 and 7 days in 96-well plates coated with 2mg/ml dECM hydrogel with and without NAP and PACAP. Then the medium was replaced with a fresh medium with MTT salt added to each well for 4 h. Finally, dimethyl sulfoxide (DMSO) was used to dissolve formazan salts, and absorbance was measured at 570 nm using the plate reader VICTOR4X Multilabel Plate Reader (Perkin Elmer, Milan, Italy). The medium alone was used as a blank.

3.4 Cell Morphology

Cells were seeded onto pre-coated (2mg/ml dECM hydrogel with and without NAP and PACAP) glass coverslips. After 3 and 7 days, cells were fixed with 4% formalin ultrapure water, rinsed and stained with phalloidin (Sigma Aldrich, Milan, Italy) and with 300 nM Diamidine-20-phenylindole dihydrochloride (DAPI) (Sigma Aldrich, Milan, Italy) for the nuclei dye. A

final rinsing was done before the samples were mounted (60% glycerol in PBS) onto glass slides and observed. Representative images were taken with the fluorescence Leica DM2500 microscope (Wetzlar, Germany) and acquired via Leica software.

3.5 Immunofluorescence

Immunofluorescence staining against Cytokeratin-3 and Cytokeratin-12 was conducted by seeding the cells, at a density of 1 x 10^4 at cm^2 on pre-coated (2mg/ml dECM hydrogel with and without NAP and PACAP) glass coverslips. After 7 days, the cells were fixed for 1 h at room temperature in 4% formalin in ultrapure water. The primary antibodies were then added, and incubation was performed overnight. The anti-cytokeratin-3 and anti-cytokeratin-12 antibodies (ThermoFisher, Milan, Italy) was each diluted 1:100 in 2% Goat Serum (Euroclone, Milano, Italy), 1% BSA (Sigma Aldrich, Milan, Italy) and 0,1% TritonX (Sigma Aldrich, Milano, Italy) in PBS at 4°C. After overnight incubation, Cytokeratin-3 and Cytokeratin-12 were revealed by secondary Texas Red-labeled anti-rabbit IgG antibodies (1:500; Vector TI2000, Vector Laboratories, CA, USA) and counterstained with 2-(4-Amidinophenyl)-6-indolcarbamidin -dihydrochloride (DAPI) (Sigma Aldrich, Milan, Italy) for the nuclei dye. The glass was then mounted (60% glycerol in PBS) and images were acquired using the Leica DM2500 fluorescence microscope (Wetzlar, Germany), while Leica Application Suite software handled image processing and analysis.

3.6 Wound healing assay

The scratch wound assay was performed and imaged using the IncuCyte Zoom Live Cell Imaging System and Scratch Wound analysis module per the manufacturer's instructions (IncuCyte, Sartorius). Briefly, cells were seeded in 96-well plates pre-coated (2mg/ml dECM hydrogel with and without NAP and PACAP) at a final density of 10 000 cells/well. The following day, scratch wounds were performed using the IncuCyte WoundMaker following the manufacturer's instructions (IncuCyte, Sartorius). The media was then replaced to remove cell debris, and the plate was imaged every 4 h using the IncuCyte for 24 hours. Wound closure was calculated using IncuCyte's wound confluence analysis module for each image over time.

3.7 Western Blotting

After 7 days of culturing, cells were lysed in boiling SDS (Tris-HCl 1 M pH 7,4, SDS 10%, PBS pH 7.4, ultrapure water), and cellular lysates were collected and stored at –20 °C. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher, Milan, Italy) according to the manufacturer protocol. Samples were prepared for electrophoresis by dissolving in Laemmli Sample Buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 5% beta-mercaptoethanol, 0,005% bromophenol blue, 2% SDS) (Sigma Aldrich, Milano, Italy). Electrophoresis was performed using Sodium Dodecyl Sulphate-PolyAcrylamide Gel (SDS-PAGE) using 7.5% N, N'-methylenebisacrylamide (acrylamide) and then electrophoretically transferred to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). Blotted proteins were blocked with 5% non-fat dried milk on PBS for 1 h at room temperature, then incubated overnight at 4 °C with using anti-rabbit cytokeratin 3 and 12 (Thermo Fisher Scientific, Italy) and mouse anti- α-tubulin (Merck Millipore, Milan, Italy). After washing, membranes were incubated with HRP-conjugated secondary antibody (1:2000; Perkin-Elmer,

Milan, Italy) for 1 h at room temperature, and bands were visualized using a chemosensitive visualizer (ChemiDocTM Touch Imaging System, Bio-Rad, Milano, Italy). Densitometric analysis was performed using ImageLab (BioRad, Hercules, CA, USA) software. Experiments were performed in triplicate.

3.8 Statistical analysis

The data were expressed as mean ± standard deviation values. The Student's t-test served to determine statistical significance. The statistical analysis produced p-values which showed significance ($p \leq 0.05$) with respect to CTR with * $p \leq 0.05$. All experiments were performed at least three times to ensure reproducibility.

4. RESULTS

4.1 dECM Hydrogel Preparation and Characterization

The dECM hydrogels underwent light transmittance evaluation for corneal applications through transparency testing at 2 mg/ml, 4 mg/ml and 9 mg/ml concentrations as shown in Figure 8. We conducted a general and macroscopical observation of the hydrogels: from left to right in the image, the samples correspond to 2 mg/ml (clear), 4 mg/ml (slightly opaque), and 9 mg/ml (most opaque), indicating that the lower concentration (2 mg/ml) has better optical clarity than 4 and 9 mg/ml. Moreover, the right panel shows the percentage of light transmittance over time (freshly prepared, 1 week and 2 weeks after preparation). The highest transmittance (above 90%) was observed in 2 mg/ml hydrogels, which remained stable up to 1 weeks, while 9 mg/ml had the lowest transmittance. These results indicate that lower dECM concentrations are more appropriate for corneal applications because of their better optical properties**.**

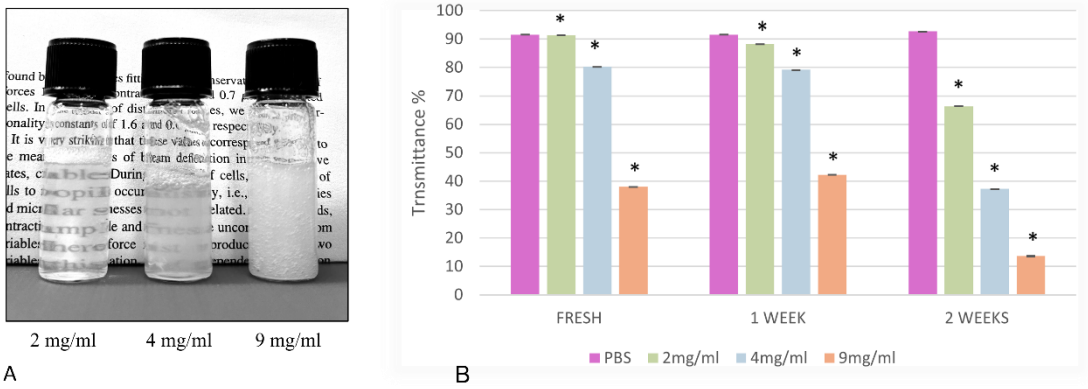


Figure 8: Transmittance of dECM hydrogels over time. A) General transparency observation of hydrogels at different concentrations. B) Light transmission of hydrogels at visible light. Results represented from three different experiments, expressed as mean ± SD. Statistical significance with respect to blank was indicated with *$p \leq 0.05$.

Then, we also evaluated the transparency of dECM hydrogels enriched with PACAP and NAP peptides, in order to determine if peptides addition affected optical clarity (Figure 9). The 2 mg/ml dECM hydrogel enriched with PACAP or NAP peptides maintained their transparency at about 83–85%, which remained within the suitable range for corneal applications. Overall, the results indicate that the lower concentration (2 mg/ml) with or without peptide enrichment maintains optical clarity.

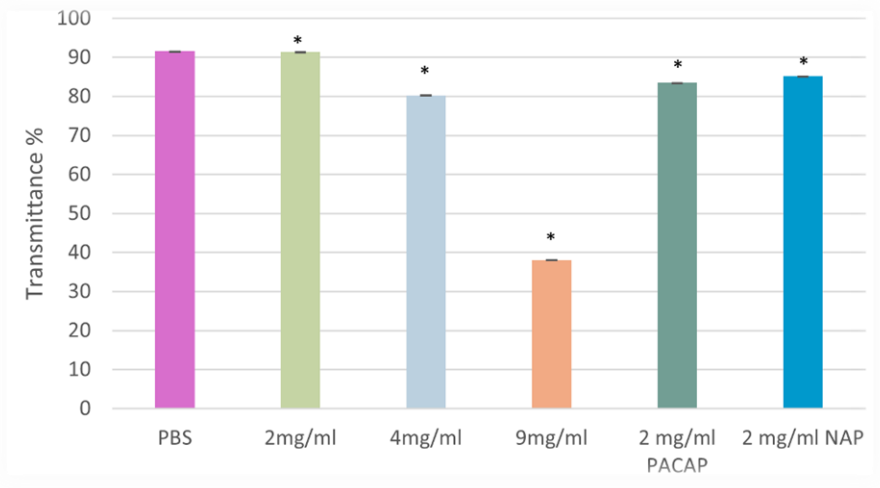


Figure 9: light transmittance (%) of dECM hydrogels at different concentrations and with peptide enrichment. The transmittance of dECM hydrogels was measured at 2, 4, and 9 mg/ml and for 2 mg/ml hydrogels enriched with PACAP or NAP peptides compared to a blank control. The values represent mean ± SD from three independent experiments (n = 3). Statistical significance with respect to blank was indicated with *p ≤ 0.05.

4.2 Effect of dECM and Peptide-Enriched Hydrogels on Corneal Epithelial Cell Viability

MTT assay evaluated primary corneal epithelial cell viability after 3 and 7 days of culture (Figure 10). The dECM-based groups including PACAP and NAP-enriched samples demonstrated equivalent absorbance values to the control group on day 7, which indicated sustained cell viability. The peptide-only groups treated with PACAP without dECM demonstrated decreased viability at a statistically significant level compared to the control. The results indicate that dECM functions as a supportive matrix for epithelial cell survival and the addition of PACAP or NAP maintain cell viability.

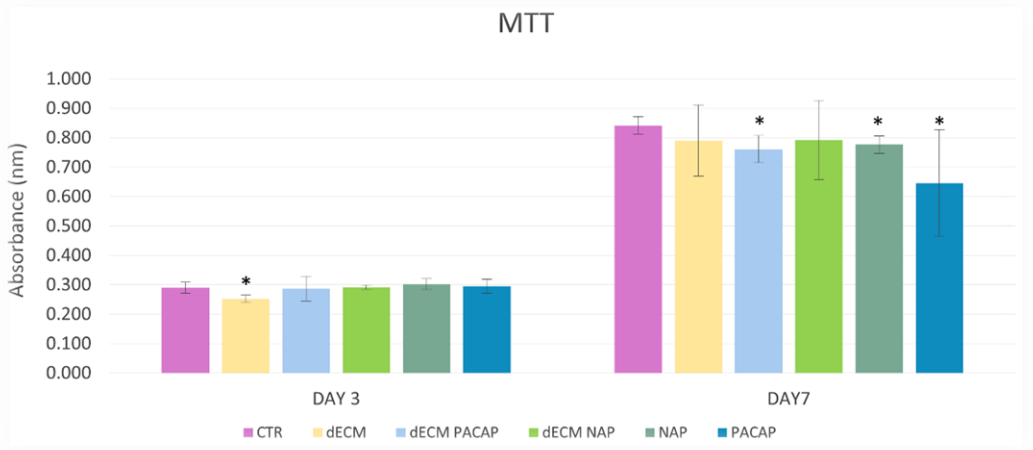


Figure 10: The MTT assay measured primary corneal epithelial cell viability across different conditions (CTR, dECM, dECM PACAP, dECM NAP, NAP, and PACAP) at day 3 and day 7. The results show absorbance values at 590 nm (mean ± SD, n = 3).

Statistical significance with respect to control was indicated with *p ≤ 0.05.

4.3 Effect of dECM and Peptide-Enriched Hydrogels on cellular morphology

Moreover, we evaluated how the hydrogel enriched with two different peptides affect corneal epithelial cell morphology after 3 and 7 days. The Figure 11 shows how these substrates enable epithelial cell structure formation, cell attachment and spatial organization. All cell cultures maintained epithelial-like cell morphology with polygonal shapes at day 3. The cells seeded on dECM NAP developed uniform organization and better spreading patterns with respect to the dECM with or without PACAP, which seemed to decrease slightly, indicating the dECM NAP as a better support for epithelial growth at day 7. However, the observations demonstrate that dECM creates an optimal environment for corneal epithelial structure and morphology when used with peptides.

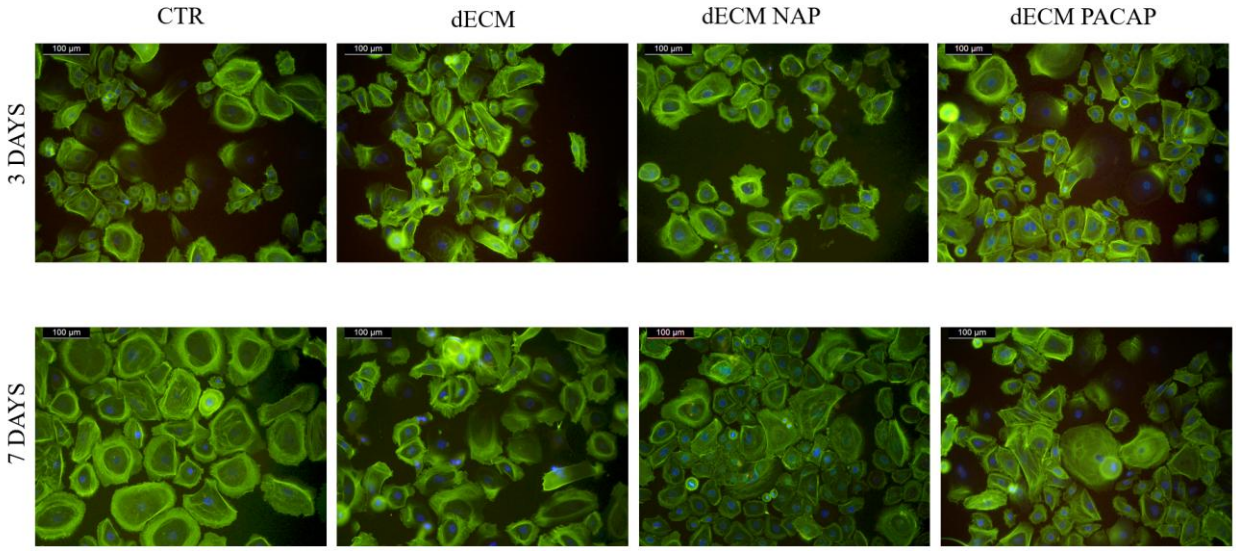


Figure 11: Fluorescence microscopy images showing the morphology of primary corneal epithelial cells cultured under different conditions after 3 and 7 days. Cells were stained with phalloidin (green) to visualize F-actin and DAPI (blue) to stain nuclei. Scale bar = 100 µm.

4.4 Migration assay for studying cell migration and wound healing:

The scratch wound migration assay evaluated how different hydrogel formulations affected corneal epithelial cell motility across four conditions: control (CTR), dECM, dECM with NAP, and dECM with PACAP (Figure 12). The images taken at 0, 8 and 16 hours showed progressive wound closure in all groups with no significant differences.

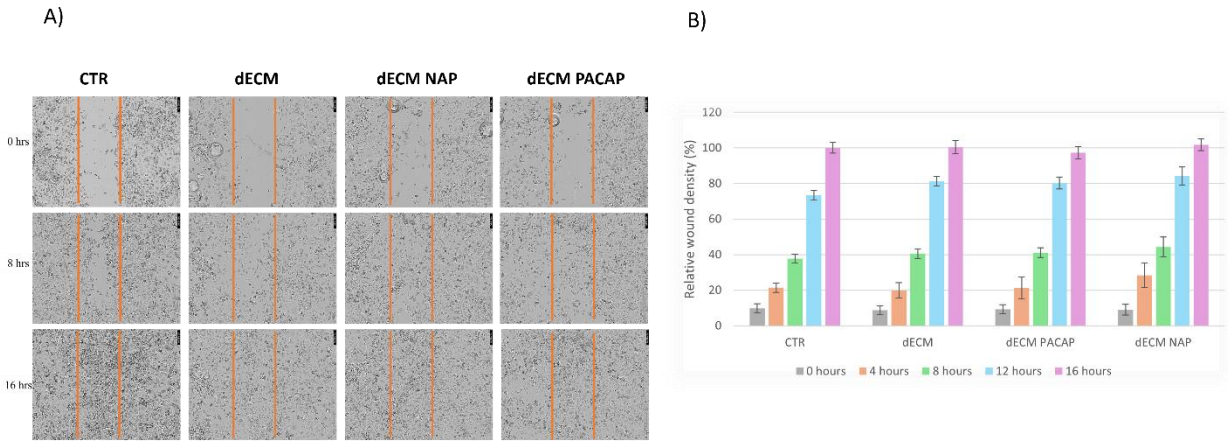


Figure 12: A) Scratch wound migration assay images of primary corneal epithelial cells under different conditions at 0, 8, and 16 hours. The images demonstrate progressive wound closure and cell migration patterns across all groups. B**)** Quantitative analysis of wound confluence (%) over time for the four tested conditions (CTR, dECM, dECM NAP, dECM PACAP). Data are expressed as mean ± SD from three independent experiments.

4.5 Effect of dECM and Peptide-Enriched Hydrogels on corneal markers expression

The immunofluorescence staining of Cytokeratin 3 (CK3) and Cytokeratin 12 (CK12) in primary corneal epithelial cells after 7 days of culture under different conditions is shown in Figures 13. CK3 and CK12, which are key markers of corneal epithelial differentiation, were expressed across all groups. A slightly but stronger fluorescence signal of CK3 was observed in the dECM NAP and dECM PACAP groups compared to the control, suggesting that these conditions may better support epithelial differentiation. The cells displayed a clear cytoplasmic localization of these markers, suggesting that the tested hydrogels contribute to maintaining the epithelial phenotype.

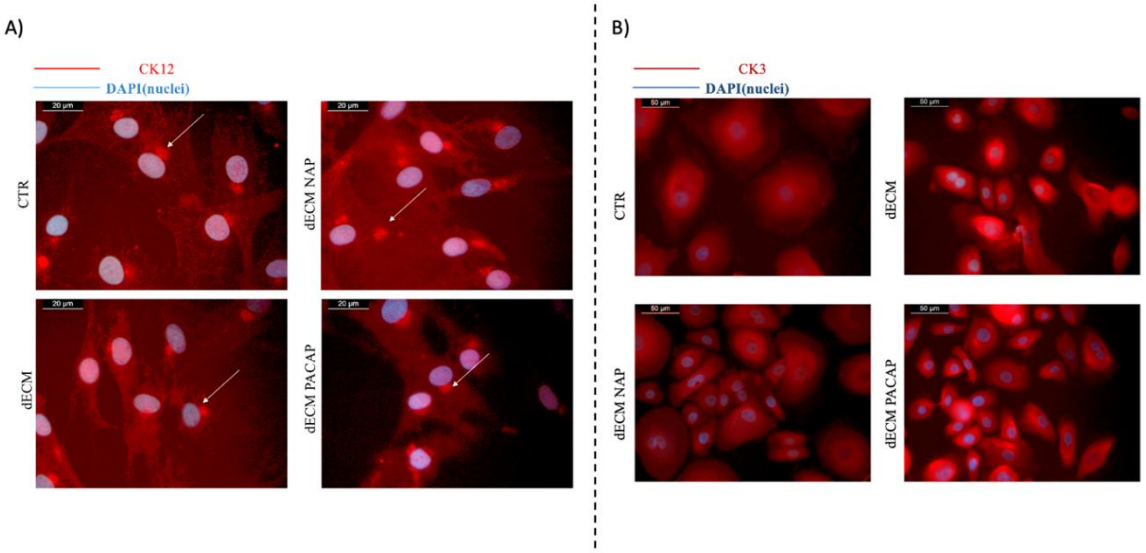


Figure 13: Immunofluorescence staining of A) Cytokeratin 12 (CK12) and B) Cytokeratin 3 (CK3) in primary corneal epithelial cells after 7 days of culture under different conditions (CTR, dECM, dECM NAP, dECM PACAP). CK3 is shown in red, and nuclei are counterstained with DAPI (blue). Scale bar = 50 and 20 µm.

In addition, western blot analysis was performed in order to evaluate corneal epithelial marker expression of the CK3 and CK12 in primary corneal epithelial cells as shown in Figure 14. Results showed a statistically significant increase in CK12 expression in the dECM with NAP compared to the control. Similarly, CK3 expression was also enhanced in both dECM peptide-enriched conditions, even though only dECM with NAP showed statistical significance.

Overall, these results indicate that the combination of dECM with NAP peptide may better support corneal epithelial phenotype and specification.

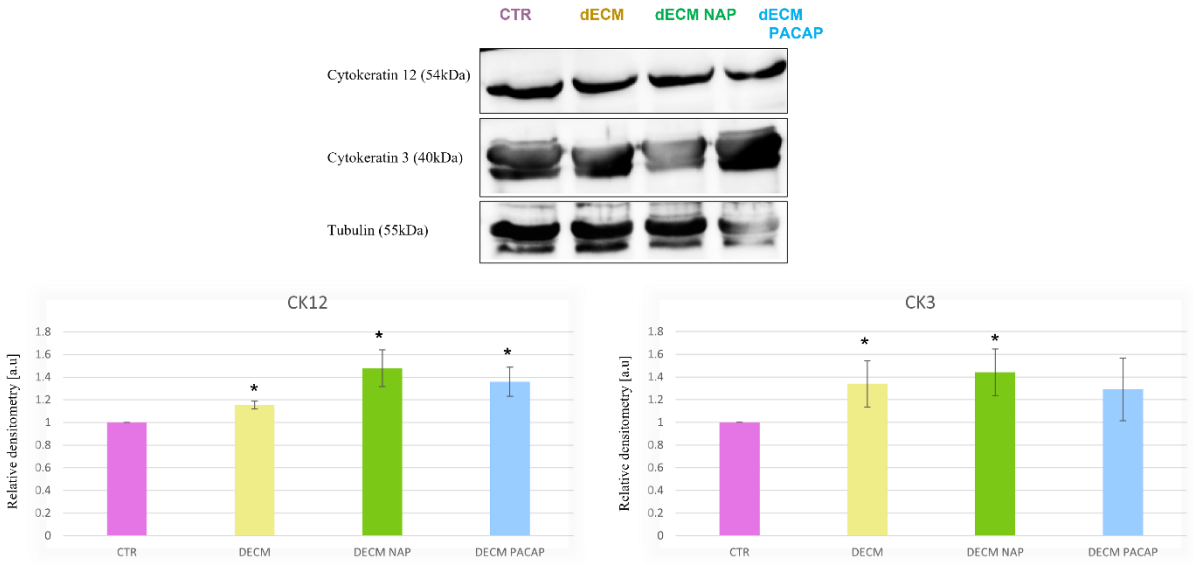


Figure 14: Western blot analyses with anti-cytokeratin 12 (CK12) and anti-cytokeratin 3 (CK3) antibodies. Tubulin expression was used to normalize the results. Proteins were revealed on total lysates and all results were normalized with respect to control. Densitometry obtained represent results from three different experiments, expressed as mean ± SD. Statistical significance with respect to control was indicated with * $p \leq 0.05$.

5. DISCUSSION

Corneal degenerative disorders and trauma still constitute today a major cause of vision loss in the world, making a strong need for effective regenerative therapies. Decellularized extracellular matrix dECM-based hydrogels have recently received attention as promising scaffolds as they are biocompatible and capable of preserving native structural and biochemical cues. In this study, we investigated the ability of dECM hydrogels supplemented by biologically active peptides (NAP and PACAP) alone or in combination to promote corneal epithelial cell proliferation and regeneration.

The research evaluated dECM-based hydrogels for corneal tissue engineering through complete assessments of their structural properties and optical characteristics and biological performance with and without PACAP and NAP bioactive peptide enrichment. The study shows that hydrogel concentration together with peptide incorporation determine the, cytocompatibility and regenerative capacity needed for corneal surface reconstruction without significantly compromise the optical clarity. In fact, 2 mg/ml dECM hydrogel showed to be the effective formulation according to quantitative transparency analysis, as it maintained light transmittance above 90% throughout two weeks. The optical clarity of 4 mg/ml and 9 mg/ml hydrogels appeared significantly lower than the other concentrations. This reduction in clarity at higher hydrogel concentrations is due to the increased matrix density, which causes more light scattering as a result of refractive index mismatches, as reported. The results obtained are in agreement with previous findings that hydrogel microstructure controls the amount of transmitted light [72], [90]. In particular, the ECM density lowers the light scattering since the refractive index heterogeneity in the network is minimized [92]. The native corneal stroma achieves transparency through its highly organized and uniformly spaced collagen fibrils [92], [93]. Thus, any deviation from this structure in dense or disorganized hydrogels matrices will result in micro-heterogeneities that scatter visible light and cause visual acuity loss. In our study, the hydrogel containing 2 mg/ml demonstrated the optimal mechanical compliance in terms of optical performance among the evaluated concentrations, and it is a promising platform for therapeutic agent delivery with maintained vision quality.

We next evaluated how the dECM, enriched with NAP and PACAP peptides, affects optical performance after optimizing concentration levels. The optical clarity of 2 mg/ml hydrogels remained high at 83–85% after adding PACAP and NAP. In fact, the clinical success of corneal grafts depends on their transparency. The small soluble molecules of PACAP and NAP distribute uniformly throughout the hydrated collagen network without causing microstructural disruption or refractive index changes [78], [94], [95] . Interestingly, PACAP and NAP have been demonstrated to enhance epithelial wound healing while simultaneously decreasing inflammation and NAP has been shown to promote corneal nerve regeneration [78], [94], [95]. The successful integration into a transparent scaffold demonstrates that multifunctional hydrogels can provide structural support while delivering targeted therapeutic effects [96], [97]. The cell viability assay showed that both PACAP- and NAP-functionalized hydrogels preserved corneal epithelial survival rates at day 7 levels equivalent to controls although the dECM-only group showed a slight decrease at day 3. Moreover, the morphological analysis

confirmed that dECM, with or without peptides, supported cell–substrate interactions and cytoskeletal arrangement [76], [94]. The results align with literature which reports that NAP is able to stabilize actin cytoskeleton and strengthen cell–cell junctions, which were demonstrated in UV-damaged corneal models for their anti-apoptotic and cytoprotective effects [92], [93]. Thus, these findings demonstrate the supportive function of dECM-derived scaffolds because they intrinsically contain native collagen, laminin and glycosaminoglycans which enhance cell adhesion and proliferation [76], [98].

Cell migration is an essential step in corneal wound healing, since epithelial closure is needed swiftly to regain barrier function and avoid infection [46], [47]. In agreement, the cell migration assays revealed that the dECM + NAP combination achieved wound closure rates at the same level as controls during the 16-hour scratch wound test which indicated better motility and re-epithelialization. In fact, literature reports that the NAP compound enables cell migration through its ability to stabilize microtubules and block JNK signaling which are vital for cytoskeletal rearrangement and directional cell movement [94], [95].On the contrary, the beneficial effects of PACAP on cell viability and morphology did not translate to significant migration promotion because it activates different signaling pathways [86], [87]. In addition, it is known that the dECM scaffold provides integrin-binding sites from fibronectin and laminin proteins, which create a biochemical environment for cell migration, while NAP enhances cellular responses to these signals [76], [97]. Notably, previous studies report that NAP promotes cell survival and movement as well as terminal differentiation and cytoskeletal specialization. The mechanisms behind these effects include oxidative stress reduction and intracellular signaling environment stabilization [86], [95], [96]. The dECM matrix works in conjunction with these effects by activating integrins and transcription factors that control keratin gene expression [76], [98] .

The intermediate filament proteins Cytokeratins serve as epithelial differentiation markers which include CK3 and CK12 as specific markers for corneal epithelial cells. The corneal epithelium expresses CK3 in its suprabasal and superficial surface layers but CK12 shows exclusive presence in corneal epithelial cells without appearing in conjunctival or limbal epithelial cells [4]. CK3 and CK12 show their proper distribution in the cytoplasm which indicates that the epithelial cells have correctly differentiated and developed their specific structures. [94], [95]. Western blot analysis confirmed the molecular evidence of differentiation through additional tests. The dECM/NAP group showed the highest CK12 expression which matches central and suprabasal corneal epithelium and displayed elevated CK3 levels that indicate more mature epithelial cells [99], [100], [101]. The CK3/CK12 expression in PACAP-enriched hydrogels showed positive results but with lower intensity than NAP-treated samples which indicates that PACAP supports cell survival and growth but NAP specifically accelerates maturation processes [94], [102]. NAP functions as a well-established compound which supports cell survival and organization and terminal differentiation through its stress-reducing properties and stabilization of intracellular signaling pathways [86], [94], [95]. The research shows that dECM scaffolds with NAP bioactive peptides create an optimal environment which enables both structural organization and phenotypic preservation of corneal epithelial cells for functional tissue engineering.

To conclude, the formulation of the dECM, combined with peptides, provides excellent optical transparency and cytocompatibility alongside structural support and promotes both enhanced migration and mature epithelial phenotype development. The results show promise but additional research with larger sample sizes and strict statistical validation must be performed to establish reproducibility and clinical application potential. The combination of structural integrity with targeted biochemical modification, especially in NAP-functionalized dECM hydrogels, makes them an attractive approach for future corneal repair applications.

6. Reference

[1] K. Cholkar, S. P. Patel, A. D. Vadlapudi, and A. K. Mitra, “Novel strategies for anterior segment ocular drug delivery.,” *J Ocul Pharmacol Ther*, vol. 29, no. 2, pp. 106–23, Mar. 2013, doi: 10.1089/jop.2012.0200.

[2] R. Gaudana, H. K. Ananthula, A. Parenky, and A. K. Mitra, “Ocular drug delivery.,” *AAPS J*, vol. 12, no. 3, pp. 348–60, Sep. 2010, doi: 10.1208/s12248-010-9183-3.

[3] L. E. Downie *et al.*, “BCLA CLEAR - Anatomy and physiology of the anterior eye,” *Contact Lens and Anterior Eye*, vol. 44, no. 2, pp. 132–156, Apr. 2021, doi: 10.1016/j.clae.2021.02.009.

[4] A. Schermer, S. Galvin, and T. T. Sun, “Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells.,” *J Cell Biol*, vol. 103, no. 1, pp. 49–62, Jul. 1986, doi: 10.1083/jcb.103.1.49.

[5] S. Masterton and M. Ahearne, “Mechanobiology of the corneal epithelium,” *Exp Eye Res*, vol. 177, pp. 122–129, Dec. 2018, doi: 10.1016/j.exer.2018.08.001.

[6] M. Notara *et al.*, “In sickness and in health: Corneal epithelial stem cell biology, pathology and therapy,” *Exp Eye Res*, vol. 90, no. 2, pp. 188–195, Feb. 2010, doi: 10.1016/j.exer.2009.09.023.

[7] D. W. DelMonte and T. Kim, “Anatomy and physiology of the cornea.,” *J Cataract Refract Surg*, vol. 37, no. 3, pp. 588–98, Mar. 2011, doi: 10.1016/j.jcrs.2010.12.037.

[8] Y. Komai and T. Ushiki, “The three-dimensional organization of collagen fibrils in the human cornea and sclera.,” *Invest Ophthalmol Vis Sci*, vol. 32, no. 8, pp. 2244–58, Jul. 1991.

[9] A. J. Rózsa and R. W. Beuerman, “Density and organization of free nerve endings in the corneal epithelium of the rabbit.,” *Pain*, vol. 14, no. 2, pp. 105–120, Oct. 1982, doi: 10.1016/0304-3959(82)90092-6.

[10] M. J. A. J. A. and W. J. Esperson. Hogan, *Histology of the human eye; an atlas and textbook* . Philadelphia: Philadelphia, Saunders 1971, 1971.

[11] C. Belmonte, M. C. Acosta, and J. Gallar, “Neural basis of sensation in intact and injured corneas.,” *Exp Eye Res*, vol. 78, no. 3, pp. 513–25, Mar. 2004, doi: 10.1016/j.exer.2003.09.023.

[12] C. E. Ghezzi, J. Rnjak-Kovacina, and D. L. Kaplan, “Corneal tissue engineering: recent advances and future perspectives.,” *Tissue Eng Part B Rev*, vol. 21, no. 3, pp. 278–87, Jun. 2015, doi: 10.1089/ten.TEB.2014.0397.

[13] H. S. Dua, “Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche,” *British Journal of Ophthalmology*, vol. 89, no. 5, pp. 529–532, May 2005, doi: 10.1136/bjo.2004.049742.

[14] M. A. Dziasko, H. E. Armer, H. J. Levis, A. J. Shortt, S. Tuft, and J. T. Daniels, “Localisation of Epithelial Cells Capable of Holoclone Formation In Vitro and Direct Interaction with Stromal Cells in the Native Human Limbal Crypt,” *PLoS One*, vol. 9, no. 4, p. e94283, Apr. 2014, doi: 10.1371/journal.pone.0094283.

[15] M. Santra, Y.-C. Liu, V. Jhanji, and G. H.-F. Yam, “Human SMILE-Derived Stromal Lenticule Scaffold for Regenerative Therapy: Review and Perspectives,” *Int J Mol Sci*, vol. 23, no. 14, p. 7967, Jul. 2022, doi: 10.3390/ijms23147967.

[16] E. M. Espana and D. E. Birk, “Composition, structure and function of the corneal stroma.,” *Exp Eye Res*, vol. 198, p. 108137, Sep. 2020, doi: 10.1016/j.exer.2020.108137.

[17] D. E. Birk and R. L. Trelstad, “Extracellular compartments in tendon morphogenesis: collagen fibril, bundle, and macroaggregate formation.,” *J Cell Biol*, vol. 103, no. 1, pp. 231–240, Jul. 1986, doi: 10.1083/jcb.103.1.231.

[18] O. Kermani, W. Fabian, and H. Lubatschowski, “Real-Time Optical Coherence Tomography-Guided Femtosecond Laser Sub-Bowman Keratomileusis on Human Donor Eyes,” *Am J Ophthalmol*, vol. 146, no. 1, pp. 42–45, Jul. 2008, doi: 10.1016/j.ajo.2008.03.003.

[19] T. F. Linsenmayer *et al.*, “Development and roles of collagenous matrices in the embryonic avian cornea.,” *Prog Retin Eye Res*, vol. 17, no. 2, pp. 231–65, Apr. 1998, doi: 10.1016/s1350-9462(97)00010-4.

[20] C. M. Tong, K. van Dijk, and G. R. J. Melles, “Update on Bowman layer transplantation,” *Curr Opin Ophthalmol*, vol. 30, no. 4, pp. 249–255, Jul. 2019, doi: 10.1097/ICU.0000000000000570.

[21] J. Germundsson, G. Karanis, P. Fagerholm, and N. Lagali, “Age-Related Thinning of Bowman’s Layer in the Human Cornea In Vivo,” *Investigative Opthalmology & Visual Science*, vol. 54, no. 9, p. 6143, Sep. 2013, doi: 10.1167/iovs.13-12535.

[22] S. E. Wilson, “Bowman’s layer in the cornea– structure and function and regeneration,” *Exp Eye Res*, vol. 195, p. 108033, Jun. 2020, doi: 10.1016/j.exer.2020.108033.

[23] I. K. Gipson, S. J. Spurr-Michaud, and A. S. Tisdale, “Anchoring fibrils form a complex network in human and rabbit cornea.,” *Invest Ophthalmol Vis Sci*, vol. 28, no. 2, pp. 212–20, Feb. 1987.

[24] A. S. Tisdale, S. J. Spurr-Michaud, M. Rodrigues, J. Hackett, J. Krachmer, and I. K. Gipson, “Development of the anchoring structures of the epithelium in rabbit and human fetal corneas.,” *Invest Ophthalmol Vis Sci*, vol. 29, no. 5, pp. 727–36, May 1988.

[25] J. M. Fitch, J. Gross, R. Mayne, B. Johnson-Wint, and T. F. Linsenmayer, “Organization of collagen types I and V in the embryonic chicken cornea: monoclonal antibody studies.,” *Proceedings of the National Academy of Sciences*, vol. 81, no. 9, pp. 2791–2795, May 1984, doi: 10.1073/pnas.81.9.2791.

[26] M. K. Gordon, J. W. Foley, D. E. Birk, J. M. Fitch, and T. F. Linsenmayer, “Type V collagen and Bowman’s membrane. Quantitation of mRNA in corneal epithelium and stroma.,” *J Biol Chem*, vol. 269, no. 40, pp. 24959–66, Oct. 1994.

[27] P. A. Faye *et al.*, “Focus on cell therapy to treat corneal endothelial diseases,” *Exp Eye Res*, vol. 204, p. 108462, Mar. 2021, doi: 10.1016/j.exer.2021.108462.

[28] L. Balyen, “The Physiology of Cornea,” *JOJ Ophthalmol*, vol. 9, no. 3, Dec. 2022, doi: 10.19080/JOJO.2022.09.555764.

[29] Y. Zhou, T. Wang, S. S. Tuli, W. A. Steigleman, and A. A. Shah, “Overview of Corneal Transplantation for the Nonophthalmologist.,” *Transplant Direct*, vol. 9, no. 2, p. e1434, Feb. 2023, doi: 10.1097/TXD.0000000000001434.

[30] K. M. Meek and C. Boote, “The organization of collagen in the corneal stroma,” *Exp Eye Res*, vol. 78, no. 3, pp. 503–512, Mar. 2004, doi: 10.1016/j.exer.2003.07.003.

[31] M. Sridhar, “Anatomy of cornea and ocular surface,” *Indian J Ophthalmol*, vol. 66, no. 2, p. 190, 2018, doi: 10.4103/ijo.IJO_646_17.

[32] H. S. Dua, L. A. Faraj, D. G. Said, T. Gray, and J. Lowe, “Human Corneal Anatomy Redefined,” *Ophthalmology*, vol. 120, no. 9, pp. 1778–1785, Sep. 2013, doi: 10.1016/j.ophtha.2013.01.018.

[33] J. A. Bonanno, “Molecular mechanisms underlying the corneal endothelial pump.,” *Exp Eye Res*, vol. 95, no. 1, pp. 2–7, Feb. 2012, doi: 10.1016/j.exer.2011.06.004.

[34] A. O. Eghrari, S. A. Riazuddin, and J. D. Gottsch, “Overview of the Cornea: Structure, Function, and Development.,” *Prog Mol Biol Transl Sci*, vol. 134, pp. 7–23, 2015, doi: 10.1016/bs.pmbts.2015.04.001.

[35] J. Zavala, G. R. López Jaime, C. A. Rodríguez Barrientos, and J. Valdez-Garcia, “Corneal endothelium: developmental strategies for regeneration,” *Eye*, vol. 27, no. 5, pp. 579–588, May 2013, doi: 10.1038/eye.2013.15.

[36] N. C. Joyce, D. L. Harris, and D. M. Mello, “Mechanisms of mitotic inhibition in corneal endothelium: contact inhibition and TGF-beta2.,” *Invest Ophthalmol Vis Sci*, vol. 43, no. 7, pp. 2152–9, Jul. 2002.

[37] W. M. Bourne, “Biology of the corneal endothelium in health and disease,” *Eye*, vol. 17, no. 8, pp. 912–918, Nov. 2003, doi: 10.1038/sj.eye.6700559.

[38] V. Vilkelyte, P. Thompson, M. Coelho, M. Woronkowicz, P. Skopinski, and H. Roberts, “Challenges and Advances in Magnetic Nanoparticle-Guided Delivery of Cultured Human Corneal Endothelial Cells—A Review,” *Applied Sciences*, vol. 14, no. 13, p. 5877, Jul. 2024, doi: 10.3390/app14135877.

[39] E. Y. Wang *et al.*, “Global Trends in Blindness and Vision Impairment Resulting from Corneal Opacity 1984-2020: A Meta-analysis.,” *Ophthalmology*, vol. 130, no. 8, pp. 863–871, Aug. 2023, doi: 10.1016/j.ophtha.2023.03.012.

[40] N. Sadanandan *et al.*, “Pituitary Adenylate Cyclase-Activating Polypeptide: A Potent Therapeutic Agent in Oxidative Stress.,” *Antioxidants (Basel)*, vol. 10, no. 3, Feb. 2021, doi: 10.3390/antiox10030354.

[41] J. Lorenzo-Morales, N. A. Khan, and J. Walochnik, “An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment,” *Parasite*, vol. 22, p. 10, Feb. 2015, doi: 10.1051/parasite/2015010.

[42] J. S. Weiss *et al.*, “IC3D Classification of Corneal Dystrophies—Edition 2,” *Cornea*, vol. 34, no. 2, pp. 117–159, Feb. 2015, doi: 10.1097/ICO.0000000000000307.

[43] T. Nakamura *et al.*, “Successful Regrafting of Cultivated Corneal Epithelium Using Amniotic Membrane as a Carrier in Severe Ocular Surface Disease,” *Cornea*, vol. 22, no. 1, pp. 70–71, Jan. 2003, doi: 10.1097/00003226-200301000-00017.

[44] H. S. Dua, J. S. Saini, A. Azuara-Blanco, and P. Gupta, “Limbal stem cell deficiency: concept, aetiology, clinical presentation, diagnosis and management.,” *Indian J Ophthalmol*, vol. 48, no. 2, pp. 83–92, Jun. 2000.

[45] D. M. Najjar, S. T. Awwad, W. M. Zein, and W. F. Haddad, “Assessment of the corneal endothelium in acute ultraviolet keratitis.,” *Med Sci Monit*, vol. 12, no. 5, pp. MT23-5, May 2006.

[46] L. Lu, P. S. Reinach, and W. W.-Y. Kao, “Corneal Epithelial Wound Healing,” *Exp Biol Med*, vol. 226, no. 7, pp. 653–664, Jul. 2001, doi: 10.1177/153537020222600711.

[47] A. V. Ljubimov and M. Saghizadeh, “Progress in corneal wound healing,” *Prog Retin Eye Res*, vol. 49, pp. 17–45, Nov. 2015, doi: 10.1016/j.preteyeres.2015.07.002.

[48] F.-S. X. Yu, J. Yin, K. Xu, and J. Huang, “Growth factors and corneal epithelial wound healing,” *Brain Res Bull*, vol. 81, no. 2–3, pp. 229–235, Feb. 2010, doi: 10.1016/j.brainresbull.2009.08.024.

[49] K. A. Diehl, J. D. Foley, P. F. Nealey, and C. J. Murphy, “Nanoscale topography modulates corneal epithelial cell migration,” *J Biomed Mater Res A*, vol. 75A, no. 3, pp. 603–611, Dec. 2005, doi: 10.1002/jbm.a.30467.

[50] S. J. Liliensiek, S. Campbell, P. F. Nealey, and C. J. Murphy, “The scale of substratum topographic features modulates proliferation of corneal epithelial cells and corneal fibroblasts,” *J Biomed Mater Res A*, vol. 79A, no. 1, pp. 185–192, Oct. 2006, doi: 10.1002/jbm.a.30744.

[51] G. A. Abrams, S. S. Schaus, S. L. Goodman, P. F. Nealey, and C. J. Murphy, “Nanoscale Topography of the Corneal Epithelial Basement Membrane and Descemet’s Membrane of the Human,” *Cornea*, vol. 19, no. 1, pp. 57–64, Jan. 2000, doi: 10.1097/00003226-200001000-00012.

[52] K. Suzuki, “Cell–matrix and cell–cell interactions during corneal epithelial wound healing,” *Prog Retin Eye Res*, vol. 22, no. 2, pp. 113–133, Mar. 2003, doi: 10.1016/S1350-9462(02)00042-3.

[53] A. V Ljubimov, R. E. Burgeson, R. J. Butkowski, A. F. Michael, T. T. Sun, and M. C. Kenney, “Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms.,” *Lab Invest*, vol. 72, no. 4, pp. 461–73, Apr. 1995.

[54] J. D. Zieske *et al.*, “Basement Membrane Assembly and Differentiation of Cultured Corneal Cells: Importance of Culture Environment and Endothelial Cell Interaction,” *Exp Cell Res*, vol. 214, no. 2, pp. 621–633, Oct. 1994, doi: 10.1006/excr.1994.1300.

[55] A. Shadmani and A. Y. Wu, “Navigating the path to corneal healing success and challenges: a comprehensive overview.,” *Eye (Lond)*, vol. 39, no. 6, pp. 1047–1055, Apr. 2025, doi: 10.1038/s41433-025-03619-2.

[56] G. Saccu *et al.*, “Regenerative Approaches and Future Trends for the Treatment of Corneal Burn Injuries,” *J Clin Med*, vol. 10, no. 2, p. 317, Jan. 2021, doi: 10.3390/jcm10020317.

[57] S. S. Tuli, G. S. Schultz, and D. M. Downer, “Science and Strategy for Preventing and Managing Corneal Ulceration,” *Ocul Surf*, vol. 5, no. 1, pp. 23–39, Jan. 2007, doi: 10.1016/S1542-0124(12)70050-2.

[58] C. Malhotra and A. K. Jain, “Human amniotic membrane transplantation: Different modalities of its use in ophthalmology,” *World J Transplant*, vol. 4, no. 2, p. 111, 2014, doi: 10.5500/wjt.v4.i2.111.

[59] P. Rosenthal and J. Cotter, “The Boston Scleral Lens in the management of severe ocular surface disease.,” *Ophthalmol Clin North Am*, vol. 16, no. 1, pp. 89–93, Mar. 2003, doi: 10.1016/s0896-1549(02)00067-6.

[60] A. L. Young, A. C. O. Cheng, H. K. Ng, L. L. Cheng, G. Y. S. Leung, and D. S. C. Lam, “The use of autologous serum tears in persistent corneal epithelial defects.,” *Eye (Lond)*, vol. 18, no. 6, pp. 609–14, Jun. 2004, doi: 10.1038/sj.eye.6700721.

[61] S. Salehi *et al.*, "[New nanofibrous scaffold for corneal tissue engineering].," *Klin Monbl Augenheilkd*, vol. 231, no. 6, pp. 626–30, Jun. 2014, doi: 10.1055/s-0034-1368533.

[62] D. Barritault, P. Desgranges, A. Meddahi-Pellé, J.-M. Denoix, and J.-L. Saffar, "RGTA ® -based matrix therapy – A new branch of regenerative medicine in locomotion," *Joint Bone Spine*, vol. 84, no. 3, pp. 283–292, May 2017, doi: 10.1016/j.jbspin.2016.06.012.

[63] S. Bonini *et al.*, "Phase II Randomized, Double-Masked, Vehicle-Controlled Trial of Recombinant Human Nerve Growth Factor for Neurotrophic Keratitis.," *Ophthalmology*, vol. 125, no. 9, pp. 1332–1343, Sep. 2018, doi: 10.1016/j.ophtha.2018.02.022.

[64] U. Elbaz, R. Bains, R. M. Zuker, G. H. Borschel, and A. Ali, "Restoration of corneal sensation with regional nerve transfers and nerve grafts: a new approach to a difficult problem.," *JAMA Ophthalmol*, vol. 132, no. 11, pp. 1289–95, Nov. 2014, doi: 10.1001/jamaophthalmol.2014.2316.

[65] B. T. Robbins, K. A. Montreuil, N. Kundu, P. Kumar, and V. Agrahari, "Corneal Treatment, Repair, and Regeneration: Exosomes at Rescue.," *Pharmaceutics*, vol. 16, no. 11, Nov. 2024, doi: 10.3390/pharmaceutics16111424.

[66] J. Liu *et al.*, "Bioactive scaffolds for tissue engineering: A review of decellularized extracellular matrix applications and innovations," *Exploration*, vol. 5, no. 1, Feb. 2025, doi: 10.1002/EXP.20230078.

[67] C. E. Ghezzi, J. Rnjak-Kovacina, and D. L. Kaplan, "Corneal tissue engineering: recent advances and future perspectives.," *Tissue Eng Part B Rev*, vol. 21, no. 3, pp. 278–87, Jun. 2015, doi: 10.1089/ten.TEB.2014.0397.

[68] Z. Chen *et al.*, "Biomaterials for corneal bioengineering," *Biomedical Materials*, vol. 13, no. 3, p. 032002, Mar. 2018, doi: 10.1088/1748-605X/aa92d2.

[69] H. Kobayashi and Y. Ikacia, "Corneal cell adhesion and proliferation on hydrogel sheets bound with cell-adhesive proteins," *Curr Eye Res*, vol. 10, no. 10, pp. 899–908, Jan. 1991, doi: 10.3109/02713689109020325.

[70] L. Lei *et al.*, "Biofunctional peptide-click PEG-based hydrogels as 3D cell scaffolds for corneal epithelial regeneration," *J Mater Chem B*, vol. 10, no. 31, pp. 5938–5945, 2022, doi: 10.1039/D2TB00983H.

[71] S. Chameettachal *et al.*, "Prevention of Corneal Myofibroblastic Differentiation *In Vitro* Using a Biomimetic ECM Hydrogel for Corneal Tissue Regeneration," *ACS Appl Bio Mater*, vol. 4, no. 1, pp. 533–544, Jan. 2021, doi: 10.1021/acsabm.0c01112.

[72] K. M. Meek and C. Knupp, "Corneal structure and transparency.," *Prog Retin Eye Res*, vol. 49, pp. 1–16, Nov. 2015, doi: 10.1016/j.preteyeres.2015.07.001.

[73] M. Fathi, J. Barar, A. Aghanejad, and Y. Omidi, "Hydrogels for ocular drug delivery and tissue engineering.," *Bioimpacts*, vol. 5, no. 4, pp. 159–64, 2015, doi: 10.15171/bi.2015.31.

[74] A. Bordbar-Khiabani and M. Gasik, "Smart Hydrogels for Advanced Drug Delivery Systems," *Int J Mol Sci*, vol. 23, no. 7, p. 3665, Mar. 2022, doi: 10.3390/ijms23073665.

[75] A. A. Golebiowska, J. T. Intravaia, V. M. Sathe, S. G. Kumbar, and S. P. Nukavarapu, "Decellularized extracellular matrix biomaterials for regenerative therapies: Advances, challenges and clinical prospects," *Bioact Mater*, vol. 32, pp. 98–123, Feb. 2024, doi: 10.1016/j.bioactmat.2023.09.017.

[76] M. Gomez-Florit *et al.*, “Natural-Based Hydrogels for Tissue Engineering Applications,” *Molecules*, vol. 25, no. 24, p. 5858, Dec. 2020, doi: 10.3390/molecules25245858.

[77] C. N. Munyiri, E. S. Madivoli, J. Kisato, J. Gichuki, and P. G. Kareru, “Biopolymer based hydrogels: crosslinking strategies and their applications,” *International Journal of Polymeric Materials and Polymeric Biomaterials*, vol. 74, no. 7, pp. 625–640, May 2025, doi: 10.1080/00914037.2024.2356603.

[78] P. Kiss *et al.*, “Effects of pituitary adenylate cyclase activating polypeptide (PACAP) in corneal epithelial regeneration and signal transduction in rats,” *Int J Pept Res Ther*, vol. 28, no. 3, p. 92, May 2022, doi: 10.1007/s10989-022-10405-1.

[79] G. Maugeri *et al.*, “Activity-Dependent Neuroprotective Protein (ADNP)-Derived Peptide (NAP) Counteracts UV-B Radiation-Induced ROS Formation in Corneal Epithelium,” *Antioxidants*, vol. 11, no. 1, p. 128, Jan. 2022, doi: 10.3390/antiox11010128.

[80] M. Muttenthaler, G. F. King, D. J. Adams, and P. F. Alewood, “Trends in peptide drug discovery,” *Nat Rev Drug Discov*, vol. 20, no. 4, pp. 309–325, Apr. 2021, doi: 10.1038/s41573-020-00135-8.

[81] D. J. Craik, D. P. Fairlie, S. Liras, and D. Price, “The Future of Peptide-based Drugs,” *Chem Biol Drug Des*, vol. 81, no. 1, pp. 136–147, Jan. 2013, doi: 10.1111/cbdd.12055.

[82] A. Miyata *et al.*, “Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38),” *Biochem Biophys Res Commun*, vol. 170, no. 2, pp. 643–648, Jul. 1990, doi: 10.1016/0006-291X(90)92140-U.

[83] M. Wei, S. Naruse, T. Ozaki, P. Hu, V. Wray, and K. Nokihara, “Differences in Action of PACAP-27 and PACAP-38 on Guinea Pig Gallbladder Smooth Muscle Using Synthetic C-terminally Modified PACAP Peptides,” *Int J Pept Res Ther*, vol. 15, no. 3, pp. 227–232, Sep. 2009, doi: 10.1007/s10989-009-9183-2.

[84] Z. Wang *et al.*, “The PACAP-derived peptide MPAPO facilitates corneal wound healing by promoting corneal epithelial cell proliferation and trigeminal ganglion cell axon regeneration,” *Int J Biol Sci*, vol. 15, no. 12, pp. 2676–2691, 2019, doi: 10.7150/ijbs.35630.

[85] S. Chameettachal *et al.*, “Human cornea-derived extracellular matrix hydrogel for prevention of post-traumatic corneal scarring: A translational approach,” *Acta Biomater*, vol. 171, pp. 289–307, Nov. 2023, doi: 10.1016/j.actbio.2023.09.002.

[86] A. Galushkin and I. Gozes, “Intranasal NAP (Davunetide): Neuroprotection and circadian rhythmicity,” *Adv Drug Deliv Rev*, vol. 220, p. 115573, May 2025, doi: 10.1016/j.addr.2025.115573.

[87] S. Scuderi *et al.*, “Davunetide (NAP) Protects the Retina Against Early Diabetic Injury by Reducing Apoptotic Death,” *Journal of Molecular Neuroscience*, vol. 54, no. 3, pp. 395–404, Nov. 2014, doi: 10.1007/s12031-014-0244-4.

[88] N. K. Sharma, N. K. Sethy, R. N. Meena, G. Ilavazhagan, M. Das, and K. Bhargava, “Activity-dependent neuroprotective protein (ADNP)-derived peptide (NAP) ameliorates hypobaric hypoxia induced oxidative stress in rat brain,” *Peptides (N.Y.)*, vol. 32, no. 6, pp. 1217–1224, Jun. 2011, doi: 10.1016/j.peptides.2011.03.016.

[89] M. Zhang *et al.*, “3D bioprinting of corneal decellularized extracellular matrix: GelMA composite hydrogel for corneal stroma engineering,” *Int J Bioprint*, vol. 9, no. 5, p. 774, Jun. 2023, doi: 10.18063/ijb.774.

[90] J. R. Hassell and D. E. Birk, “The molecular basis of corneal transparency,” *Exp Eye Res*, vol. 91, no. 3, pp. 326–335, Sep. 2010, doi: 10.1016/j.exer.2010.06.021.

[91] K. Y. Wu, S. Y. Qian, A. Faucher, and S. D. Tran, “Advancements in Hydrogels for Corneal Healing and Tissue Engineering.,” *Gels*, vol. 10, no. 10, Oct. 2024, doi: 10.3390/gels10100662.

[92] P. Fagerholm *et al.*, “Stable corneal regeneration four years after implantation of a cell-free recombinant human collagen scaffold,” *Biomaterials*, vol. 35, no. 8, pp. 2420–2427, Mar. 2014, doi: 10.1016/j.biomaterials.2013.11.079.

[93] C. E. Ghezzi, J. Rnjak-Kovacina, and D. L. Kaplan, “Corneal Tissue Engineering: Recent Advances and Future Perspectives,” *Tissue Eng Part B Rev*, vol. 21, no. 3, pp. 278–287, Jun. 2015, doi: 10.1089/ten.teb.2014.0397.

[94] G. Maugeri *et al.*, “Activity-Dependent Neuroprotective Protein (ADNP)-Derived Peptide (NAP) Counteracts UV-B Radiation-Induced ROS Formation in Corneal Epithelium,” *Antioxidants*, vol. 11, no. 1, p. 128, Jan. 2022, doi: 10.3390/antiox11010128.

[95] G. Maugeri, A. G. D’Amico, B. Magrì, G. Musumeci, and V. D’Agata, “Activity-Dependent Neuroprotective Protein (ADNP): An Overview of Its Role in the Eye,” *Int J Mol Sci*, vol. 23, no. 21, p. 13654, Nov. 2022, doi: 10.3390/ijms232113654.

[96] H. Choi, W.-S. Choi, and J.-O. Jeong, “A Review of Advanced Hydrogel Applications for Tissue Engineering and Drug Delivery Systems as Biomaterials,” *Gels*, vol. 10, no. 11, p. 693, Oct. 2024, doi: 10.3390/gels10110693.

[97] R. Wei *et al.*, “Self-healing adhesive oxidized guar gum hydrogel loaded with mesenchymal stem cell exosomes for corneal wound healing,” *J Nanobiotechnology*, vol. 23, no. 1, p. 321, Apr. 2025, doi: 10.1186/s12951-025-03366-2.

[98] A. A. Golebiowska, J. T. Intravaia, V. M. Sathe, S. G. Kumbar, and S. P. Nukavarapu, “Decellularized extracellular matrix biomaterials for regenerative therapies: Advances, challenges and clinical prospects,” *Bioact Mater*, vol. 32, pp. 98–123, Feb. 2024, doi: 10.1016/j.bioactmat.2023.09.017.

[99] K. Theerakittayakorn *et al.*, “Differentiation Induction of Human Stem Cells for Corneal Epithelial Regeneration,” *Int J Mol Sci*, vol. 21, no. 21, p. 7834, Oct. 2020, doi: 10.3390/ijms21217834.

[100] J. F. Jameson, M. O. Pacheco, H. H. Nguyen, E. A. Phelps, and W. L. Stoppel, “Recent Advances in Natural Materials for Corneal Tissue Engineering,” *Bioengineering*, vol. 8, no. 11, p. 161, Oct. 2021, doi: 10.3390/bioengineering8110161.

[101] T. Ramos *et al.*, “Specific decellularized extracellular matrix promotes the plasticity of human ocular surface epithelial cells,” *Front Med (Lausanne)*, vol. 9, Nov. 2022, doi: 10.3389/fmed.2022.974212.

[102] G. Maugeri *et al.*, “Pituitary Adenylate Cyclase-Activating Polypeptide Protects Corneal Epithelial Cells against UV-B-Induced Apoptosis via ROS/JNK Pathway Inhibition,” *Applied Sciences*, vol. 12, no. 7, p. 3435, Mar. 2022, doi: 10.3390/app12073435.

7. ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor Boccafoschi for her constant guidance and support and for giving me the opportunity to be part of her research group. Her dedication, positive energy, and encouragement have played a fundamental role in shaping my academic journey.

Working in her laboratory has been an invaluable experience that allowed me to develop not only scientific and professional skills but also confidence and personal growth. Her trust and mentorship have inspired me to pursue my future career in research with even greater passion and determination.

I would also like to sincerely thank Dr. Simona, who has been a true guide and support throughout my experiments and thesis work. With her patience, knowledge, and kindness, she was always there to answer my questions, guiding me step by step with the care and dedication of a compassionate teacher. Her presence made this journey smoother and far more encouraging. I wish her all the very best in every aspect of life and in her professional path.

I would like to express my deepest gratitude to my dear parents and my brother. On the hard and exhausting path of migration, there is only one person who truly understands the difficulties of leaving one's homeland, and that person is a mother. My mother experienced every emotion alongside me, walked beside me, and gave me the strength to keep going.

My brother Mohammad, you may be younger than me, but you have shown such beautiful maturity. I am deeply grateful for all your love and support.

To my beloved Saicharan, your presence came to me like a rescue ship in the turbulence of the sea of my life and brought the color of peace to my world. Thank you for being there.

I am also thankful to all my friends who stood by me and made this path easier to walk. Especially my long-standing friend, like a Persian carpet that becomes more valuable with age, my dear Heliya; thank you for these 17 years of friendship. May you achieve everything you desire in the best possible way.

On my first day arriving in Italy, my world was filled with calm thanks to the presence and help of you two. Dear Amin and Sajjad, I sincerely thank you and wish you all the best. I also thank dear Sepideh, Atefeh and Sahel, who may be newer in my life but are just as precious.

I would also like to thank dear Alireza and Walter, who were always present in every moment. And my dear Vato and Dr. Paolo, I have not forgotten you, and I am grateful for all the support you gave me.

I would like to thank all my aunts, uncles, and cousins for giving me hope and lighting a path for me. I hope God never takes these joys away from us.

Finally, I am deeply grateful to everyone who made this academic journey possible.